FLUORINATED CARBOHYDRATES AS PROBES OF MECHANISM AND SPECIFICITY IN GLYCOSYL TRANSFERASES

By

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ABSTRACT

The Compounds 2-deoxy-2-fluoro-β-D-glucosyl fluoride (**1**), 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucoside (**2**), 2-deoxy-2-fluoro-β-D-galactosyl fluoride (**3**) and 2-deoxy-2-fluoro-β-D-mannosyl fluoride (**4**) were all found to be potent covalent inhibitors of β-glucosidase from *Alcaligenes faecalis* (pABG5 β-glucosidase), which function through the accumulation of a stable glycosyl-enzyme intermediate. The mechanism of action for these inhibitors was investigated and found to be both specific and active site directed, involving a 1:1 stoichiometric formation of an enzyme inhibitor complex.

Investigation of the pre-steady state kinetics for the inhibition reaction provided values for the rates of formation (k_{on}) and hydrolysis (k_{off}) of the glycosylated-enzyme intermediate. The values of k_{on} determined in this manner are 5.9, 25, 3.6 and 5.6 $min.^{-1}$ for compounds **1**, **2**, **3** and **4** respectively, while the values of k_{off} were found to be much smaller; $1.5x10^{-5}$, 5.4×10^{-3} and 1.0×10^{-3} $min.^{-1}$, respectively for the intermediates formed from compounds **1**, **3** and **4**.

The isolated intermediates were also found to be competent in the catalysis of transglycosylation reactions and evidence for the participation of a specific binding site for the acceptor compound in this process was gained. The results of these kinetic experiments were corroborated by data obtained from ^{19}F-NMR spectra of the glycosyl-enzyme intermediate and by isolation and subsequent characterization of the transglycosylation products. The stereochemistry of the inhibition reaction was investigated by a simple ^{19}F-NMR experiment, and was found to be consistent with a double displacement reaction mechanism, as would be expected from the generally accepted reaction mechanism for this type of enzyme.

The compound D-glucal was found to be a substrate for pABG5 β-glucosidase (k_{cat} = 2.28 $min.^{-1}$, K_m = 0.85 mM). Hydration of this compound by pABG5 β-glucosidase in deuterated buffers demonstrated that the double bond of D-glucal was deuterated stereo-specifically from below the α-face. The compound 2-fluoro-D-glucal was found to be a weak competitive inhibitor (K_i = 30 mM) of pABG5 β-glucosidase.

Glycogen phosphorylase catalyses the reversible phosphorolysis of glycogen. A series of deoxy analogues of the enzymes natural substrate, α-D-glucose 1-phosphate have been prepared and along with a number of deoxyfluoro analogues tested as substrates. All were found to act as substrates but at exceedingly slow rates. The large rate reductions when compared with the normal substrate can be attributed to a deleterious combination of electronic and binding effects in the modified substrates reducing the stability of the enzymic transition states. A linear free energy relationship between k_{cat} and the first order rate constant for the acid catalysed hydrolysis of the same series of deoxy and deoxyfluoro glucopyranosyl phosphates was demonstrated, suggesting similar transition states for the two reactions and implicating an oxocarbonium ion-like transition state in the enzymic reaction. The binding data obtained from the steady state kinetics of these analogues suggests that hydrogen bonding interactions are qualitatively conserved in the glucopyranose binding site during the T- to R-state conformational transition of the enzyme and that interactions between the enzyme and the hydroxyl groups at the 3- and 6-positions of the glucopyranose ring of the substrate are potentially important for stabilization of the enzymic transition state.

The specificity of the substrate phosphate binding site has been probed using the compounds 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate (**5**), (1-deoxy-α-D-glucopyranosyl) methylphosphonate (**6**) and 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphofluoridate (**7**). The results suggested that phosphorylase *b* can bind both the monanionic and dianionic forms of its substrate with approximately equal affinity. NMR studies of the ternary enzyme-ligand complexes formed with glycogen phosphorylase *b* and **5** or **6** indicated that no proton donation occurred in the ground-state active site complex.

A preliminary investigation into the ability of the cellulase complex from a number of different wood-degrading fungi to hydrolyse p-nitrophenyl β-glucoside has been carried out. This work is aimed at producing environmentally safe fungicides, which are activated by the β-glucosidase component of the cellulase complex in these organisms and this study was carried out in conjunction with Forintek Canada.

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List of Abbreviations and Definitions.

Carbohydrate Structure:

2FβGlcF	2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride
2FβGalF	2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride
2FβManF	2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride
2FαGlcF	2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride
2FβGlcDNP	2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside
βGclPNP	p-nitrophenyl β-D-glucopyranoside
βGalPNP	p-nitrophenyl β–D-galactopyranoside
1dβGlcϕ	1-deoxy-β–D-glucopyranosyl benzene
1dαGlcCP	(1-deoxy-α-D-glucopyranosyl) methylphosphonate
2FαGlcP	2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate
3FαGlcP	3-deoxy-3-fluoro-α-D-glucopyranosyl phosphate
4FαGlcP	4-deoxy-4-fluoro-α-D-glucopyranosyl phosphate
6FαGlcP	6-deoxy-6-fluoro-α–D-glucopyranosyl phosphate
2FαGlcPF	2-deoxy-2-fluoro-α-D-glucopyranosyl phosphofluoridate
2dαGlcP	2-deoxy-α-D-arabinohexopyranosyl phosphate
3dαGlcP	3-deoxy-α-D-ribohexopyranosyl phosphate
4dαGlcP	4-deoxy-α-D-xylohexopyranosyl phosphate
6dαGlcP	6-deoxy-α-D-gluopyranosyl phosphate
αGlcP	α-D-glucopyransoyl phosphate
2FGlc	2-deoxy-2-fluoro-D-glucose
2dGlc	2-deoxy-D-arabinohexose

Other Abbreviations.

PLP	pyridoxal 5'-phosphate
PL	pyridoxal
PLPPαGlc	pyridoxal 5'-diphospho-α-D-glucose
AMP	adenosine monophosphate
AMPS	adenosine monophosphorothioate
ADP	adenosine diphosphate
ATP	adenosine triphosphate

Kinetic and Physical Constants

T	Temperature (K)
k	The Boltzman constant
h	Planck's constant.
k_{on}	The first order rate constant for glycosylation
k_{off}	The first order rate constant for deglycosylation
K_m	The Michalis-Menten constant (the substrate concentration which gives half maximal rate)
V_{max}	The maximal rate of an enzyme-catalysed reaction
k_{cat}	The first order rate constant for catalysis
K_d	The dissociation constant for the non-covalent enzyme-substrate complex
K_i	The inhibitor constant (the dissociation constant for the non-covalent enzyme-inhibitor complex)

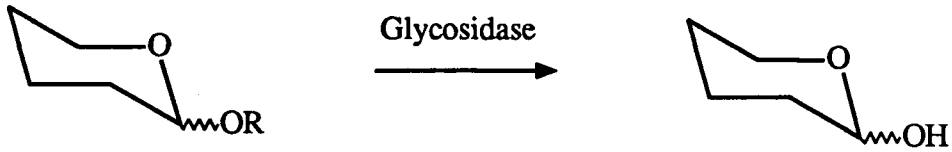
CHAPTER 1.

Glycosidases and Glycoside Hydrolysis

Introduction

The Catalytic Mechanism of Glycosidases.

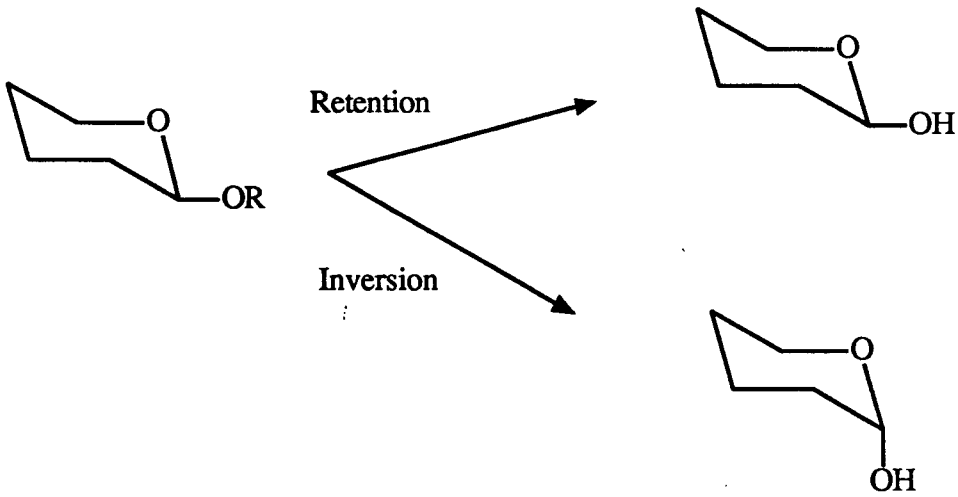
Glycosidases (glycoside hydrolases) are a broad class of enzymes which catalyse hydrolysis of glycosidic linkages, hydrolysis occurring by scission of the C-O bond between sugar and aglycone.



Scheme 1-1. Reaction catalysed by a glycosidase.

For the enzyme's naturally occurring substrate the aglycone is usually a second sugar, but many glycosidases show little or no specificity for this part of the substrate and in practice will also hydrolyse simple alkyl and aryl glycosides.

This extremely large class of enzymes may be divided into a number of sub classes based upon substrate specificity and the stereochemistry of their products. Firstly, glycosidases are usually very specific for the anomeric configuration of their substrate, a β-glycosidase will only hydrolyse β-glycosides and an α-glycosidase only α-glycosides. Secondly, hydrolysis can occur at the anomeric centre with retention or inversion of configuration and glycosidases like most other enzymes produce products with a well defined stereochemistry (Scheme 1-2). Lastly, these enzymes may again be subdivided based on their specificity for the type of sugar ring, glucose, galactose, pyranose or furanose etc. A single glycosidase may in fact be active against a variety of different sugars, that is a glucosidase may show some galactosidase and mannosidase activity as well, but the enzyme is usually classified from the name of the substrate against which it is most active.



Scheme 1-2. Stereochemical consequences of glycosidase-catalysed hydrolysis.

By far the most widely studied subclass is that of the "retaining" β-glycopyranosidases and the following discussion will largely be restricted to results obtained from this class of enzymes. A general mechanism for "retaining" glycosidases was first proposed over 30 years ago by Koshland (1953) (Scheme 1-3). Since then it has been refined by results from a great deal of experimental work, but its essential features have withstood the test of time. The main features of this mechanism are:

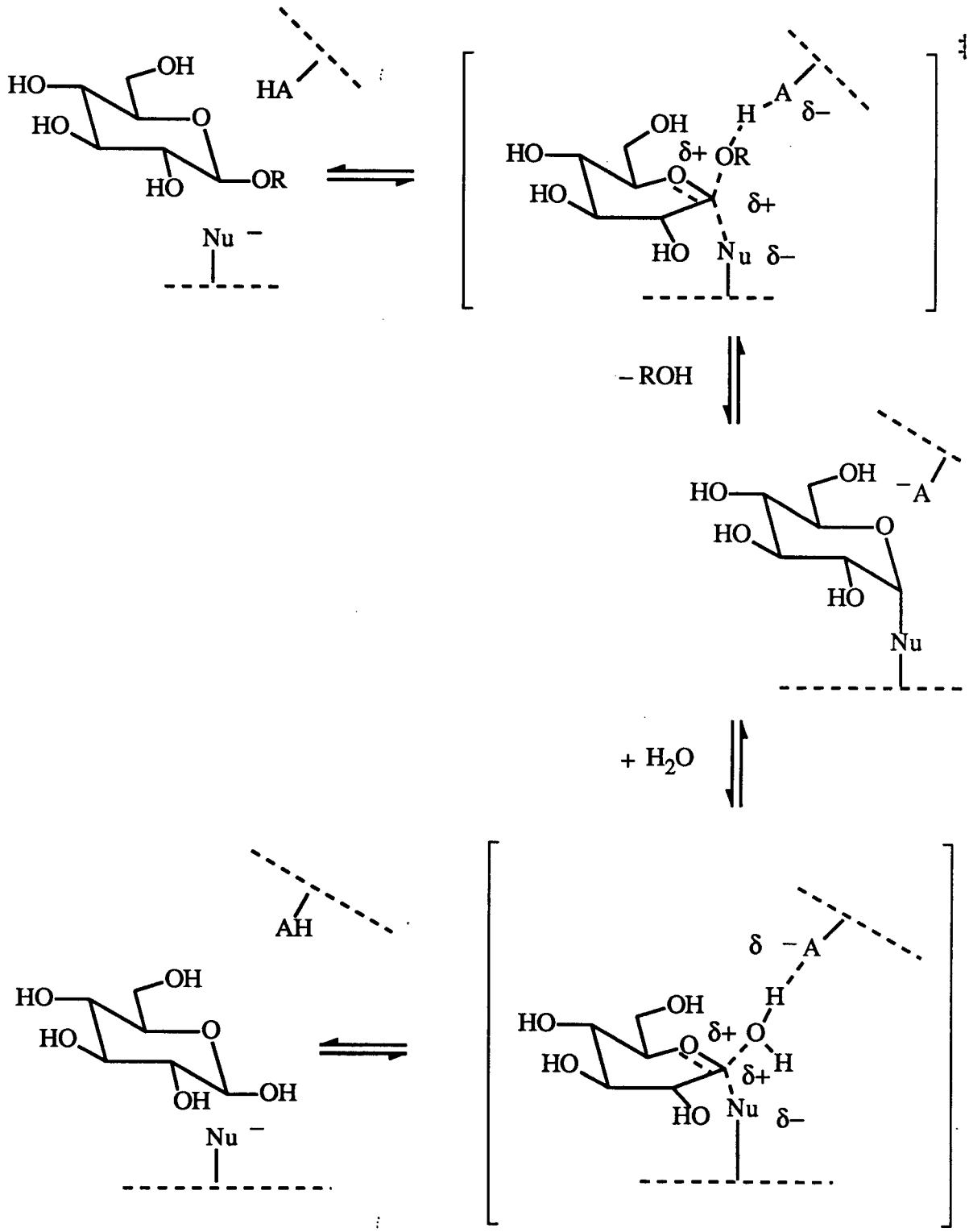
1) The reaction proceeds via formation of a covalent enzyme intermediate.

2) This covalent intermediate is reached from both directions through oxocarbonium ion-like transition states.

3) Acid catalysis may assist aglycone departure.

4) Non-covalent interactions provide most of the rate acceleration.

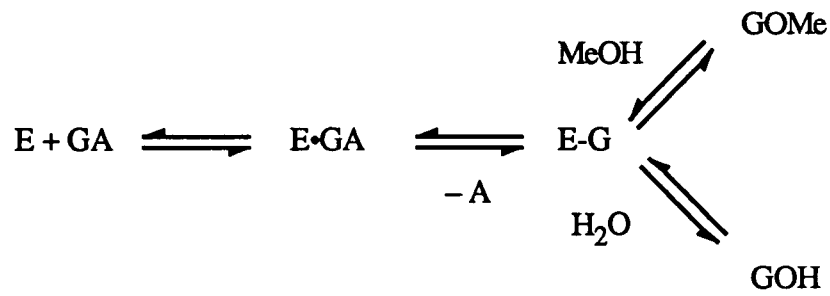
Features 1 and 4 will be addressed in some detail in the following Results and Discussion section and therefore will not be covered here. For a recent review on the mechanistic aspects of glycosidase chemistry the reader is referred to Sinnott (1987).



Scheme 1-3. A general reaction mechanism for a "retaining" β-glucosidase.

Glycosyl-Enzyme Intermediate.

Much of the kinetic evidence for the intermediacy of a glycosylated-enzyme was derived by observing partitioning ratios between normal hydrolysis and methanolysis products formed when β-galactosidase and aryl β-galactosides were incubated in buffers containing methanol (see Scheme below and Table 1-I).



Scheme 1-4 A kinetic model for partitioning of products between water and methanol from a common intermediate. Where GA is the glycoside substrate, E•GA is the non covalent enzyme-substrate complex, E-G is the covalent glycosyl-enzyme intermediate, GOMe is the methanolysis product and GOH is the hydrolysis product.

Although substrate reactivity varies over a wide range a constant partitioning between methanolysis and hydrolysis products was observed, a result which is consistent with the reaction proceeding through a common intermediate. The formation of an intermediate is also consistent with the relative V_{max} values seen in this table. These suggest that for weakly activated substrates glycosylation is rate limiting as these values are variable. However, for the highly activated substrates V_{max} is independent of aglycone structure which infers that for these substrates a process other than bond cleavage has become rate determining, this being hydrolysis of the glycosyl-enzyme.

Although Koshland's original proposal suggested that the glycosyl-enzyme was a covalent species (and there is a great deal of evidence that suggests this is so), Phillips and co-workers have proposed a glycosyl cation in this role (Imoto et al., 1972). This proposal was based on the X-ray crystal structure of lysozyme which seemed to possess many features which would stabilize this putative oxocarbonium ion intermediate. Most notable was the potential to form an intimate ion pair with Asp-52.

Table 1-I. Partitioning of galactosyl-β-galactosidase between water and methanol [a].

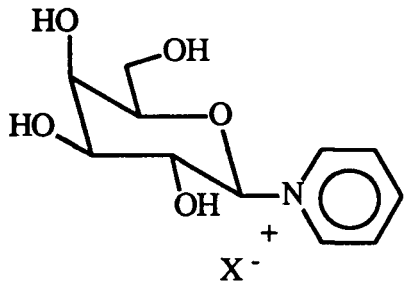
β-galactoside	Methanolysis / Hydrolysis	V_{max} (relative)	Rate-determining step
2,4-Dinitrophenyl	-	1.3	Degalactosylation
3,5-Dinitrophenyl	-	1.1	Degalactosylation
2,5-Dinitrophenyl	-	1.1	Degalactosylation
2-Nitrophenyl	1.97	1.0	-
3-Nitrophenyl	1.96	0.9	-
3-Chlorophenyl	2.08	0.5	Galactosylation
4-Nitrophenyl	1.99	0.2	Galactosylation
Phenyl	1.94	0.1	Galactosylation
4-Methoxyphenyl	2.14	0.1	Galactosylation
4-Chlorophenyl	2.13	0.02	Galactosylation
4-Bromophenyl	2.02	0.02	Galactosylation
Methyl	2.2	0.06	Galactosylation

[a] Data from Fersht (1984),Stokes and Wilson (1972), Sinnott and Viratelle (1973) and Sinnott and Souchard (1973).

However, it has recently been estimated that tertiary alkyl cations which are of comparable stability to glycosyl cations in water (Cocker et al. 1973; Young and Jencks, 1977), have a lifetime in the order of 10^{-10} seconds (Chiang and Kresge, 1985). It is difficult to see how even a very favourable environment as might be provided by lysozyme could prolong the lifetime of such a reactive species sufficiently for reaction to occur. The lifetime of the ion-pair would have to be sufficient for the leaving group to diffuse away and the glycosyl acceptor to diffuse in and react before the ion-pair collapsed to form a covalent intermediate.

While the plausibility of an ion-pair functioning as a stable intermediate appears remote, there exists a great deal of evidence to support the hypothesis that the transition states for formation and hydrolysis of the glycosylated enzyme have substantial oxocarbonium ion-like character. With careful choice of substrate it is possible to obtain rate limiting glycosylation or deglycosylation with many different glycosidases. Hence it is plausible to

investigate the enzymic transition states of these individual steps using kinetic isotope effects (KIE). Large secondary α-deuterium isotope effects (1.15 - 1.20) were observed during hydrolysis of C-1 deuterated β-galactosyl pyridinium salts by *E. coli (lac Z)* β-galactosidase (Sinnott and Withers, 1974) and a slightly smaller effect (1.10) for hydrolysis of O-aryl β–galactosides with the magnesium-free enzyme (Sinnott et al., 1978).



β-galactosyl pyridinium salt.

For both of these enzyme-substrate combinations glycosylation is rate determining, therefore the large isotope effect indicates substantial sp^2 character in the transition state for this step. Similar conclusions have been formed for the glycosylation steps in *E. coli (ebg)* β-galactosidase (Sinnott and Burton, 1983), β-glucosidase A_3 (from *Aspergillus wentii*) (Legler et al., 1980) and for lysozyme (Dahlquist et al., 1969; Smith et al., 1973). The observation of a large oxygen-18 isotope effect (1.0467±0.0015) which is approximately its theoretical maximum of 1.0425 (Rosenberg and Kirsch, 1981a) during hydrolysis of p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-1,4-β-D-glucopyranoside by lysozyme indicated that bond cleavage was all but complete in the transition state (Rosenberg and Kirsch,1981b). This along with the previously mentioned α-deuterium KIE's led these authors to propose that glycosylation proceeded via an almost S_N1 like transition state in this enzyme. However, the p-nitrophenyl glucoside used in this study is a very unnatural substrate for this enzyme and consequently may have produced a transition state which is atypical of its normal reaction. Since no substrates are known for which deglycosylation is rate limiting in lysozyme these authors were unable to draw any conclusions about the nature

of the glycosyl intermediate which would lie along the reaction co-ordinate beyond this transition state.

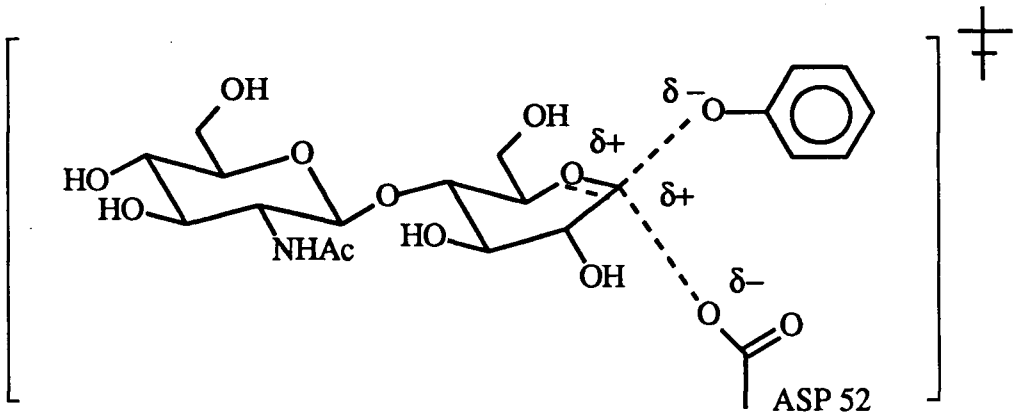
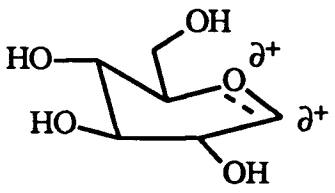


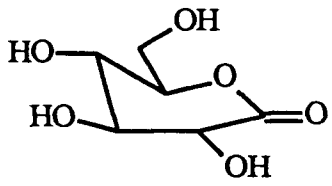
Figure 1-1. The proposed transition state structure for glycosylation of lysozyme.

For many other enzymes large positive α-deuterium KIE's have also been observed for the deglycosylation step, indicating a substantial increase in sp^2 character on going from the glycosylated enzyme to transition state (Sinnott et al., 1978; Sinnott and Souchard, 1973; Van Doorsler et al., 1984). This indirectly allows the nature of the stable intermediate to be inferred as a largely covalent (sp^3) species. If the intermediate had been a stable ion-pair an inverse isotope effect would have been seen on going to the transition state.

There is also a considerable body of evidence for oxocarbonium ion-like transition states in the form of tight-binding transition state analogues. There are a number of features which distinguish a glycosyl cation from its parent glycoside; O-5 and C-1 share a full positive charge between them, and C-5, O-5, C-1 and C-2 are all coplanar (a half-chair or boat conformation). Many stable monosaccharide derivatives possess one or both of these structural features and in many cases show enhanced binding to glycosidase when compared with a normal glycoside. Such compounds have often been postulated to be acting as a transition state "mimics" and much supportive evidence for the involvement of glycosyl cation-like transition states has been advanced based on this type of observation. A summary of results obtained from a number of studies is presented in Table 1-II.



A Glucosyl Cation



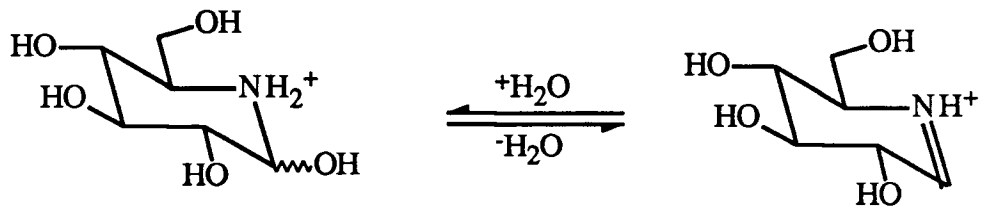
Glucono-1,5-lactone

Table 1-II. Inhibition of glycosidase by different "transition state analogues".

		K_i (mM) for competitive inhibition		
Inhibitor	Enzyme	Inhibitor	Glucose	Reference
	A. wentii β-glucosidase A_3		2.8	Legler et al. (1980)
β-Glucosylamine		0.0016		
Glucono-1,5-lactone		0.0096		
Glucono-1,5-lactam		0.036		
	Sweet Almond β-glucosidase		189	Dale et al.
Glucono-1,5-lactone		0.2		(1985)
Glucono-1,5-lactam		0.037		
Gluconohydroximino -1,5-lactone		0.098		Beer & Vasella (1986)
Gluconohydroximino N-methyl urethane		0.0023		
	Beef Liver β-glucuronidase			Niwa et al (1972)
Glucurono-1,5-lactam		3.9×10^{-5}		
Nojirimycin	β-glucosidase			Legler
	A. wentii (A_3)	0.00036	2.8	and Julich
	Almonds	0.0009	60.0	(1984).
	Helix pomatia	0.0011	46.0	

The scope and uses of these and other glucosidase inhibitors is reviewed by Lalegerie, Legler and Yon (1982).

Compounds which possess a positively charged group (or a potentially charged group in the form of a protonatable amine) generally bind more tightly to glucosidases than simple glucose. Most notable is the compound nojirimycin, which probably exists as a number of different forms in solution all of which may be to some degree inhibitory. Inspection of Scheme 1-5 shows that the N-protonated amine is isolectronic with a glucosyl cation while the N-protonated imine is both isoelectronic and isosteric.



Scheme 1-5. Different solution structures of nojirimycin.

Through a technique known as "cryoenzymology" (the study of enzyme mechanism at sub-zero temperatures) Fink and Good (1974) were able to trap a glucosyl-enzyme intermediate. This was accomplished with β-glucosidase A from sweet almonds using a cryosolvent of 50% aqueous dimethyl sulphoxide at - 20°C. Under these conditions turnover of substrate (p-nitrophenyl β-glucoside) by this enzyme is essentially stopped after the initial glucosylation step. It was found that the amount of aglycone released was stoichiometric with the concentration of enzyme, and thus all of the enzyme in the experiment was trapped as the glucosyl-enzyme intermediate.

Acid Catalysis.

Acid catalysis is not usually considered to be an essential step in the catalytic mechanism of glycosidases. Indeed in many cases it is not even required at all. Many glycosidases hydrolyse glycosyl pyridinium salts and for these substrates acid catalysis is a structural impossibility. Despite this, if the rates of S_N1 hydrolysis are compared to the rates of bond cleavage in the glycosidase-catalysed reaction, rate accelerations of 10^8-10^{13} are still

observed (Jones et al., 1977). For hydrolysis of O-aryl β-glucosides by β-glucosidase A_3 (from *A. wentii*) an analysis of the contribution from acid catalysis to aglycone departure has been made (Legler et al., 1980). In this example bond cleavage was found to be rate limiting (as evidenced by observation of a secondary deuterium KIE) but only small random variations of k_{cat} and k_{cat} / K_m with aglycone structure were observed ($\beta_{lg} = 0$), thus indicating that extensive proton donation even to the relatively acidic aglycone of 2,4-dinitrophenol was occurring. Again in this instance, a comparison of spontaneous and enzyme-catalysed hydrolysis rates for glucosyl pyridinium salts demonstrated that the enzyme was able to bring about a sizable rate acceleration for these substrates in the absence of acid catalysis. By a further comparison of enzymic hydrolysis rates for the glucopyranosyl pyridinium salts and those obtained for the O-aryl β–glucosides, acid catalysis was estimated to provide a rate acceleration in the order of $10^{(0.1 + 0.7[pKa])}$ where pKa is that of the free phenol. For the enzyme (*lac Z*) β-galactosidase operation of acid catalysis is associated with the presence of magnesium. Removal of this metal ion has little effect on the rate of hydrolysis for β-galactosyl pyridinium salts, but reduces the rate of hydrolysis for O-aryl β-galactosides.

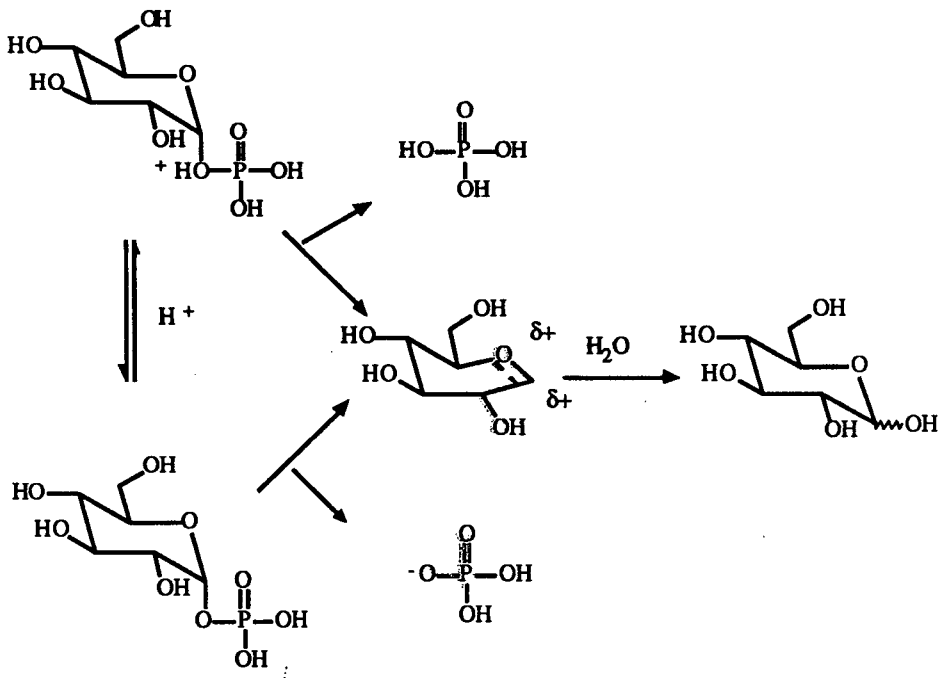
In a number of different enzymes residues which could potentially act as acid catalysts have been identified. Replacement of all of the tyrosines in (*lac Z*) β-galactosidase by m-fluorotyrosine (pK_a is 1.7 units lower than tyrosine) changes the alkaline limb of the pH profile by 1.5 units (Ring et al., 1985). Recent mutagenesis experiments (Ring et al., 1988) and a study involving a number of different isosteric charged inhibitors (Loeffler et al., 1979) also support the conclusion that the acid catalyst is a tyrosine residue. In hen's egg white lysozyme a carboxylate group (Glu 35) is seen in the X-ray crystal structure to be appropriately placed to donate a proton to the departing aglycone (Imoto et al., 1972) In α-glucosidase from yeast, affinity-labelling studies with cyclitol epoxides have implicated a histidine residue in the role of acid catalyst (Legler et al., 1973).

Substituent Effects Upon the Stability of Oxocarbonium Ion-like Transition States.

The acid catalysed hydrolysis of glucosides and glucopyranosyl phosphates is thought to proceed through transition states with substantial oxocarbonium ion-like character. Tu et al. (1971) and Firsov et al. (1974) have determined a secondary α-deuterium KIE (k_H / k_D in the range 1.10 to 1.13) for the acid catalysed hydrolysis of α-glucopyranosyl phosphate, which is consistent with this reaction preceding through transition states with substantial oxocarbonium ion-like character. Thus, this reaction has provided a non-enzymic system with which to study the electronic effects of different substitutions on the glucopyranosyl ring upon the rates of reaction.

The electronic effects on the acid catalysed rates of hydrolysis of a series of deoxy and deoxyfluoro α-D-glucopyranosyl phosphates have been probed in some detail. (Withers et al., 1986; Withers, S.G., Percival, M.D. and Street I.P. *Carbohydr. Chem.* In press). The mechanism of acid-catalysed hydrolysis of α-D-glucopyranosyl phosphate has been elucidated by Bunton et. al.,1958; Bunton et al., 1969). Under conditions of acid catalysis (pH 2 - 4) the species undergoing hydrolysis are the neutral phosphate and to a lesser extent its conjugate acid. These two species are present in relative amounts which reflect both the pH and the relevant ionization constants (Scheme 1-6). At pH values between 2 and 5 hydrolysis via the neutral species is the dominant pathway proceeding with C-O bond cleavage through an oxocarbonium intermediate or at least a transition state having substantial oxocarbonium ion-like character. A true oxocarbonium ion intermediate may not actually be involved, as it has been suggested that all glycoside hydrolyses proceed through a pre-association mechanism (Sinnott, 1984; Jencks, 1981). However, any species with substantial oxocarbonium ion character will have to adopt a conformation in which C-5, O-5 and C-2 approach coplanarity in a boat or half chair conformation.Two different electronic factors must be considered, inductive and dipolar. Based on a simple inductive argument it would be expected that the hydrolysis rate would increase as the number of bonds between the reaction

centre and electronegative substituent increases. That is, the expected order of reactivity for the fluorinated sugars would be 6 > 4 > 3 >2.



Scheme 1-6. A reaction mechanism for hydrolysis of α-D-glucopyranosyl phosphates.

Table 1-III. Hydrolysis rates for deoxy and deoxyfluoro α-D-glucopyranosyl phosphates [a].

D-glucopyranosyl Phosphate	Hydrolysis Temperature (°C)	Rate constant ($x10^5$ s^{-1})	[b] Relative Rate
2FαGlc P	25	0.068	1.00
4FαGlc P	25	0.270	3.97
3FαGlc P	25	0.480	7.05
6FαGlc P	25	1.12	16.5
αGlc P	25	4.10	60.30
3dαGlc P	25	31.0	455.9
4dαGlc P	25	111.0	1632.4
2dαGlc P	*25	11100.0	163235.3

* Rate calculated by extrapolation from data at other temperatures. [a] Data From Withers et al., (1986); Withers, S.G., Percival, M.D. and Street, I.P. *Carbohydr. Chem.* in press. Percival, M.D and Withers, S.G. *Can J. Chem.* in press. [b] Relative rate with respect to 2FαGlcP.

From the data in Table 1-III it can be seen that the order of reactivity for the 3- and 4-substituents is the reverse of that expected from the simple inductive argument, that is the 3-deoxyfluoro sugar hydrolyses faster than the 4-deoxyfluoro. This reversal of effects can be explained on the basis of the following dipolar contributions. The dipolar effect relates to the relative orientation of dipoles associated with C-OH and C-F in the ground state and in the half chair conformation of the transition state. The importance of these dipolar effects is amply illustrated by the anomeric effect. Assuming that a greater dipole is associated with the C-F bond than the C-OH the following conclusions can be drawn. The 2-deoxy-2-fluoro substituent should be the least reactive, because there is a large increase in dipole alignment on going to the transition state (see Figure 1-2). For the 3-deoxy-3-fluoro substituent there is a slight decrease in overall dipole alignment. Based on this criterion this compound should be hydrolysed slightly faster. For the 4-deoxy-4-fluoro substituent there is no net change in overall dipolar alignment, consequently no effect on the rate should be observed. Therefore on the basis of dipolar arguments the order of reactivity should be $3 > 4 > 2$. Superimposition of the dipolar effects onto the inductive effects mentioned earlier would account for the observed hydrolysis rates given in Table 1-III. As there is no significant dipole associated with the C-H bond the converse order should be observed for the deoxy glucosides.

These effects are also relevant to hydrolysis of aryl and alkyl glucosides. The similarity of these substituent effects for the two different reactions is best illustrated by Figure 1-3. The logarithm of first order rate constant for the acid-catalysed hydrolysis of the deoxy glucopyranosyl phosphates is plotted against the same parameter for a series of phenyl deoxy-D-glucopyranosides (Mega and Matsushima, 1983). A good free energy relationship is seen, ($R = 0.995$, slope = 0.94) indicating that the structure of the transition state is very similar in both cases. The slope of 0.94 indicates a greater sensitivity of the phosphate ester to substitution and would suggest that either the transition state for hydrolysis of the phosphate esters has slightly more oxocarbonium ion character or that the percentage of the conjugate acid form is a little different in the two cases.

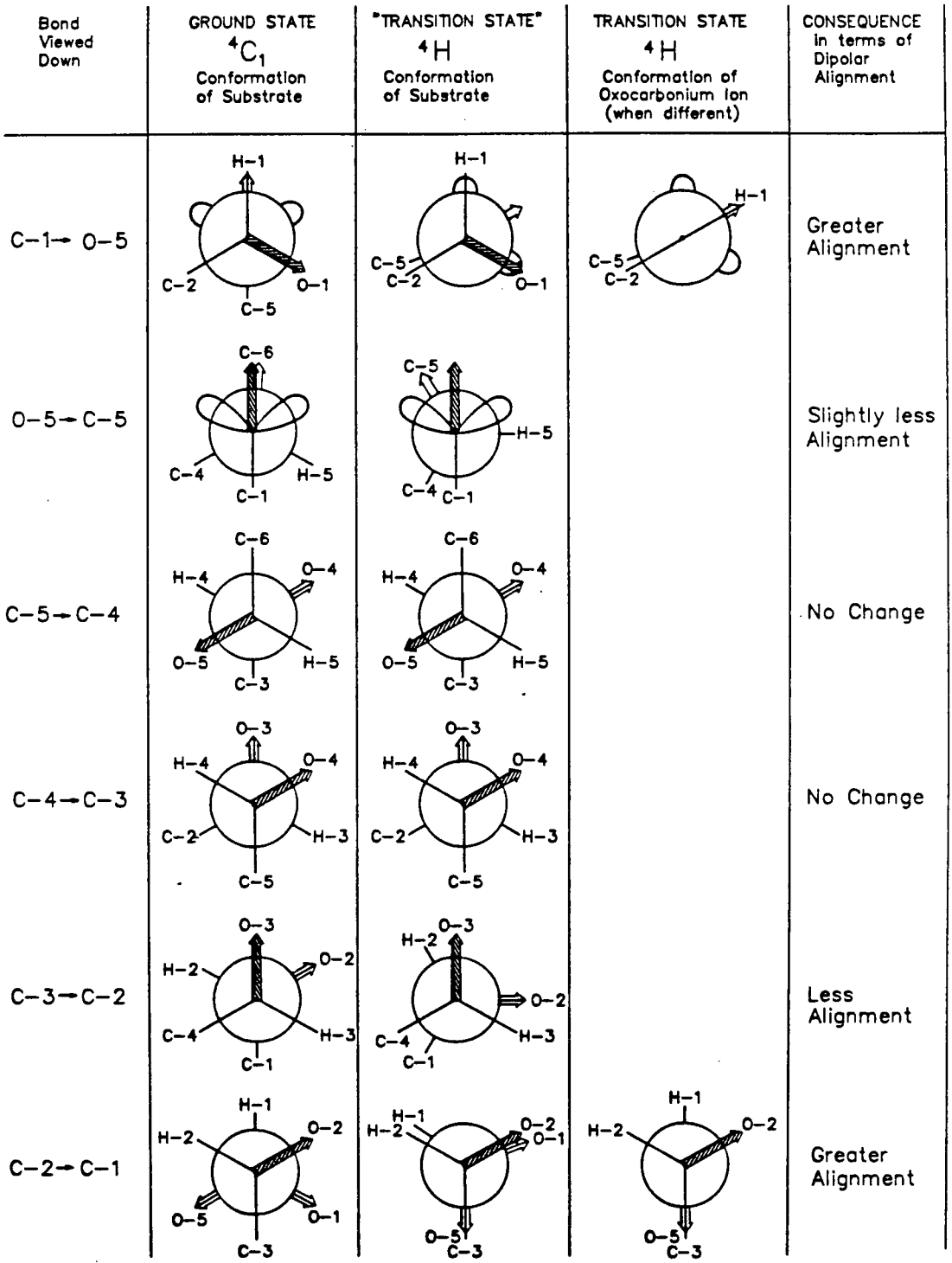


Figure 1-2. Relative dipole alignments in ground and transition states: From Withers et al, (1985).

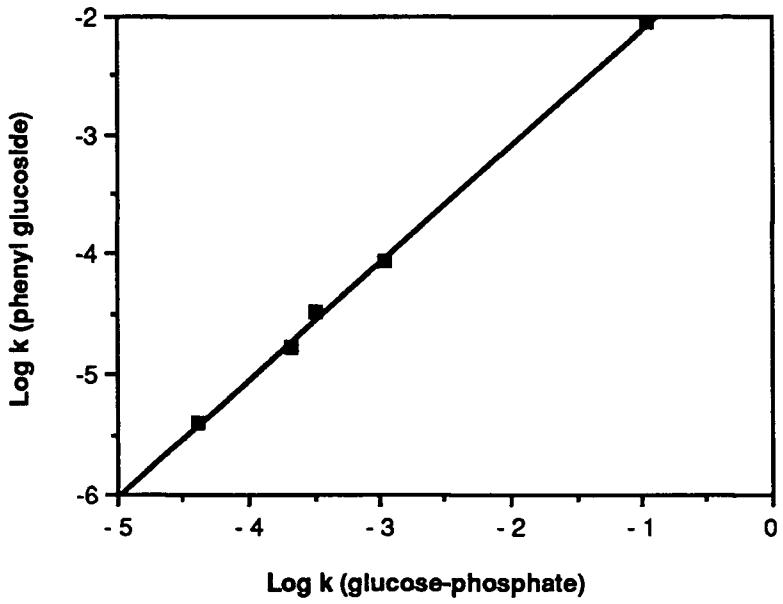


Figure 1-3. A linear free energy relationship between acid-catalysed hydrolysis of phenyl glucopyranosides and glucopyranosyl phosphates.

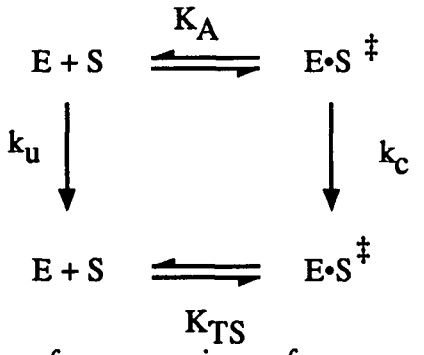
Data from Withers , S.G.; Percival, M.D. and Street, I.P. Carbohydr. Res. in press.

Binding Energy and Enzyme Catalysis.

The idea that the catalytic power of an enzyme is directly related to the stability of the enzymic transition state is now a well accepted principle. This basic idea was first advanced by Pauling (1946), but since has been elaborated by a great deal of theoretical and experimental work to the point where some authors consider that transition state stability is the only way in which the catalytic power of enzymes can be and should be described. This fundamentalist point of view is expressed by R.L.Schowen (1978)

> . . . that the entire and sole source of catalytic power is the stabilization of the transition state; that reactant-state interactions are by nature inhibitory and only waste catalytic power.

The merits of this point of view can be seen by considering the free energy changes which accompany the following processes:



Scheme 1-7. A kinetic scheme for comparison of enzyme-catalysed and non-catalysed processes. K_A and K_{TS} are the binding constants respectively for the enzyme-substrate and enzyme-transition state complexes, k_u and k_c are the rate constants for the uncatalysed and enzyme-catalysed processes.

The enzyme catalysed process proceeds firstly through formation of an enzyme bound substrate complex and then to the enzyme-bound transition state complex. The uncatalysed reaction proceeds through the free transition state. The following free energy profile can be constructed for these processes.

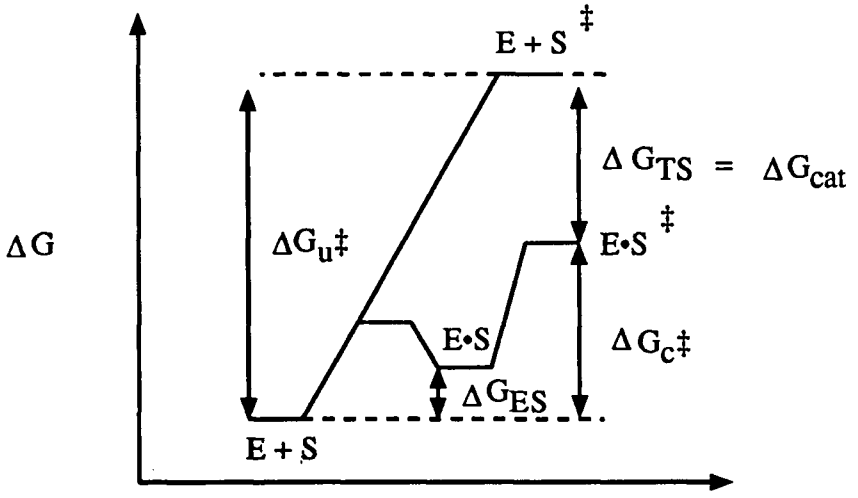


Figure 1-4. A comparison of free energy changes for non-catalysed and catalysed reactions.

The quantity ΔG_{cat} is defined as the catalytic free energy which is a positive number which will become larger as the enzyme is more effective as a catalyst

$$\Delta G_{cat} = \Delta G_u^{\ddagger} - \Delta G_c^{\ddagger}.$$

For the case shown in Figure 1-4 where only a small portion of the enzyme is occupied as

the enzyme substrate complex, ΔG_{cat} is equal to the free energy realized upon binding the transition state to the enzyme (ΔG_{TS}). Thus the entire rate enhancement of the enzyme-catalysed process can be directly attributed to the stability of the transition state gained upon binding to the enzyme.

That the enzyme achieves this crucial stabilization by providing structural complementarity to the transition state (rather than the ground-state) can be shown by considering the free energy profiles in Figure 1-5 which are derived for the following simple enzyme-catalysed process.

$$E + S \underset{}{\overset{K_d}{\rightleftharpoons}} E{\bullet}S \xrightarrow{K_{cat}} E + P$$

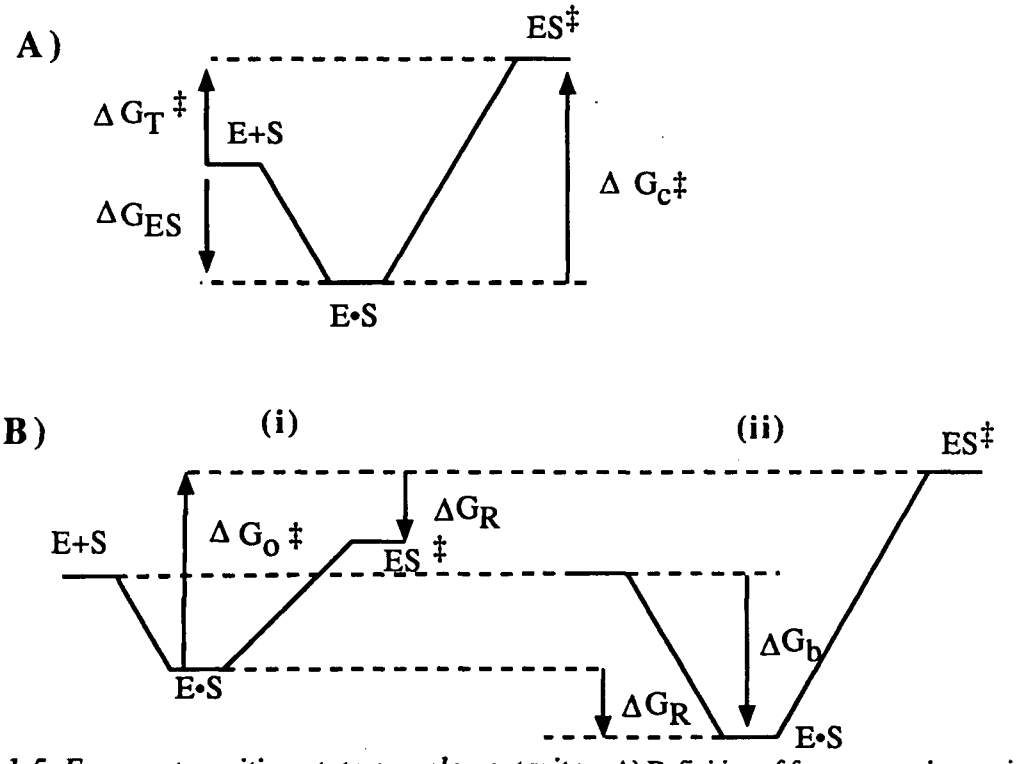


Figure 1-5. Enzyme-transition state complementarity. A) Definition of free energy changes in terms of the commonly measured parameters of enzymology. $\Delta G_T^{\ddagger}$, k_{cat} / K_m; ΔG_{ES}, $1/ K_d$; $\Delta G_c^{\ddagger}$, k_{cat}. B) Enzyme structure is complementary to (i) Transition state, the full binding energy (ΔG_b) is expressed at the transition state lowering the activation energy for this step by ΔG_R. (ii) Enzyme structure is complementary to the ground-state substrate; the full binding energy , ΔG_b is expressed at the ground state and the activation energy for the catalytic step is increased by ΔG_R. ΔG_R is the intrinsic binding energy.

From Figure 1-5A the free energies of the various species can be related to the relevant rate and equilibrium constants from the following relationships:

$$\Delta G_c^{\ddagger} = RT \ln (\mathbf{k}\, T / h) - RT \ln (k_{cat})$$

$$\Delta G_{ES} = RT \ln (K_d)$$

$$\Delta G_T^{\ddagger} = RT \ln (\mathbf{k}\, T / h) - RT \ln (k_{cat} / K_d)$$

and $$\Delta G_T^{\ddagger} = \Delta G_c^{\ddagger} + \Delta G_{ES} \qquad (1)$$

Where $\Delta G_T^{\ddagger}$ and $\Delta G_c^{\ddagger}$are unfavorable and algebraically positive and ΔG_{ES} is favourable and algebraically negative.

Enzyme structure complementary to the ground state of the substrate: in this case the maximum binding energy is realized on binding of the substrate, therefore any changes which occur on going to the transition state can only result in a decrease in binding energy. For this case:

$$\Delta G_c^{\ddagger} = \Delta G_o^{\ddagger} + \Delta G_R \qquad (2)$$

and $$\Delta G_{ES} = \Delta G_b \qquad (3)$$

The Gibbs free energy of activation for k_{cat} / K_m is given by equation **1**, substituting from **2** and **3**:

$$\Delta G_T^{\ddagger} = \Delta G_o^{\ddagger} + \Delta G_R + \Delta G_b \qquad (4)$$

Enzyme Complementarity to Transition State: here the full binding energy (ΔG_b) is realized at the transition state. There will be an adverse term (ΔG_R) in the initial formation of the enzyme-substrate complex which will increase K_d but the gain in binding energy as the reaction reaches the transition state will increase k_{cat}. Thus:

$$\Delta G_c^{\ddagger} = \Delta G_o^{\ddagger} - \Delta G_R \qquad (5)$$

and $$\Delta G_{ES} = \Delta G_b + \Delta G_R \qquad (6)$$

Substituting terms from **5** and **6** into equation **1:**

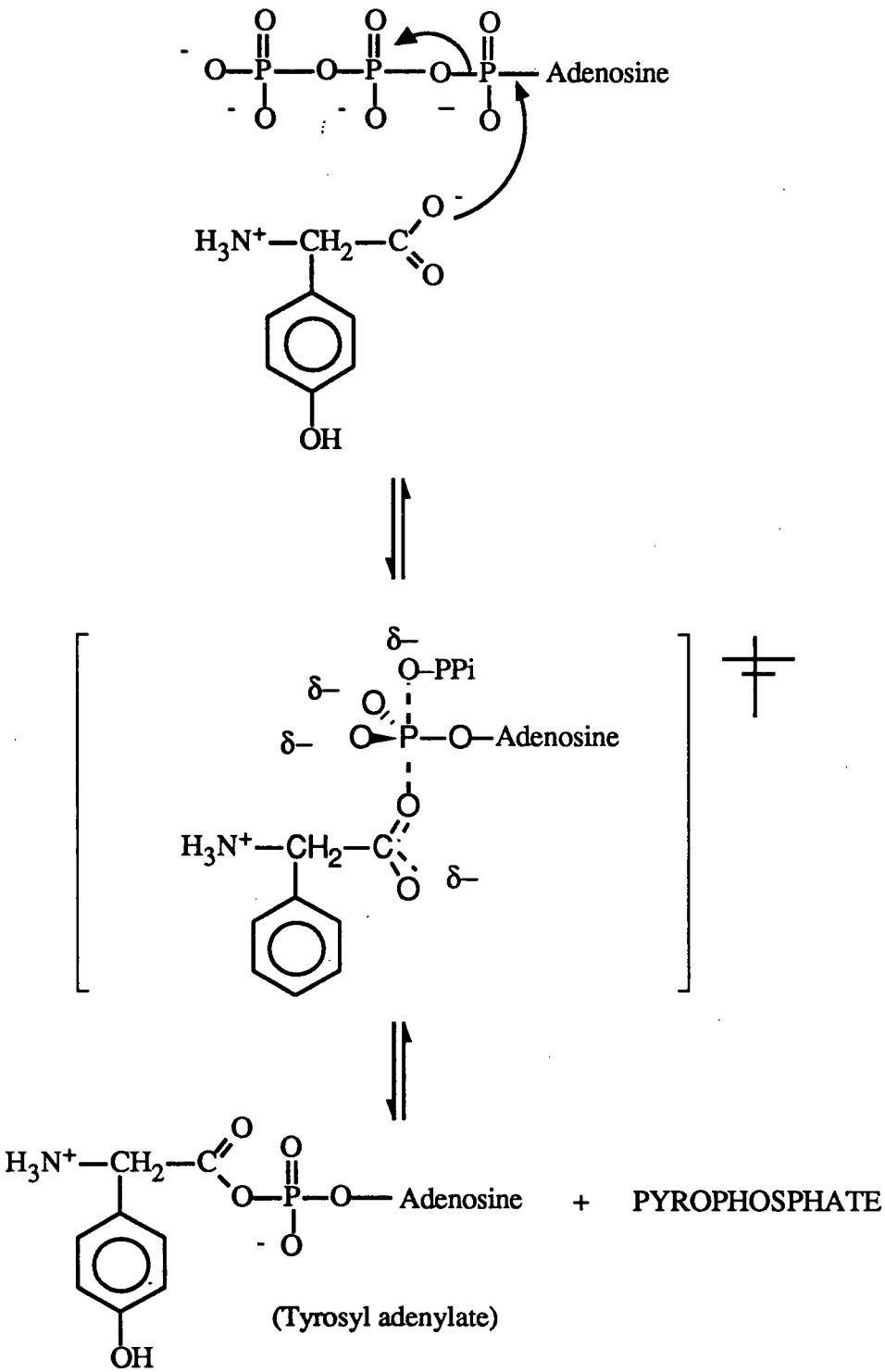
$$\Delta G_T^{\ddagger} = \Delta G_o^{\ddagger} + \Delta G_b \qquad (7)$$

The term ΔG_R cancels out and a comparison of equations **4** and **7** shows that k_{cat} / K_m is higher for the enzyme being complementary to the transition state rather than the initial structure of the substrate by a factor of exp ($- \Delta G_R / RT$).

Thus, by binding the ground state of the substrate only as tightly as is necessary for physiological function, and stabilizing the transition state of the catalytic step by providing tight and specific interactions, the enzyme has evolved a mechanism whereby binding energy can be converted to a rate enhancement for a chemical reaction. As mentioned these ideas were first postulated approximately 40 years ago, but it is only relatively recently that this hypothesis has gained experimental support. A recent series of papers from A.R.Fersht and his colleagues have very elegantly demonstrated the validity of these ideas. For recent reviews see Fersht, 1987; Fersht et al., 1986a; Fersht et al., 1986b.

The system that they have studied is tyrosyl tRNA synthetase, an enzyme which *in vivo* catalyses the very specific attachment of tyrosine residues to ribosomal bound tRNA. (see Scheme 1-8). Kinetically the enzyme is extremely well characterized and conforms to a ping pong kinetic mechanism, the product of the first half reaction being tyrosyl adenylate which remains tightly bound to the enzyme. Using a combination of steady state and pre-steady state kinetics the rate and equilibrium constants for each individual step can be determined. Structurally this enzyme is also well characterized, with the crystal structure at medium resolution being available (Blow and Brick, 1985).

Using the technique of site directed mutagenesis, Fersht and his group have prepared a series of mutant enzymes in which specific substrate-protein interactions (as identified from the X-ray crystallographic structure) have been deleted. After a thorough kinetic investigation of each enzyme mutant, they are able to prepare free energy profiles for the enzymic reaction and by comparison with the wild type enzyme deduce the importance of each interaction as the catalytic reaction progresses.



TYROSYL ADENYLATE + tRNA ⇌ AMINOACYL-TYROSYL-tRNA

Scheme 1-8. The reaction catalysed by tyrosyl tRNA synthetase.

They found that the enzyme used a complex network of hydrogen bonds and steric interactions to attain specificity in the ground state enzyme-substrate complex, but as the reaction progressed through the transition state these interactions became more numerous and stronger. This was particularly true of residues adjacent to the reaction centre, and it was suggested that the enzyme had maximized these interactions to stabilize the pentacoordinate geometry of the transition state for the phosphoryl transfer step. Interactions which occurred between enzyme and substrate at positions remote from the reaction centre (with the ribose ring of ATP) were also found to contribute to some extent to the stabilization of the transition state. These binding effects were found to be so important to catalysis that Fersht has proposed that they are the only significant way in which the enzyme exerts its catalytic effect. This is very likely to be the case for this particular enzyme as no other specific groups (acid catalysts, nucleophiles etc.) which could assist in catalysis have been identified.

β-Glucosidase from *Alcaligenes faecalis*.

The wild type of the enzyme used in the following chapter (Results and Discussion) is a β-glucosidase obtained from *Alcaligenes faecalis* originally isolated by Han and Srinivasan (1968) and later re-investigated by Day and Withers (1986). This β-glucosidase was later cloned into an *E. coli* expression vector (Wakarchuck et al., 1986) and this clone (pABG5 β-glucosidase) was used for the following experimental work. The wild type β-glucosidase was extensively characterized by Day and Withers (1986) and found to have a monomer molecular weight of approximately 50,000 Daltons. The active form of this enzyme was also found to be a dimer. This enzyme readily hydrolysed a wide variety of different substrates including β-glucosides, β-mannosides, β-galactosides, cellobiose, β-glucosyl fluoride and thio-glucosides (see Table 1-IV). Wakarchuck et al., (1986) found that the cloned enzyme was identical to wild type in amino acid composition and showed cross reactivity to antibodies which had been raised against the wild type. In addition we have carried out a

kinetic characterization of the clone with a number of the substrates shown in Table 1-IV and found it to be essentially indistinguishable from the data presented here.

Table 1-IV. Substrate profile for wild type A. faecalis β-glucosidase [a].

Substrate	K_m (mM)	V_{max} (μmol. min^{-1} mg^{-1})
βGlcPNP	0.083 ± 0.006	31.6 ± 0.48
βGalPNP	2.9 ± 0.5	39.2 ± 2.1
βManPNP	0.02 ± 0.002	0.024 ± 0.0005
βGlcDNP	0.031± 0.002	35.1± 0.9
βGlc pyridinium	4.9 ± 1.2	0.54 ± 0.9
βGlc azide	3.40 ± 0.33	1.55 ± 0.6
βGlcF	3.7 ± 0.2	41.1 ± 1.2
Cellobiose	0.70 ± 0.06	30.3 ± 1.1
Cellotriose	0.39 ± 0.014	25.6 ± 0.3
Cellotetraose	0.33 ± 0.0012	23.8 ± 0.2
Cellopentaose	0.32 ± 0.008	21.3 ± 0.2
Lactose	73.2 ± 6.0	23.6 ±1.5
Sucrose	3.82 ± 0.80	4.71 ± 0.42
Salicin	0.38 ± 0.025	10.4 ±0.21
Gentiobiose	3.85 ± 0.68	2.24 ± 0.18

[a] Data from Day and Withers (1986).

Table 1-V. Inhibitors of A. faecalis β-glucosidase [a].

Inhibitor	K_i (mM)	Inhibition Type
β–Cellobioside PNP	0.029	Competitive
β-glucosylamine	0.4	Competitive
Glucono-1,5-lactone	0.0017	Competitive

[a] Data from Day and Withers (1986).

Our Strategy for Development of Novel Mechanism-Based Glycosidase Inhibitors.

From the foregoing discussion it can be seen that there is a great deal of evidence from kinetic experiments to support the intermediacy of a glycosylated-enzyme in the reaction pathway. However, at the outset of this project such a catalytically competent intermediate had never been isolated in a sufficiently stable form to allow its characterization.

Our strategy for development of a mechanism-based inhibitor which would allow us to accomplish this aim is based on a key feature of the catalytic mechanism; both formation and decomposition of the intermediate proceeds through electron deficient transition states Introduction of electron withdrawing groups adjacent to the reaction centre would likely destabilize an already electron deficient transition state. For glycosidases, both glycosylation and deglycosylation processes pass through similar transition states, therefore introduction of an electronegative substituent at C-2 would be expected to reduce the rate of both of these steps producing a very slow substrate. Incorporation of a highly reactive leaving group into this deactivated substrate might then increase the rate of glycosylation sufficiently to allow trapping of the 2-deoxy-2-fluoroglucosyl intermediate and inhibit the enzyme in a temporary covalent fashion. In addition the fluorine-substituent would provide an active site probe which would allow spectroscopic characterization of the trapped intermediate.

The following chapter of this thesis describes some initial studies on the synthesis of 2-deoxy-2-fluoro glucosyl fluorides from partially protected derivatives of mannose. The testing of a number of different 2-deoxy-2-fluoro glucosides and glycosyl fluorides as inhibitors of pABG5 β-glucosidase is also described along with a series of kinetic experiments which were used to determine their specificity and mechanism of action. The results from the kinetic experiments were corroborated by experiments using ^{19}F-NMR the results from which allowed the determination of the stereochemical course of the inhibition mechanism.

CHAPTER 2.

Mechanism-Based Glucosidase Inhibitors
Results and Discussion

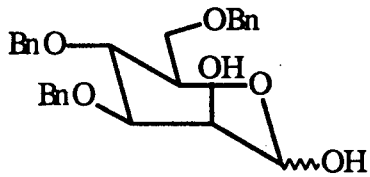
Synthesis of 2-Deoxy-2-Fluoro-D-Glucosyl Fluorides.

Considerable effort has been expended in the synthesis of fluorinated sugars particularly of 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose (Penglis, 1981). One reason for this has been the widespread use of ^{18}F-labelled carbohydrates as probes for studying energy metabolism in the brain by positron emission tomography (Wolf, 1981). In addition fluorinated carbohydrates have been suggested to have anti-tumor activity (Shatton et al., 1969) and have also been used as probes of the active sites of enzymes and other carbohydrate binding proteins.

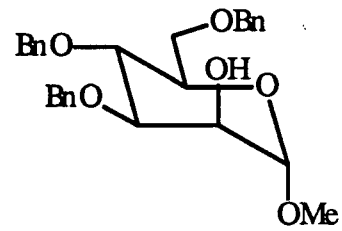
Several different approaches to the synthesis of 2-deoxy-2-fluoro carbohydrates have been adopted, and these fall into the two categories of addition to glycals and of nucleophilic displacements (or epoxide ring opening) using fluoride. A particularly promising approach has been the use of diethylaminosulfur trifluoride (DAST) to convert a hydroxyl group to a fluorine with inversion of stereochemistry (Sharma and Korytnyk, 1977; Somawardhana and Brunngraber, 1983; Middleton, 1975; Tewson and Welch, 1978; Card, 1983). In connection with our project to develop 2-deoxy-2-fluoro glycosyl fluorides as potential glycosidase inhibitors we embarked upon a study of the use of DAST to effect fluorinations of 3,4,6-tri-O-benzyl-D-mannopyranose {tribenzylmannose} and methyl 3,4,6-tri-O-benzyl-α-D-manno-pyranoside {methyl tribenzylmannoside} to try to achieve a rapid and convenient preparation of these compounds.

Since characterization of these compounds relies to a considerable extent upon ^{19}F-NMR data of the fluorinated products it is pertinent to review some relevant information on this subject prior to discussion of the results. In general, a fluorines attached to the anomeric centre of a sugar will resonate well downfield (δ = 136 - 151 ppm) of fluorine attached elsewhere on the sugar ring. In addition, α-pyranosyl fluorides tend to resonate at lower field strengths than the corresponding β-anomer. Fluorines attached elsewhere on the sugar ring generally resonate in the range of δ195 to 205 ppm for fluorine attached to a secondary carbon while a fluorine attached to a primary centre will resonate well upfield at

approximately δ 220 ppm. Geminal couplings to fluorine tend to be large (≈ 50 Hz) and vicinal couplings range from 0 Hz up to ≈ 30 Hz in a trans diaxial relationship.



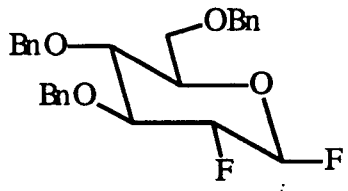
tribenzylmannose



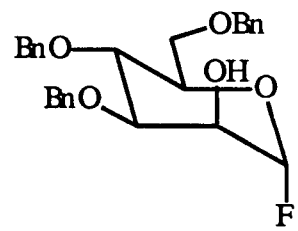
methyl tribenzylmannoside

Reaction of DAST with 3,4,6-tri-O-benzyl-D-mannopyranose.

Treatment of tribenzylmannose with three equivalents of DAST for 24 hours at room temperature results in the formation of two major products, which could be separated by "flash chromatography". The chromatographically more mobile fraction was identified as 3,4,6-tri-O-benzyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (30% isolated yield) while the slower product was shown to be 3,4,6-tri-O-benzyl-α-D-mannopyranosyl fluoride (18% isolated yield).



3,4,6-tri-O-benzyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride

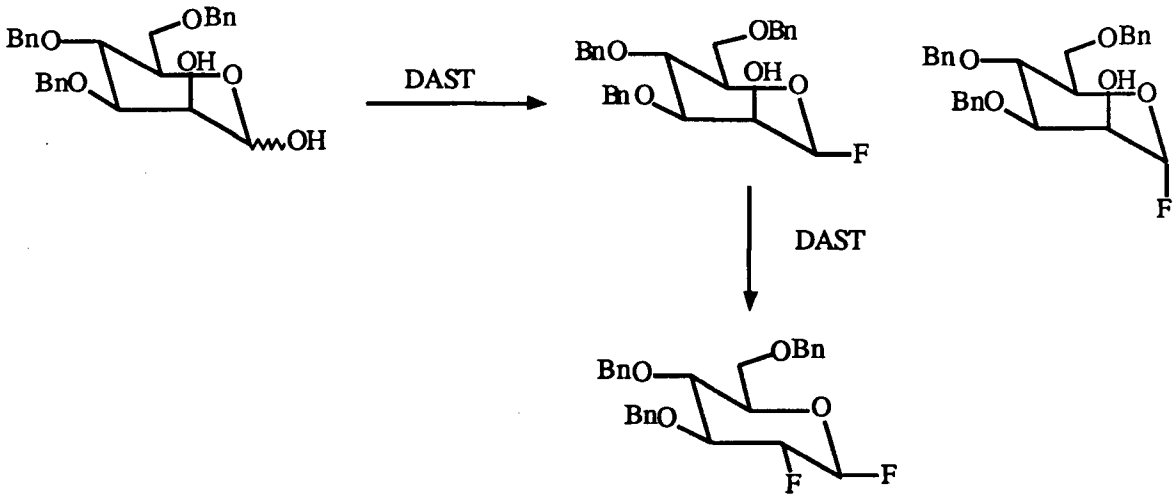


3,4,6-tri-O-benzyl-α-D-mannopyranosyl fluoride

Proof of structure for the former compound was obtained from mass spectral data (*m / z* = 454) and from both proton and ^{19}F-NMR spectroscopic data, since for example, two fluorine resonances were observed (δ = 141.05 and 197.78 ppm) consistent with a 2-deoxy-2-fluoro-β-D-hexopyranosyl fluoride. Further the fluorine resonance at C-2 showed only relatively small ^{1}H-^{19}F and ^{19}F-^{19}F couplings, other than the geminal coupling. All NMR data were very similar to those published for 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-

glucopyranosyl fluoride (Hall et al., 1971). Additional proof was obtained by removal of the benzyl blocking groups via catalytic hydrogenolysis with a palladium catalyst to yield the free sugar. This compound was found to be identical to an authentic sample of 2-deoxy-2-fluoro β-D-glucosyl fluoride prepared by an alternative route (Hall et al., 1971) as judged from thin layer chromatography and NMR data. No difluorinated product possessing the α-configuration was observed. Only one resonance was observed in the ^{19}F-NMR spectrum (δ = 140.98 ppm) of the second compound, showing it to be a glycosyl fluoride, probably α-linked. The proton NMR data suggested it to be a mannosyl fluoride, since no large coupling was observed between H-2 and F-1 and between H-2 and H-3 as would be expected for a glucosyl fluoride. Further, the ^{19}F-NMR chemical shift corresponded very closely to the reported value (δ = 138.8 ppm) for the compound 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl fluoride (Hall et al. 1969) and for α-D-mannopyranosyl fluoride itself (δ = 138.8 ppm). Thus the compound was identified as 3,4,6-tri-O-benzyl-α-D-mannopyranosyl fluoride.

This mixture of products suggests that the initial reaction involves the formation of an anomeric mixture of mannopyranosyl fluorides of which the β-anomer only can then undergo a second, much slower, reaction at C-2 to produce a difluorinated product (Scheme 2-1).



Scheme 2-1. Reaction of DAST with tribenzylmannose.

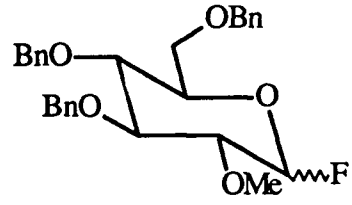
Indeed, studies (Rosenbrook et al., 1985; Posner et al., 1984) have shown that DAST is a very efficient reagent for synthesis of the appropriately protected glycosyl fluorides. It has also been noted by several authors (Miljokovic et al., 1974, Franks and Montgomery, 1968) that displacement reactions at C-2 of mannopyranosyl derivatives with a charged nucleophile such as fluoride or azide, occur much more readily on sugars of the β-configuration than those of the α-configuration. This is exemplified by the reaction of DAST with benzyl 3,4,6-tri-O-benzyl-β-D-mannopyranoside (Dessinges et al., 1984), which gave the desired displacement product in 80% yield with a reaction time of only 5 minutes. Similar success has been achieved in effecting displacement of trifluoromethanesulphonate from C-2 of β-mannopyranosides using cesium fluoride (T. Haradahira et al., 1985) and tris-(diethylamino)sulphonium difluorotrimethyl silicate (Szarek et al., 1985). In contrast, an attempt to displace trifluoromethanesulphonate from C-2 of methyl α-mannopyranoside using azide gave only a 30% yield of the desired 2-azido-2-deoxy-α-glucosyl derivative (Vos et al., 1984). In this case the major product was a result of a competing elimination reaction.

The reluctance of α-mannosides to undergo displacement at C-2 has been attributed to a variety of factors. These include the high electron density around the required path of the incoming nucleophile due to the lone pair present on the α-anomeric substituent and secondly, unfavorable dipolar interactions that occur in the transition state between the axial anomeric substituent and the approaching nucleophile. Given the highly polar nature of the carbon-fluorine bond , it would be expected that the presence of an axial fluorine substituent at the anomeric centre would increase the magnitude of this latter effect. However, since the electron density associated with the fluorine would be held very closely to the nucleus relative to the case for oxygen, it might be expected on this basis that such displacements would occur more readily for glycosyl fluorides than for α-linked O-glycosides. Since all attempts to effect a displacement at C-2 of the tribenzylmannosyl fluoride by DAST were unsuccessful, as were attempts to force the reaction of tribenzylmannose towards a greater

yield of the difluorinated sugar by extending the reaction time or heating, it must be concluded that the dipolar argument is probably the more reasonable in this case.

Reaction of DAST with Methyl Tribenzylmannoside.

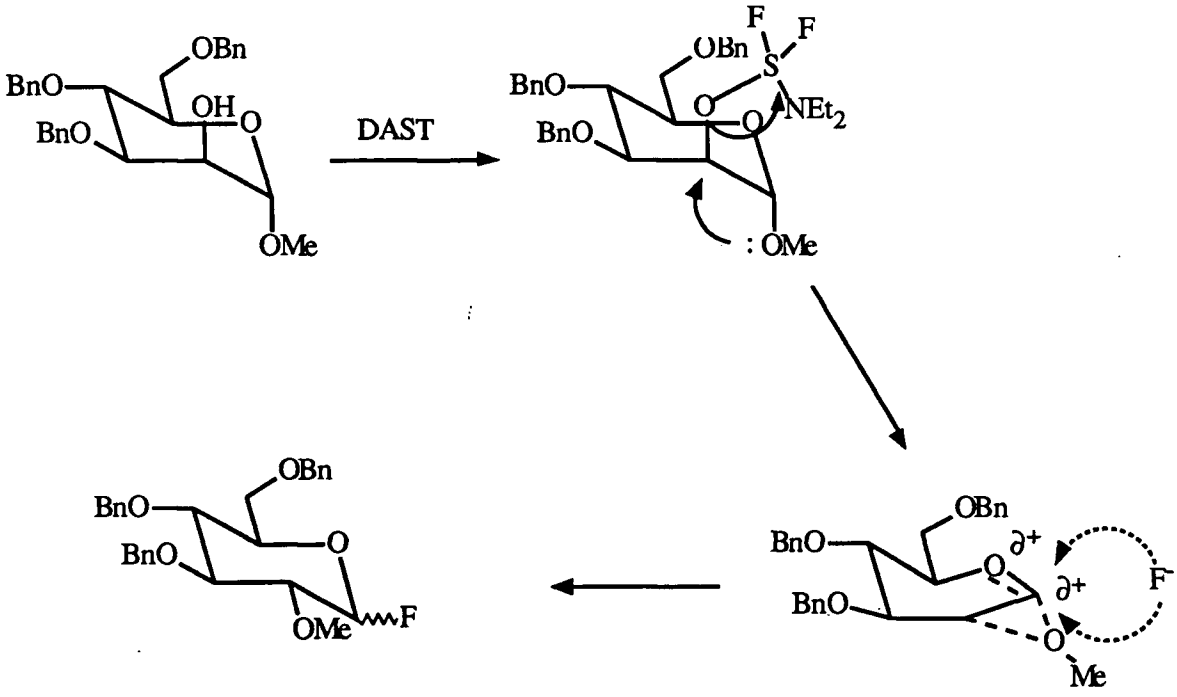
In an effort to see if DAST could effect a displacement from C-2 of an α-mannopyranoside, methyl tribenzylmannoside was treated with three equivalents of DAST in dichloromethane at reflux (≈ 40 - 45°C) for 90 minutes. Instead of the expected displacement products, a 46% yield of a 1:1 anomeric mixture of 3,4,6-tri-O-benzyl-2-O-methyl-D-glucopyranosyl fluorides was obtained.



3,4,6-tri-O-benzyl-2-O-methyl-D-glucopyranosyl fluorides

These were identified by a number of techniques as follows. The data are consistent with a fluorine at C-1 rather than C-2 since ^{19}F-NMR resonances appear well downfield in a region characteristic of glycosyl fluorides. A *gluco* rather than a *manno* configuration was assigned, since in the case of the α-fluoride a large coupling (25.61 Hz) is observed between H-2 and F-1, a magnitude which is only observed from *trans*-diaxially related substituents, particularly at C-2. Similarly, for the β-anomer a large H-1, H-2 coupling of 6.7 Hz is observed which is again consistent only with a *trans*-diaxial arrangement of the two protons. Additionally, a long-range coupling (1.1 Hz) over 5 bonds between fluorine and the protons of the 2-O-methyl substituent is observed in the β-anomer. A similar proton-fluorine coupling is observed in the two 3,4,6-tri-O-acetyl-2-O-methyl-D-glucosyl fluorides (Hall et al., 1969). Mass spectral data are also consistent with these structures since the parent ion mass (*m* / *z* =466) is identical for the two compounds isolated, and in agreement with the proposed structure. Moreover a major peak (parent - 20) due to loss of hydrogen fluoride is observed,

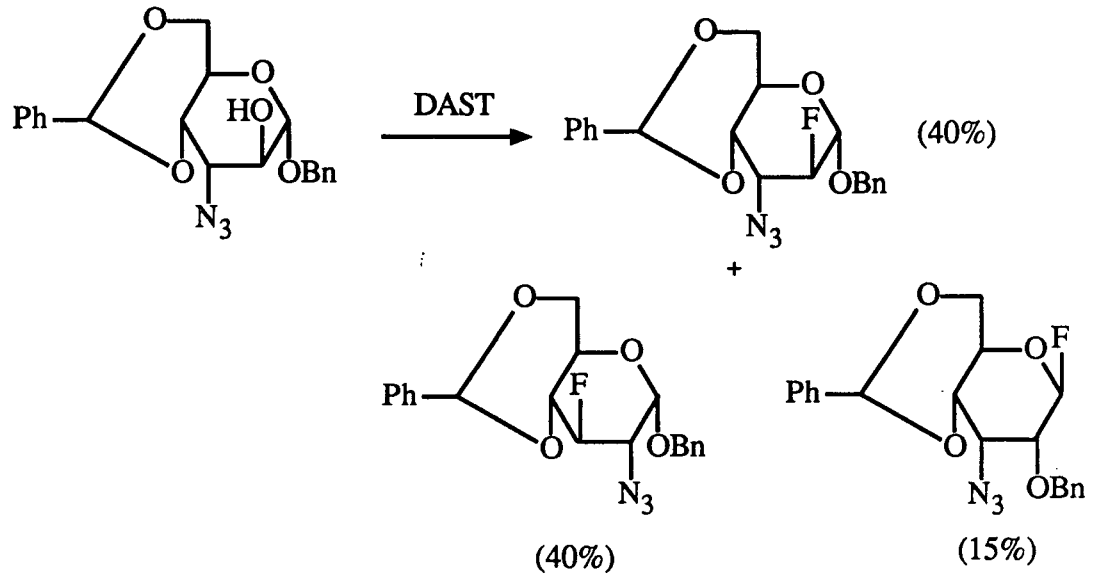
consistent with the ready loss of fluoride from the anomeric centre. Further, acid catalysed hydrolysis of the product produces a single compound which no longer contains any fluorine, as evidenced by the lack of fluorine-proton couplings in the proton NMR. Such behaviour would be expected for a glycosyl fluoride but not for a 2-deoxy-2-fluoro sugar. Thus rather than effecting a displacement reaction at C-2, a methyl ether migration has occurred with the concomitant or subsequent attack of fluoride at the anomeric centre. A likely mechanism for this would involve the initial formation of the sulfoxo derivative at the axial hydroxyl group (see Scheme 2-2). Rather than suffer displacement by free fluoride ion, this highly reactive leaving group is instead displaced by intramolecular attack of the glycosidic oxygen atom to produce a 2-O-methyl glycosyl oxocarbonium ion intermediate.



Scheme 2-2. Reaction of DAST with methyl tribenzylmannoside.

This intermediate then undergoes attack by fluoride at the anomeric centre, apparently with equal ease from either face to produce the 1:1 mixture of glucosyl fluorides. It is not therefore a thermodynamically controlled reaction. A mechanism in which the sulfoxy derivative at C-2 provides a concerted intramolecular delivery of fluoride to the anomeric centre would not

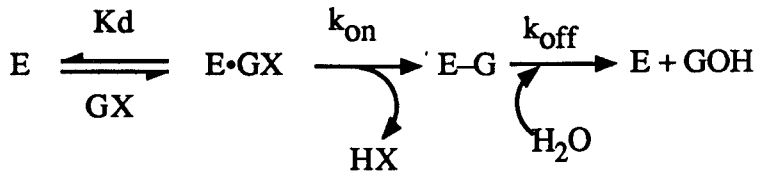
appear to be likely in this case since this would result in exclusive formation of the β-fluoride. Migrations of this type are quite rare in fluorination attempts using DAST, usually elimination is the major competing reaction. However, a further example of this type of behaviour has appeared in the literature, in this case both the migration of a benzyl ether and a neighbouring azido-substituent were observed during the synthesis of 2'-C-fluoro-β-daunomycin (Castillion et al., 1985).



Scheme 2-3. Functional group migrations encountered in the synthesis of 2'-C-fluoro daunomycin (Castillion et al., 1985).

Inhibition of β-Glucosidase by 2-Deoxy-2-Fluoro Glycosides.

The general reaction mechanism which has been proposed for retaining glycosidases is depicted in Scheme 1-3. From this mechanism it is possible to derive a rate expression as follows. Assuming that only the chemical steps are kinetically important, then the mechanism becomes that of a two step hydrolase.



Scheme 2-4. A kinetic model for a two step hydrolase. Where E is the enzyme, GX is the glycoside, XH is the aglycone, E•GX is the non covalent Michaelis complex, E-G is the covalent glycosyl-enzyme intermediate and GOH is glycose. The kinetic constants are: K_d the dissociation constant for the E•GX complex and k_{on} and k_{off} are respectively the first order rate constants for glycosylation and deglycosylation.

Assuming that the concentration of enzyme is negligible relative to the concentration of added substrate and assuming a rapid equilibrium formation of E•GX then:

$$[E\bullet GX] = \frac{[E]\,[GX]}{K_d}$$

For slow substrates like 2-deoxy-2-fluoro glycosides this assumption should be particularly valid, as the rate of covalent bond formation may be many orders of magnitude slower than for its non-fluorinated counterpart. Therefore under these conditions it is probable that the dissociation rate of the non covalent enzyme-substrate complex is much greater than the rate of covalent bond formation.

Assuming that a steady state concentration of the E-G complex is attained during the reaction:

$$\frac{d\,[E\text{-}G]}{dt} = k_{on}\,[E\bullet GX] - k_{off}\,[E\text{-}G] = 0$$

$$k_{on}[E\bullet GX] = k_{off}\,[E\text{-}G]$$

$$\frac{k_{off}}{k_{on}}[E\bullet GX] = [E\text{-}G].$$

Substituting for [E•GX]

$$[E\text{-}G] = \frac{k_{on}[E][GX]}{k_{off} K_d}$$

at steady state the fraction of the total enzyme (E_o) occupied by the E-G complex will be given by:

$$\frac{[E\text{-}G]}{[E_o]} = \frac{[E\text{-}G]}{[E] + [E\bullet GX] + [E\text{-}G]}$$

substituting for [E•GX], [E-G] and dividing by [E]

$$\frac{[E\text{-}G]}{[E_o]} = \frac{\frac{k_{on}}{k_{off} K_d}[GX]}{1 + \frac{[GX]}{K_d} + \frac{k_{on}}{k_{off} K_d}[GX]}$$

$$= \frac{\frac{k_{on}}{k_{on} + k_{off}}[GX]}{\left(\frac{k_{off} K_d}{k_{off} + k_{on}}\right) + [GX]} \qquad (1).$$

The rate of production of GOH is then given by

$$\frac{d[GOH]}{dt} = k_{off}[E\text{-}G].$$

Therefore at steady state the rate of GOH production (v_{GOH}) / unit enzyme concentration (E_o) will be given by:

$$\frac{v_{GOH}}{[E_o]} = \frac{\frac{k_{on}\, k_{off}}{k_{off}+k_{on}}[GX]}{\left(\frac{k_{off} K_d}{k_{off}+k_{on}}\right) + [GX]} \qquad (2)$$

following the standard form of Michaelis-Menten equation $\frac{v_{GOH}}{[E_o]} = \frac{k_{cat}[GX]}{K_m + [GX]}$ then,

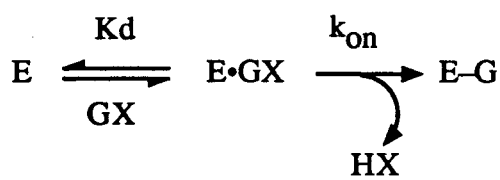
$K_m = \frac{k_{off} K_d}{k_{off}+k_{on}}$ and $k_{cat} = \frac{k_{on}\, k_{off}}{k_{on} + k_{off}}$.

The constant k_{cat} can be defined as the limiting rate constant for turnover of the enzyme. In this case it can be seen that at steady state the rate of aglycone production (v_X) will be given by the same expression. The derivation of this steady state rate expression shows how the observed rate of an enzyme-catalysed reaction can be defined in terms of the individual rate or equilibrium constants for each step. In the following sections of this chapter it will be shown how these individual rate constants can be determined experimentally, and how they relate to the mechanism of inhibiton of pABG5 β-glucosidase by 2-deoxy-2-fluoro glycosides.

Under the standard assay conditions employed in this work, enzyme was incubated with inhibitor and at appropriate time intervals aliquots were removed and diluted into a large excess of substrate. This essentially halts the inactivation both by providing a large dilution and high concentrations of a competing ligand. Under these conditions any inhibitor which is not covalently bound to the enzyme will be competed out of the enzyme's active site by the large excess of substrate, consequently making this fraction of the enzyme available for catalysis.

Using this method pABG5 β-glucosidase was tested for time dependent inactivation by 2FβGlcF and 2FβGlcDNP at pH 6.8 and 37°C. In both cases pre-incubation of the enzyme with these compounds led to an extremely rapid decrease in activity, the half life of inhibition at saturating concentrations of inhibitor was estimated 1.6 seconds for 2FβGlcDNP and slightly longer, 7 seconds, for 2FβGlcF.

These results are consistent with the kinetic model when the rate of deglycosylation is very slow compared with the rate of glycosylation and all of the enzyme accumulates rapidly as the E-G complex (see below).



Scheme 2-5. A kinetic model for the rapid accumulation of the E-G complex.

Under these conditions a number of testable experimental criteria can be applied to show that inhibition is occurring at the active site.

When $[GX] >> [E_o]$ that is $[GX] - [E_o] \approx [GX]$ the reaction kinetics will be pseudo first order with respect to enzyme:

$$\frac{d[E\text{-}G]}{dt} = k_{obs}\,[E_o] \qquad (6)$$

where $k_{obs} = \frac{k_{on}\,[GX]}{K_d + [GX]}$. It can also be seen from this expression that at saturating concentrations of inhibitor ($K_d + [GX] \approx [GX]$) then $k_{obs} = k_{on}$.

The disappearance of E (decrease in activity) with time can be related to the total enzyme concentration (initial activity) by

$$[E] = [E_o] \exp(-k_{obs}\,t).$$

For both of these inhibitors time course residual activity could be fitted to a single exponential using a computer-assisted least squares regression analysis, or from a semi logarithmic plot of residual activity versus time. Values for the kinetic constants k_{on} and K_d could be determined by extrapolating the rate data to saturating concentrations of inhibitor using a computer aided fitting procedure based on the statistical method of Wilkinson (1961). This program fits the data to the non-linear form of the standard Michaelis-Menten equation. Alternatively a plot of reciprocal k_{obs} versus reciprocal glycoside concentration, could be used. In this case the X-intercept yields $-K_d^{-1}$ and the Y intercept gives k_{on}^{-1}. Because of the hazards associated with curve fitting to this type of plot (Wilkinson, 1961; Johansen and Lumry, 1961; Matyska and Kovar, 1985) kinetic constants and the errors associated with them are determined from the computer aided fitting procedures. Graphical representation of the data is provided for visual inspection only and the lines presented on these graphs are determined using a simple linear least squares regression analysis. The results obtained in this manner for pABG5 β-glucosidase with 2FβGlcF and 2FβGlcDNP are presented in Figure 2-1 and 2-2 and Table 2-I.

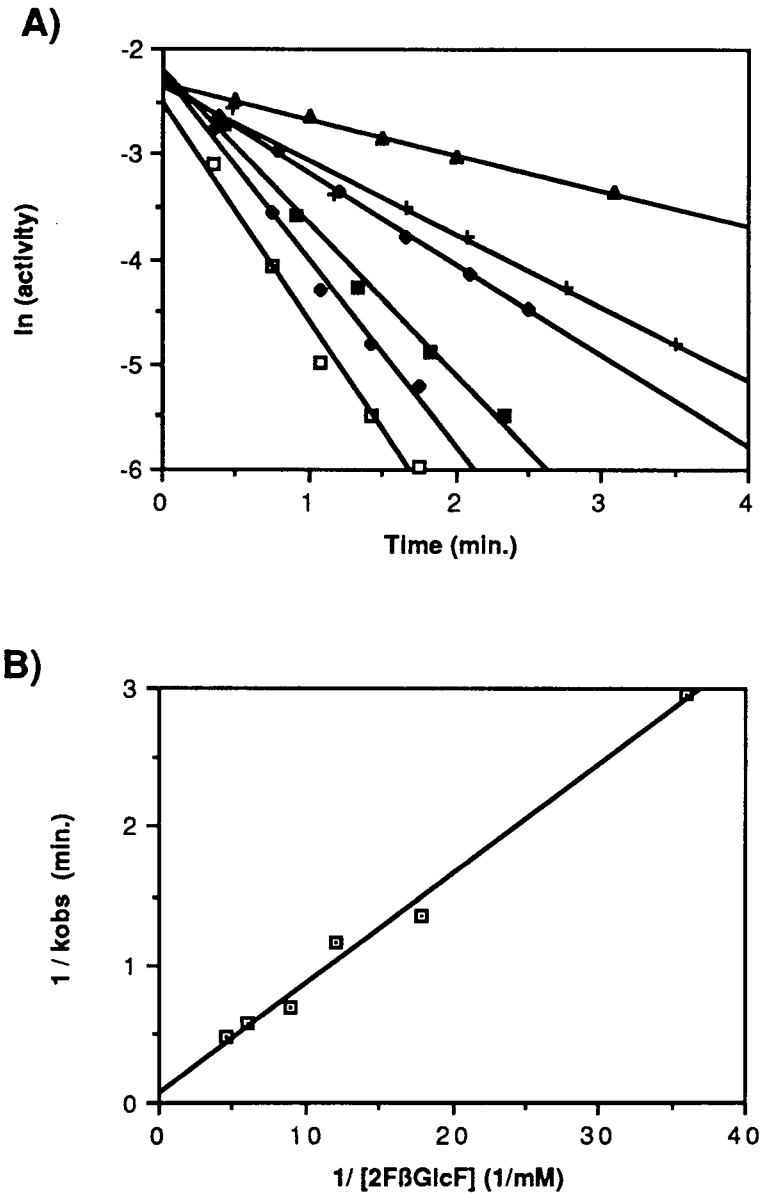


Figure 2-1. Inhibition of pABG5 β-glucosidase by 2FβGlcF.

A) A semilogarithmic plot of activity versus time at 37°C, pH 6.8 in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. The following inhibitor concentrations were used (mM): ❑ 0.22,◆ 0.167, ■ 0.11, ◊ 0.083, + 0.056,▲ 0.029. B) A double reciprocal plot of the following values obtained for k_{obs} at their respective inhibitor concentration 2.078, 1.763, 1.441, 0.866, 0.736 and 0.337 min^{-1}. These values were obtained from a non-linear least squares regression analysis.

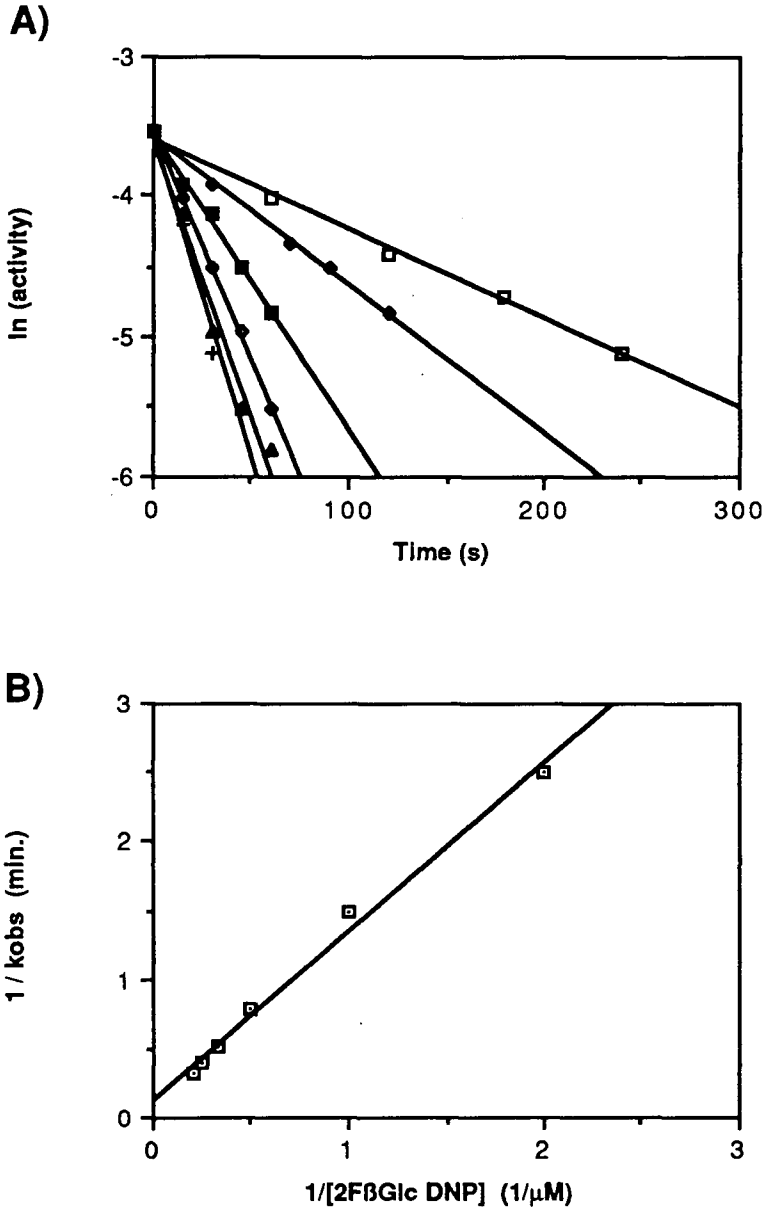
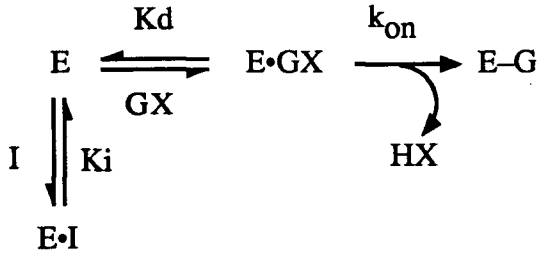


Figure 2-2. Inhibition of pABG5 β-glucosidase by 2FβGlcDNP.

A) A semilogarithmic plot of activity versus time at 37°C, pH 6.8 in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. The following inhibitor concentrations were used (μM):❑ 0.5,◆ 1.0, ■ 1.9, ◊ 3.04, ▲ 4.0,+ 5.0. B) A double reciprocal plot o f the following values obtained for k_{obs} at their respective inhibitor concentration 0.40, 0.66, 1.27, 1.96, 2.50, 3.06 min^{-1}. These values were obtained from a non-linear least squares regression analysis.

Inhibition with 2FβGlcDNP was so rapid that measurements could not be made at concentrations approaching saturation, consequently the value given for K_d is imprecise. However it is quite similar to the K_m value of 30μM which had been measured previously for βGlcDNP (Day and Withers, 1986), suggesting that the fluorine at C-2 has a negligible effect on binding.

A second test for the active site–directed nature of the time dependent loss of activity is whether the presence of a competitive ligand diminishes the rate of inactivation at a given inhibitor concentration. A competitive ligand will bind specifically but reversibly only to the free enzyme (See below).



Scheme 2-6. A kinetic model for competitive inhibition. K_i = the dissociation constant of the enzyme-competitive inhibitor complex.

Intuitively it can be seen that while E is tied up as the E•I complex it is not able to bind GX. This has the effect of reducing the effective concentration of the E•GX complex which in turn will reduce the rate of irreversible inactivation. The effect of these competitive ligands is thus seen as a relative reduction in the pseudo first order rate constants for irreversible inhibition. However, because the competitive ligand will only bind reversibly to the enzyme, inhibition will eventually go to completion. Isopropylthio β-D-glucopyranoside was used as the competitive ligand.This thio-glucoside binds relatively well to pABG5 β-glucosidase (K_i = 4 mM) but is hydrolysed only slowly. The results of these "protection" experiments with pABG5 β-glucosidase, 2FβGlcF and 2FβGlcDNP are given in the form of a semi-logarithmic time course (Figure 2-3) for the irreversible inhibition reaction in the presence and absence of competitive ligand (βGlcS-iPr). The marked reduction in inhibition rate which

occurs in the presence of βGlcS-iPr provides strong evidence that irreversible inhibition is occurring only at the active site of the enzyme.

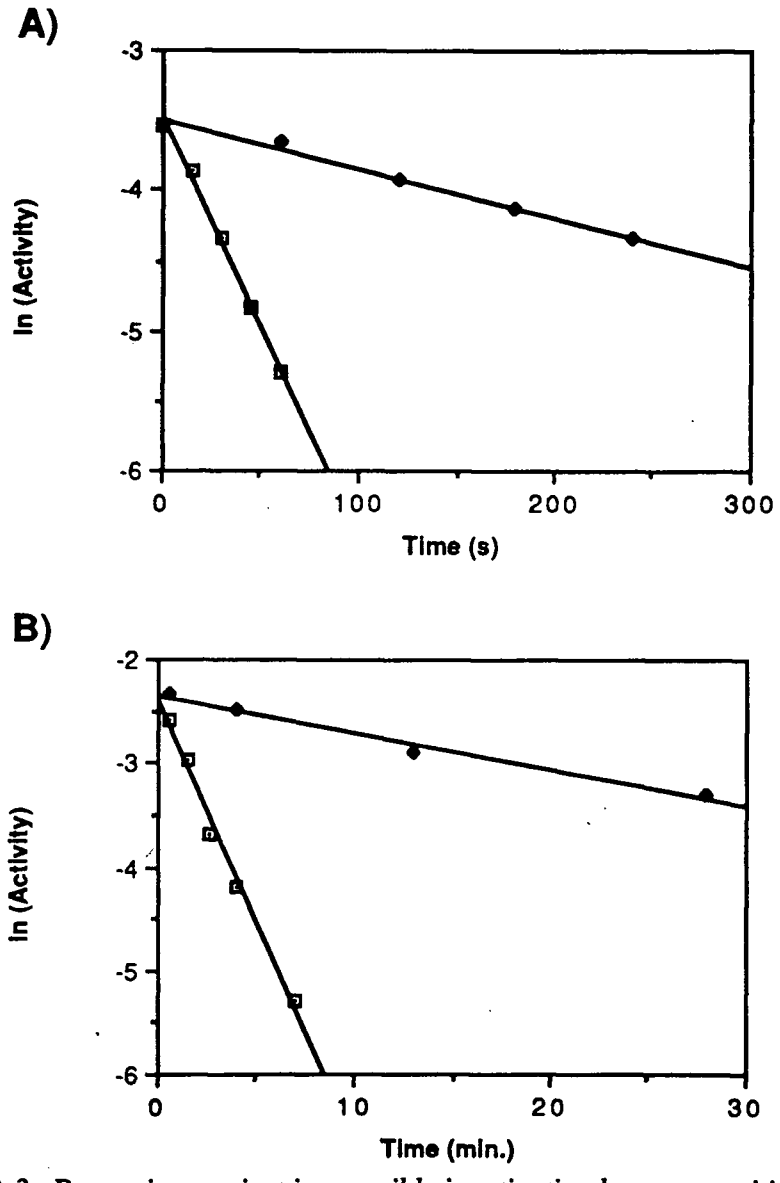


Figure 2-3 . Protection against irreversible inactivation by a competitive ligand.

Inactivation of pABG5 β-glucosidase at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. A) Inactivation at a single fixed concentration of 2FβGlcDNP (0.003 mM) with the following concentrations of βGlcS-iPr: ❑ 0 mM, (k_{obs} = 1.7 min^{-1}), ▲ 20 mM, (k_{obs} = 0.195 $min.^{-1}$). B) Inactivation at a single fixed concentration of 2FβGlcF (0.067 mM), with the following concentrations of βGlcS-iPr: ❑ 0 mM, (k_{obs} = 0.425 $min.^{-1}$); ▲, 20 mM, (k_{obs} = 0.035 $min.^{-1}$).

A further criterion can be applied to determine the specificity of the inhibitor in that the efficiency of the inhibitors should closely resemble the selectivity of the enzyme for its natural substrates. Previously, studies on pABG5 β-glucosidase (Day and Withers, 1986) have shown that this enzyme possesses β-mannosidase and β-galactosidase as well as β-glucosidase activity. The specificity of the enzyme was also found to be essentially absolute for β-glycosides, compounds such as αGlcPNP showing no detectable substrate activity. The same authors also indicated that the aglycone site possessed a marked affinity for aromatic groups. This conclusion was formed on the basis of K_m values, βGlcDNP (K_m = 30 μM) bound to the enzyme tighter than βGlcPNP (K_m = 80 μM), both of which possessed much greater affinity than βGlcF (K_m = 3.69 mM). The results of inactivation experiments with pABG5 β-glucosidase and a number of different 2-deoxy-2-fluoro glycosides are given in Table 2-I and in the form of double reciprocal plots for pseudo first order rate constants of inhibition by 2FβMan F and 2FβGal F are presented in Figures 2-4 and 2-5.

Table 2-I. Inhibition constants for inactivation of pABG5 β-glucosidase by 2-deoxy-2-fluoro-glycosides [a].

Inhibitor	K_d (mM)	k_{on} ($min.^{-1}$)	k_{on}/K_d ($min.^{-1}\ mM^{-1}$)
2FβGlcDNP	0.05	25	500
2FβGlcF	0.39 ± 0.12	5.9 ± 1.4	15.1
2FβGalF	2.58 ± 0.5	3.62 ± 0.49	1.40
2FβManF	1.29±0.12	5.6 ± 1.0	4.34
2FαGlcF	69 ± 7	0.93 ± 0.06	0.013

[a] All reactions conducted at 37°C, pH 6.8 in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA.

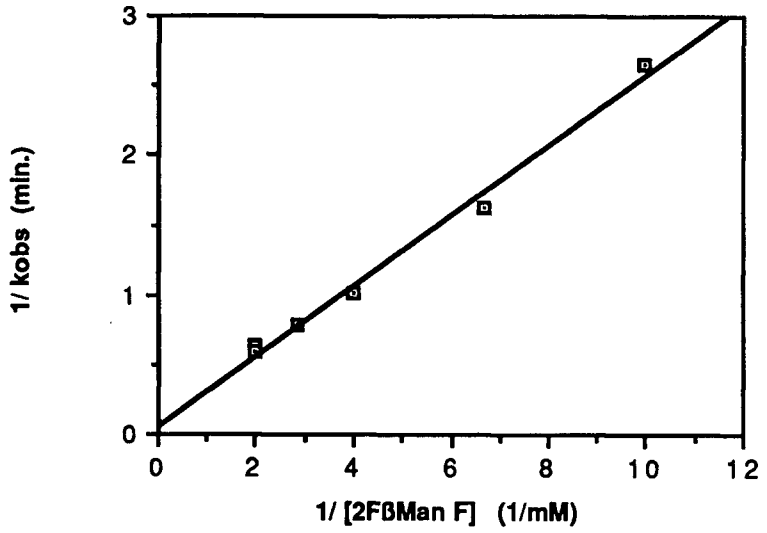


Figure 2-4. Inhibition of pABG5 β-glucosidase by 2FβManF.

Reaction conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. The following inhibitor concentration (mM) gave the values of k_{obs} ($min.^{-1}$) presented in parentheses: 0.1, (0.368); 0.15, (0.616); 0.25, (0.981); 0.35, (1.22); 2.0, (1.679).

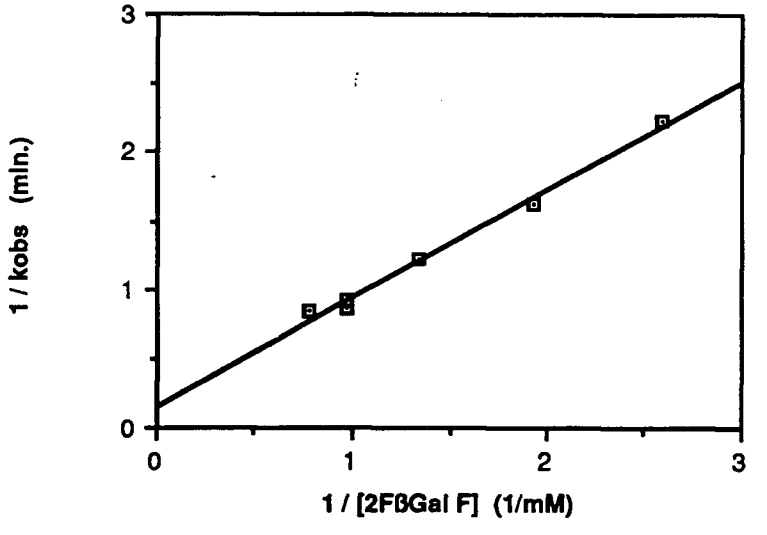


Figure 2-5. Inhibition of pABG5 β-glucosidase by 2FβGalF.

Reaction conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. Activity was monitored by dilution into 1 mL of 3.0 mM βGalPNP.The following inhibitor concentrations (mM) gave the values of k_{obs} ($min.^{-1}$) presented in parentheses: 1.29, (1.18); 1.03 (1.17); 0.52, (0.615); 0.77, (0.822); 0.39 (0.449).

The data in Table 2-I clearly reflects a number of these trends. The enzyme was inhibited efficiently by 2FβManF, 2FβGalF and 2FβGlcF, thus the enzyme appears to be exhibiting its characteristic specificity for the glycose portion of the inhibitor. The relative efficacies of inactivation (as best expressed by k_{on} / K_d) show that the enzyme is also very specific for anomeric configuration of the inhibitor. The α-fluoride inactivates some 1,000 fold less efficiently than the β-fluoride. The affinity of the aglycone site for aromatic groups can also be seen by comparison of dissociation constants for 2FβGlcF and 2FβGlcDNP, the dinitrophenyl glucoside binding some eight fold tighter than the glucosyl fluoride. Thus the relative effectiveness of these inhibitors appears to reflect the specificity of the enzyme for its normal substrates.

Turnover of the E-G Complex.

The inactivation process represents the first step of turnover of a very slow substrate. Thus when freed from excess inhibitor the inactivated glycosyl-enzyme should slowly undergo the second step and suffer hydrolysis liberating free enzyme. The presence of free and active enzyme could then be detected by reaction with a substrate under the appropriate assay conditions.

Samples of pABG5 β-glucosidase were inactivated with the various 2-deoxy-2-fluoro glycosides and then freed from excess inhibitor by gel filtration chromatography on a small column of Sephadex G-10 (19.5 cm x 1 cm). This separation was particularly easy for 2FGlc-enzyme prepared from 2FβGlcDNP, as both the 2,4-dinitrophenyl glucoside and the released phenol had abnormally long retention times on the column. However, for the glycosyl fluorides the difference between void and inclusion volumes of this column was sufficient to ensure complete separation of excess inhibitor and the enzyme-bound intermediate. For calculation of the first order rate constants for the reactivation process it was necessary to determine the activity of the fully reactivated sample. This was accomplished simply by calculating a dilution factor from the ratios of optical density (at 280 nm) of the

enzyme sample before and after chromatography and multiplying by the initial activity of the sample (before inhibition). Return of activity was monitored by dilution of small aliquots from this preparation into saturating concentrations of substrate. The results of these experiments are displayed in Table 2-II.

Table 2-II. Turnover constants for 2-deoxy-2-fluoro-glycosyl-pABG5 β-glucosidase [a].

Enzyme Intermediate	[b] k'_{off} ($min.^{-1}$)	[c] k_{cat} (inhibitor) ($min.^{-1}$)	[d] k_{cat} (substrate) ($min.^{-1}$)
Gluco	1.2×10^{-5}.	1.2×10^{-5}	7,272
Galacto	5.4×10^{-3}	5.4×10^{-3}	9,017
Manno	1.0×10^{-3}	1.0×10^{-3}	5.4

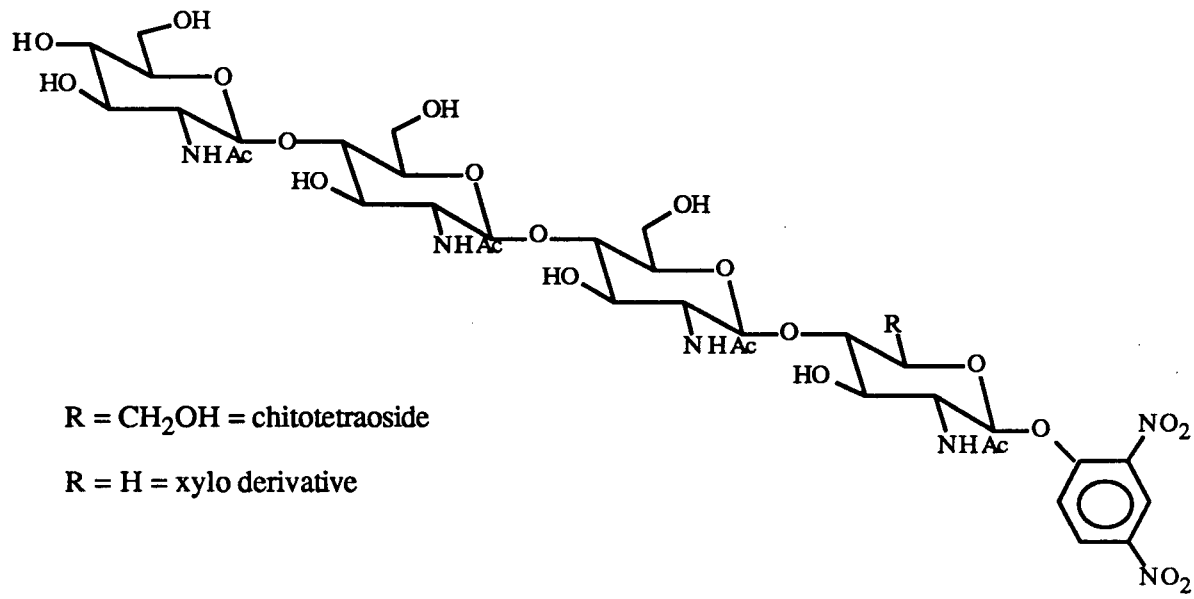
[a] All reactivation reactions were carried out at 37°C in 50 mM sodium phosphate buffer pH 6.8 containing 1mg mL^{-1} BSA. [b] $k'_{off} = k_{off}[H_2O]$. [c] $k_{cat} = \frac{k_{on}\, k'_{off}}{k_{on} + k'_{off}}$ see equation (2). [d] k_{cat} values are for the appropriate p-nitrophenyl β-glycoside. The value for βGlcPNP was determined experimentally at 37°C in 50 mM sodium phosphate buffer pH 6.8.

For the glycosylated enzymes, return of activity followed a single exponential time course and values for k'_{off} were calculated using a computer-assisted least squares non-linear regression analysis. For 2FGlc-pABG5 β-glucosidase reactivation was extremely slow, having a half life of approximately 500 hours. In this experiment careful controls to account for enzyme denaturation of both native and glycosylated enzyme under the assay conditions had to be run. After an initial loss of 15 - 20 % of the activity in the control over the first 24 hours, the residual activity remained stable for a further 5 days. The rate constant for hydrolysis of 2FGlc-enzyme was thus determined from readings taken after the first 24 hours. For both 2FMan- and 2FGal-enzymes, again hydrolysis rates were slow but considerably faster than for 2FGlc intermediate. Half lives for reactivation were calculated at 11.6 and 2.1 hours respectively.

A reasonable measure of the reduction in turnover rate caused by introduction of fluorine at the 2-position of the sugar ring, can be obtained by a comparison of k_{cat} values for

the 2-deoxy-2-fluoro glycosides (Table 2-II) with the k_{cat} value for the corresponding unsubstituted p-nitrophenyl glycoside. Ideally, a comparison of turnover rate should be made between non-fluorinated and fluorinated compounds which possess the same leaving group. Unfortunately, there is no steady state data available for βManF and βGalF therefore a direct comparison of this nature could not be made. However a comparison of k_{cat} values between fluorinated-glycosyl fluorides and the corresponding unsubstituted p-nitrophenyl β-glycosides will provide a reasonable estimate of the rate reduction caused by fluorination, since it can be seen from the data of Day and Withers (1986) that most of the effect of an aromatic aglycone is manifested as a reduction of K_m rather than increasing k_{cat}. Obviously the cost of fluorine substitution is highest for 2-deoxy-2-fluoro glucosides, turnover rate being reduced by over eight orders of magnitude. For the other glycosides, fluorine substitution at C-2 reduces turnover rate by over seven orders of magnitude for galactosides and four orders of magnitude for mannosides. The comparatively small rate reduction seen for turnover of the mannosyl inhibitors is a reflection of slow turnover rate for the non-fluorinated substrate rather than a comparatively rapid turnover of fluorinated inhibitor. The origins of these dramatic rate reductions seen upon fluorination of the sugar ring are probably two-fold: firstly, an electronic factor associated with destabilization of an oxocarbonium ion-like transition state. Secondly, a factor associated with poorer binding at the transition state. From discussion in Chapter 1 (Binding energy and enzyme catalysis), it can be seen that enzyme ligand inteactions are very important to the stability of the enzymic transition state.

There is now a considerable amount of experimental evidence which suggests that much of the catalytic effect of glycosidases arises from non-covalent interactions between the substrate and enzyme (Sinnott, 1987). Ballardie et al. (1977), have shown that while lysozyme catalyses the hydrolysis of 2,4-dinitrophenyl chitotetraoside, the xylo derivative which lacks a C-5 hydroxymethyl group (see below) exhibited no detectable substrate activity.



These authors were able to place a lower limit of 4.3 kcal mol.$^{-1}$ on the stabilization of the enzymic transition state due to interaction with the C-5 hydroxymethyl group. A number of deoxyglycoside derivatives have been tested as substrates with β-glucosidase A3 from *A. niger* (Roeser and Legler, 1981). They found that deletion of the C-2 hydroxyl group caused at least a 10^6 fold decrease in hydrolysis rate, whereas deletion of the C-4 hydroxyl or the C-5 hydroxymethyl groups caused smaller (approximately 10^4 fold) but still substantial rate decreases. An earlier study on the same enzyme had also shown that 6-deoxy glucosides were hydrolysed at approximately one tenth of the rate for the corresponding normal glucoside (Legler, 1967). Interactions with the C-2 hydroxyl group also accounted for a rate decrease of 10^4 fold in the hydrolysis of galactosides by *lac Z* β-galactosidase (Brockhaus et al., 1979).

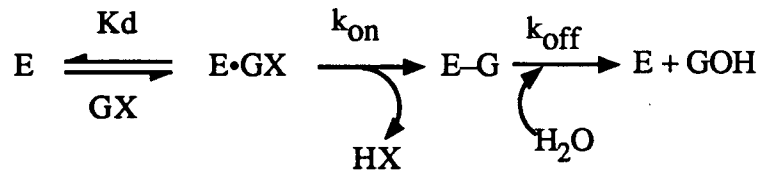
In concurrence with modern theories of enzyme catalysis most of the effect of deleting these substrate hydroxyl groups is shown as a large reduction in k_{cat} and not as an increased K_m. It is also worth noting that with respect to deletion of the C-2 hydroxyl group these rate factors are in fact a minimum estimate as removal of this electronegative group will introduce a favourable electronic effect . Based on the acid catalysed hydrolysis of phenyl glucosides another reaction which involves oxocarbonium ion-like transition states, this opposing effect could contribute as much as a 2000 fold rate increase (Mega and Matsushima, 1983).

From the data given in Table 2-I and 2-II again it can be surmised that most of the consequences of fluorination are observed as a decrease in k_{cat}. A comparison of K_m * for βGlcF (3.69 mM) taken from Day and Withers (1986) and K_d for 2FβGlcF (0.39 mM) shows that the inhibitor actually binds almost an order of magnitude tighter, but k_{cat} is reduced by many million fold. This apparently tighter binding of the difluorinated inhibitor is worthy of some note. Fersht et al (1985), have noted that an increase in binding affinity can occur upon deletion of a weak and possibly strained hydrogen bond. This was seen on mutation of threonine-51 to alanine-51 which caused deletion of a hydrogen bond in E-tyrosyl-ATP complex in tyrosyl tRNA synthetase.

If this latter example is pertinent to the increase in affinity observed here, then deletion of a relatively weak hydrogen bond in the ground state E•GX complex, but which is potentially much more important to stabilization of the transition state might explain some of the observed decrease in k_{cat}. There is also evidence for different hydrogen bonding interactions at C-2 of 2FβManF when compared with βManPNP. In the non-fluorinated glycosides βManPNP (K_m = 20 μM) binds with four fold greater affinity than βGlcPNP (80 μM) (Day and Withers, 1986), whereas the order is reversed in the fluorinated inhibitors suggesting that a hydrogen bonding interaction has been lost for the *manno* sugar.

The electronic effects of C-2 fluorination can largely be described in terms of destabilization of already electron deficient transition states (Figure 2-6). Substitution of fluorine has relatively little effect on binding, therefore the main effects of C-2 fluorination in decreasing the rate of turnover relative to the non-fluorinated substrates must derive from the increase in the height of the activation barriers of the glycosylation and deglycosylation steps ($\Delta G_g^{\ddagger}$ and $\Delta G_{off}^{\ddagger}$).

* A cautionary note about interpretation of K_m and K_d. It can be seen from the derivation of the kinetic expression for the two step hydrolase that $K_m \neq K_d$ when $k_{on} \approx k_{off}$. K_d thus represents an upper limit to the value of K_m.



FLUORINATED SUBSTRATES

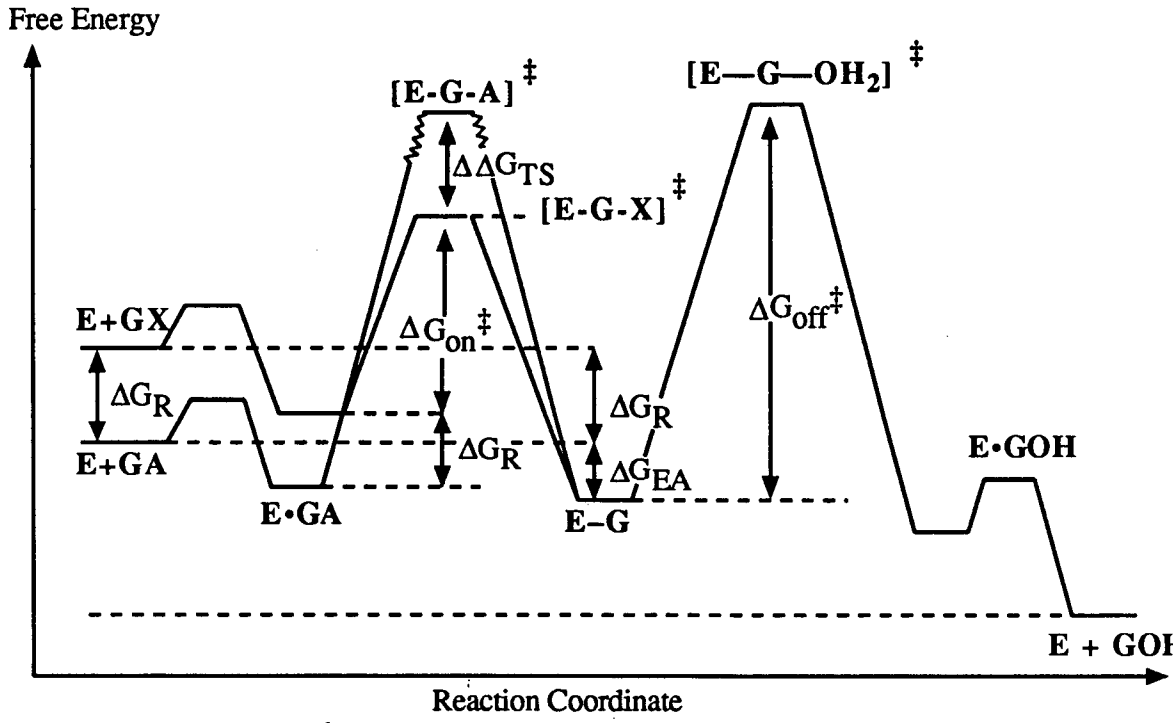


Figure 2-6. Hypothetical Gibbs free energy reaction profiles for β-glucosidase catalysed hydrolysis of fluorinated substrates.

The standard state chosen is that of saturating concentrations of substrate, 55.5 M water and a temperature of 37°C . Transition states are denoted by the symbol ‡. It is assumed that the rate of binding for all ligands to the enzyme is diffusion controlled and that both GX and GA show the same affinity for the enzyme, that is the free energy of binding is the same in both cases. Reaction profile for: GA, a 2-deoxy-2-fluoro-β-glucoside with a weakly activating aglycone; for this substrate deglycosylation may or may not be rate limiting. GX a 2-deoxy-2-fluoro β-glucoside with strongly activating aglycone showing rate limiting deglycosylation. The energies are defined as follows: ΔG_R = the difference in ground-state free energy of the β-glucosides GX and GA: $\Delta G_{on}^{\ddagger}$ = the free energy of activation for the glycosylation step from E•GX: $\Delta\Delta G_{TS}$ = the difference in free energies of the transition states formed from GA and GX: ΔG_{EA} = the free energy change for the formation of the glucosyl-enzyme from GA: $\Delta G_{off}^{\ddagger}$ = the activation free energy for the deglycosyation step.

However, if the substrate does not contain a reactive leaving group (GA) these changes alone may not be sufficient to trap a significant amount of the enzyme as the covalent complex. Increasing the leaving group ability of the aglycone has the effect of decreasing the activation energy for the glycosylation step by both destabilizing the ground state of the substrate and stabilizing the transition state. For GX the activation barrier of the glycosylation process from the non-covalent enzyme-substrate complex ($\Delta G_g^{\ddagger}$) is equal to $\Delta G_{on}^{\ddagger}$. For GA the activation barrier for the same process is given by:

$$\Delta G_g^{\ddagger} = \Delta G_{on}^{\ddagger} + \Delta G_R + \Delta\Delta G_{TS}$$

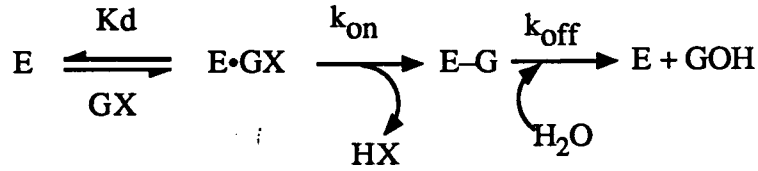
Thus the rate for the glycosylation step for the substrates GA and GX will differ by a factor of exp [-(ΔG_R+ $\Delta\Delta G_{TS}$) / RT]. It can also be seen from Figure 2-6 the free energy change on going from the free enzyme and the free substrate to the glycosyl intermediate is also more favourable for the glycoside GX, again by a factor determined by the magnitude of ΔG_R.

For both GA and GX, as the reaction passes through a common intermediate the energetics of the deglycosylation step are the same. Thus the effect of incorporating a reactive leaving group into the fluorinated substrate is of creating a thermodynamic pit in which the covalent intermediate becomes trapped.

Aglycone "Burst" and Active Site Titration.

For many purposes in enzymology it is often essential to determine accurately the enzyme concentration in an experiment. For preparations that are not totally homogeneous or not totally active this often presents a problem, as standard colorimetric or spectrophotometric techniques will overestimate the enzyme concentration under these circumstances. This problem has been overcome in some cases by the introduction of the technique of active site titration, whereby the concentration of the enzyme is related to an initial burst of product formation. This type of situation occurs when the second step is slow and an enzyme–bound intermediate accumulates during the reaction. The first mole of substrate will react rapidly to form 1 mole of product and 1 mole of enzyme bound intermediate, however subsequent

reaction then requires breakdown of the intermediate to release free enzyme. This can be seen readily from the original kinetic Scheme:



initially

$$\frac{d[X]}{dt} = k_{on}\,[E{\bullet}GX].$$

Once all of the free enzyme has accumulated as the E-G complex then the rate of production of X will be determined by k_{cat}

$$k_{cat} = \frac{k_{on}\,k_{off}}{k_{on} + k_{off}}.$$

In the case where $k_{on} >> k_{off}$ or where k_{off} tends towards zero the enzyme will rapidly accumulate as the E-G complex (rate dependent on k_{on}) and under these circumstances the amount of aglycone released will be equal to the amount of active sites in the sample. However, where k_{off} is not insignificant the initial exponential burst is followed by a slow linear increase in the quantity of aglycone released (Figure 2-7).

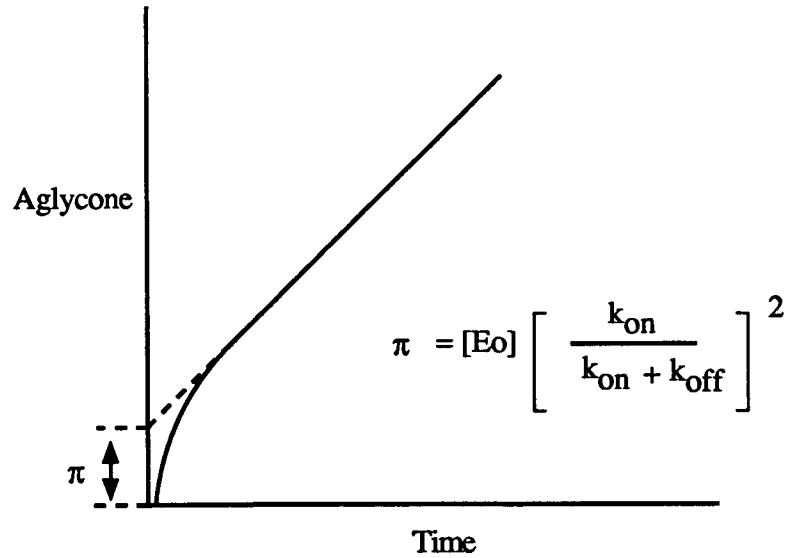


Figure 2-7. Active Site Titration.

Under these circumstances the enzyme concentration can be determined by extrapolation from the linear portion of the curve providing values are known for k_{on} and k_{off}. This latter situation has often been observed during the hydrolysis of ester substrates by a number of serine proteases (Hartley and Kilby 1954). Again these enzymes conform to the mechanism of a two step hydrolase and for hydrolysis of esters decomposition of the covalent acyl-enzyme complex is known to be rate limiting.

In a number of instances burst phase kinetics have been observed with glucosidases. This phenomenon was observed in magnesium free *lac Z* β-galctosidase at 4°C with the reactive substrate βGalDNP (Withers S.G., 1977 Ph. D. thesis University of Bristol). Initial attempts to observe a burst phase with β-glucosidase A (from sweet almonds) using βGlcPNP at a reaction temperature of 35°C were unsuccessful (Legler, 1975). However, using the same enzyme at low temperatures (-20°C in a cryosolvent of 50% aqueous DMSO) where turnover is essentially halted, Fink and Good (1974) were able to observe a pre-steady state burst which was proportional to enzyme concentration. These apparently contradictory results were later explained in terms a change in rate determining step which occurred at 24°C (Weber and Fink, 1980). A burst from the same enzyme-substrate pair was later observed at 20°C using rapid reaction techniques (Takahashi, 1975). A stable room temperature burst which lasted on the order of minutes has been seen with β-glucosidase A_3 from *A. wentii* using a modified substrate 4-methylumbelliferyl 2-deoxy-β-D-glucopyranoside (Roeser and Legler, 1981).

A burst effect should be readily observed during formation of 2FGlc-pABG5 β-glucosidase as in this case the rate of deglucosylation is truly negligible compared with the rate of glucosylation. The results of such an experiment with pABG5 β-glucosidase and 2FβGlcDNP are displayed in Figure 2-8. Enzyme concentration was determined from optical density readings at 280 nm using an absorptivity index $E^{0.1\%}$ 2.20 mL mg^{-1} cm^{-1} which in turn had been determined from a quantitative amino acid analysis. The amount of 2,4-

dinitrophenol released was determined at 400 nm using a molar extinction coefficient of 11,300 M^{-1} cm^{-1} (Day, A.G.(1985), M.Sc. Thesis, University of British Columbia).

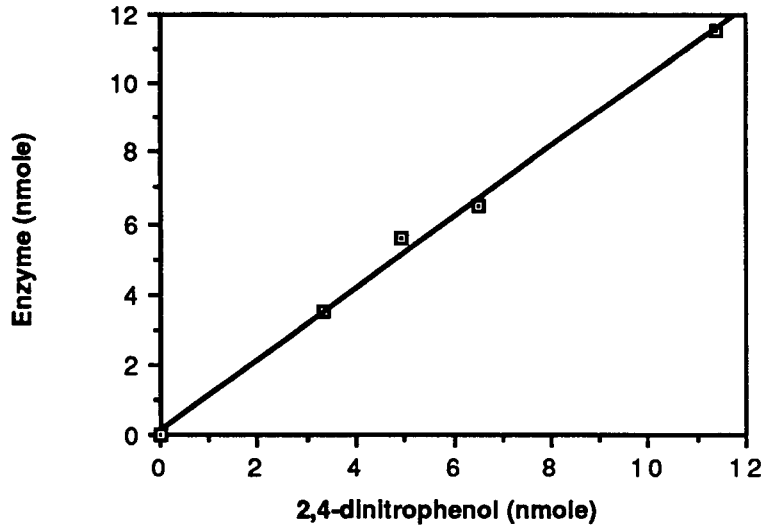


Figure 2-8. Stoichiometry of aglycone release from 2FβGlcDNP by pABG5 β-glucosidase.

Upon introduction of excess 2FβGlcDNP to the enzyme the optical density at 400 nm rapidly achieved a steady value which did not alter appreciably over the next hour. Addition of a further excess of 2-deoxy-2-fluoro-glucoside also did not alter this final value. A plot of enzyme concentration versus 2,4-dinitrophenol concentration (Figure 2-8) is linear (correlation coefficient R =1.00) giving a ratio of 1.0 mol. of dinitrophenol released per mol. of enzyme. Similarly 0.93±0.05 mol. of fluoride per mol. of enzyme were released when pABG5 β-glucosidase was inhibited with 2FβGlcF (K.Ruptiz personal communication). Fluoride concentration was determined using the colorimetric fluoride assay of Megregian (1978). This assay is based on the ability of fluoride to destroy the orange coloured zirconium eriochrome cyanide complex and is sensitive in the 0.5 to 1.0 μM range. The enzyme was prepared in a buffer containing 10 mM HEPES at pH 6.8 to avoid interference from the phosphate buffer system which is normally used. In addition careful blanks had to

be prepared to compensate for both the enzyme interference and breakdown of 2FβGlcF under the assay conditions, thus this number is probably somewhat less reliable.

Inhibition of pABG5 β-glucosidase by both of these compounds appears, within experimental error to involve a stoichiometry of 1 adduct per active site, providing further evidence for the specificity and mechanism–based nature of these inhibitors. In addition the 2,4-dinitrophenolate burst seen from 2FβGlcDNP provides a fast and accurate method of active site titration for this enzyme.

Transglycosylation as a Decomposition Route for the Glycosylated Enzyme Intermediate. Initially experiments aimed at looking for breakdown of the glycosylated-enzyme intermediate were performed by simply taking aliquots from an inhibition reaction through a large dilution into buffer containing a substrate. Under these conditions reactivation of pABG5 β-glucosidase intermediates was quite rapid, particularly for 2FGal-enzyme, but most notably relatively rapid reactivation from 2FGlc-enzyme was also seen. To further investigate this, 2FGlc-enzyme was prepared and freed from inhibitor by gel permeation chromatography. Aliquots of this preparation were incubated at 37°C in 50 mM sodium phosphate buffer at pH 6.8. Various ligands were also added to this mixture at a fixed concentration of 20 mM and the return of activity monitored as described before.The results are given in Table 2-III.

Reactivation appeared to be quite specific in its structural requirements of the ligand, as compounds which do not bear any resemblance to a sugar, such as p-nitrophenol and methanol failed to reactivate the enzyme. Sugars possessing an α-anomeric linkage also failed to cause any significant reactivation. These observations suggest that a binding site which is specific for sugar residue and anomeric linkage plays an important role in the reactivation process.

Table 2-III. Reactivation of 2FGlc-pABG5 β-glucosidase by various ligands [a].

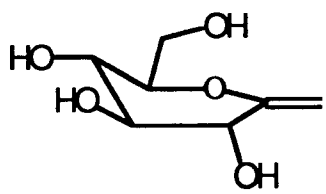
Ligand	k_{obs} ($min.^{-1}$)	[c] Relative Rate
βGlc PNP	0.027	1
βGlc S-iPr	0.0081	0.3
Salicin	0.026	0.96
Cellobiose	0.0006	0.02
1dβGlc ϕ	0.0013	0.05
αGlc PNP	N.R.	-
βGal PNP	N.R.	-
D-glucose	N.R.	-
Methanol (1M)	N.R.	-
p-nitrophenol	N.R.	-

[a] All reactions run at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. Ligands were added at a concentration of 20 mM, unless stated otherwise.[b] Relative rate compared with βGlcPNP. N.R no reactivation detected over a period of 120 minutes.

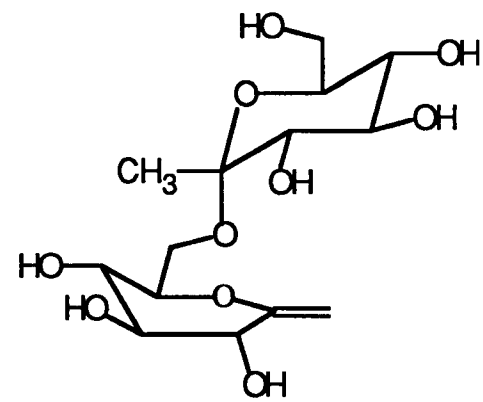
Based on this hypothesis the role of a regulatory site "or half of sites" reactivity might be proposed, however there is no evidence for allosteric behaviour from the normal steady state kinetics of this enzyme, and the observation of an aglycone burst which is stoichiometric with enzyme concentration effectively discounts the possibility of "half of sites reactivity". A second and more reasonable idea is that the enzyme is utilizing a transglycosylation reaction as a route for decomposition of the glycosylated-enzyme. That is, transfer of the 2-deoxy-2-fluoro glucosyl residue occurs onto a hydroxyl group of the incoming ligand to produce a disaccharide. There are many precedents for this type of behaviour in the literature. Gusakov et al. (1984) noted that small amounts of isocellotriose and getiobiose were formed alongside of the normal hydrolysis product when a β-glucosidase from *Aspergillus foetidus* was incubated with high concentrations of cellobiose. The disaccharide 1-deoxy-α-D-glucoheptulosyl-(2-7)-D-glucoheptulose was formed during the hydration of heptenitol by the purified α-glucosidase from *Candida tropicalis* and an

inverting exo-glucanase from *Arthrobacter globiformis* (Schlesselmann et al., 1982). It was also observed by the same authors that an α-glucosidase from rice formed both the (2-7) and the (2-5) linked heptulosyl disaccharides. Transglycosylation has also been observed during the hydrolysis of glycosyl fluorides by glucodextranase (Kitihata et al., 1981) and by β-xylosidase from *Bacillus pumulus* (Kasumi et al., 1987).

Examples of intramolecular transglycosylation have also been observed, the best example being the formation of allolactose (a β-1-6 linked disaccharide) during hydrolysis of lactose (β-1-4 linked) by *E. coli (lac Z)* β-galactosidase (Jobe and Bourgeois, 1972; Huber et al., 1976; Burstein et al., 1965). In this case the formation of allolactose has a physiological function in that it acts as an inducer for the *lac* operon (Burstein et al., 1965) and up to 50 % of the lactose hydrolysed can be converted to this intramolecular transgalactolysis product (Huber et al., 1976). The same enzyme also catalyses intermolecular transgalactolysis of a wide variety of other alcohols (Huber et al., 1984).



heptenitol



1-deoxy-α-D-glucoheptulosyl-(2-7)-D-glucoheptulose

There is evidence that transferase activity occurs in glycosidases largely because in many enzymes there is a second binding site situated adjacent to the normal active site, in which the glycosyl acceptor can bind. This provides a high local concentration of the acceptor molecule at the active site, which will then allow the transfer process to compete favourably

with the normal hydrolysis reaction. These multiple binding sites have been identified in the X-ray crystallographic structures of hen's egg white (Imoto et al., 1972) and T4 lysozyme (Anderson et al., 1981) and it seems entirely reasonable that in a class of enzymes which have evolved to handle oligomeric substrates that this will be a common structural feature. Recently, a model has been proposed for the active site cleft of pABG5 β-glucosidase (Day, A.G., 1985 M.Sc thesis, University of British Columbia). This model was constructed on the basis of the kinetics of hydrolysis for a series of cellooligosaccharides. The model suggested that the active site was composed of three separate binding sites into which a trisaccharide could fit (Figure 2-9A), hydrolysis occuring between the first and second residues situated in the two innermost subsites. Based on the relative affinities observed for glucose oligomers and the corresponding p-nitrophenyl glucosides (βGlcPNP, K_m = 80 μM and cellobiose K_m = 700 μM, p-nitrophenyl cellobioside, K_i = 29 μM and cellotriose K_m =387 μM) it was suggested that the two aglycone subsites possessed a high affinity for aromatic groups.

This model is also consistent with the reactivation data in Table 2-III as ligands which have an aromatic aglycone are much more efficient at promoting reactivation than ligands possessing a second carbohydrate moiety (compare βGlcPNP and Cellobiose). Transfer would thus occur as depicted in Figure 2-9B, the 2-deoxy-2-fluoro glycose residue would be covalently attached to the active site while the second ligand bound into the aglycone sites. The transglycosylation process would then proceed by nucleophilic attack of one of the sugar hydroxyl groups, presumably the one situated at C-4 onto C-1 of the intermediate. Transfer to the 4-position is also supported by the data, as a β-galactoside was observed to be a lot less efficient at promoting reactivation than the corresponding β-glucoside. Presumably this is due to the axial hydroxyl group at C-4 of the galactoside being poorly located to act as a nucleophile.

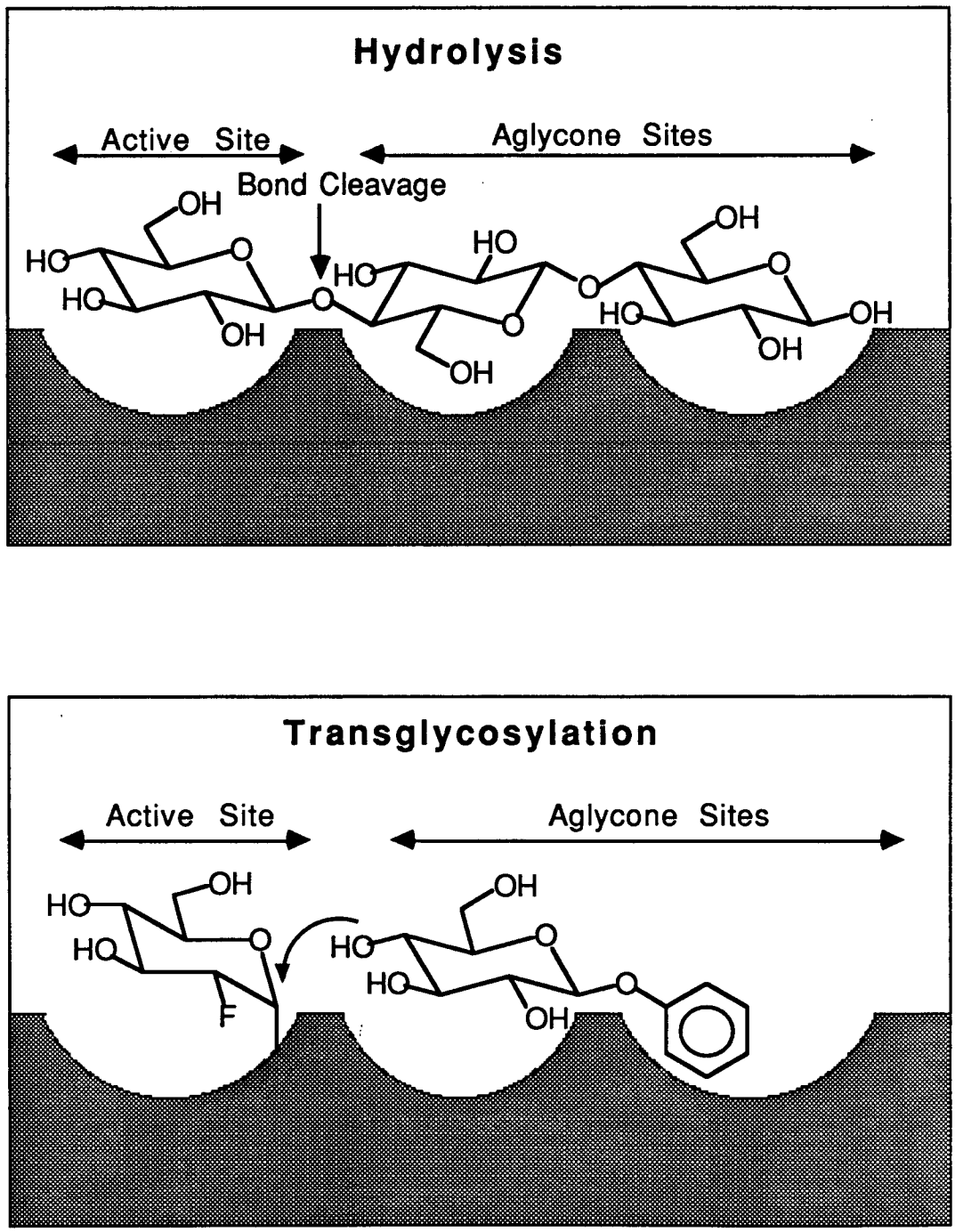
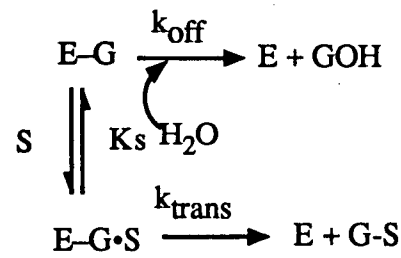


Figure 2-9. Comparative roles for the aglycone binding sites in hydrolysis and transglycosylation.

Turnover of the isolated 2-deoxy-2-fluoro glycosyl intermediate in the presence of a ligand is described by the following kinetic scheme.



Scheme 2-7. A kinetic model for transglycosylation of the isolated glycosyl-enzyme complex. Where E-G and k_{off} are as defined previously: E-G•S is the non covalent glucosylated-enzyme- ligand complex and k_{trans} is the rate constant for the transglycosylation process.

When all of the enzyme is initially present as the E-G complex, the rate of its decay will be given by:

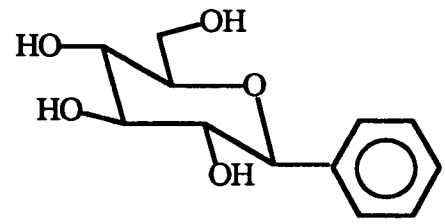
$$-\frac{d\,[E\text{-}G]}{dt} = (k'_{off} + k'_{trans})\,[E\text{-}G]$$

where $k'_{trans} = \frac{k_{trans}\,[S]}{K_s + [S]}$ and $k'_{off} = k_{off}\,[H_2O]$.

Again when the reaction is carried out with a large excess of S, the reaction will become pseudo–first order with respect to [E-G] and at saturating concentrations the observed first order rate constant (k_{obs}) will be equal to ($k'_{off} + k_{trans}$). Under these circumstances the increase in activity (increase in free enzyme) with time can be related to the full activity of the sample by

$$[E] = [E_o]\,(1 - \exp\,[-k_{obs}\,t]\,).$$

Initial attempts to quantify the transfer process were carried out using βGlcPNP as the reactivation substrate, however the situation was complicated by rapid hydrolysis of the substrate caused by the reactivated enzyme. To circumvent this problem a carbon linked glycoside 1-deoxy-β-D-glucosyl benzene (1dβGlcϕ) was used.



1-deoxy-β-D-glucosyl benzene

This compound is a reasonable competitive inhibitor of pABG5 β-glucosidase, binding to the active site with a dissociation constant of 3.4 mM. However the data in Table 2-III would suggest that it is some 50 fold less efficient at promoting reactivation than βGlcPNP. This is probably because 1dβGlc ϕ is not isosteric with a normal oxygen linked glycoside and this will presumably affect its binding to the transglycosylation sites.

Isolated glycosylated-pABG5 β-glucosidase was incubated in 50 mM sodium phosphate buffer at pH 6.8 in the presence of 1dβGlcϕ. Return of activity followed a single exponential time course (Figure 2-10A), the rate constant of which was dependent on the concentration of 1dβGlcϕ present in the reaction. Values for K_s and (k_{off} + k_{trans}) were obtained by extrapolating to saturating concentrations of 1dβGlcϕ using the normal computer fitting procedure. Replots of reciprocal 1dβGlcϕ concentration versus reciprocal rate constant are given in Figure 2-8B. The necessary end point activity (full activity) used in calculation of the first order rate constants was estimated for samples of 2FGlc- and 2FMan-enzymes from ratios of the optical density at 280 nm before and after Sephadex chromatography multiplied by the initial activity of the enzyme before inhibition. For 2FGal-enzyme samples were simply allowed to reactivate until a constant activity was obtained over several assays, the results of these experiments are given in Table 2-IV. Transglycosylation rates were extremely rapid for 2FGal-enzyme. In the presence of 23 mM 1dβGlcϕ approximately 97% of the expected enzyme activity had returned to the sample within 46 minutes. Although reactivation was considerably slower, again up to 90 to 95% of the original activity could be recovered from both 2FGlc- and 2FMan-enzyme upon incubation with 1dβGlcϕ.

Table 2-IV. Kinetic constants for transglycosylation of 1dβGlc φ by pABG5 β-glucosidase [a].

Enzyme Intermediate	[b] $k'_{off} + k_{trans}$ ($min.^{-1}$)	K_S (mM)	$\frac{k_{trans}}{k'_{off}}$
2FGlc	$(5.3 \pm 0.1) \times 10^{-3}$	59 ± 3.1	441
2FMan	$(7.4 \pm 0.9) \times 10^{-3}$	64 ± 14	7.4
2FGal	0.355 ± 0.004	69 ± 9.5	66

[a] All reactions were conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. [b] Calculated with the values given for k'_{off} in Table 2-5.

In all three cases classical saturation kinetics with respect to 1dβGlcφ were observed. This fact along with the similar values obtained for K_S in all three systems provides further evidence for involvement of a single specific binding site in the reactivation process. Indeed it must be the presence of this second binding site which allows transglycosylation to compete effectively with hydrolysis.

It is interesting to note the increased rates of the transglycosylation process as compared to hydrolysis (k_{trans} / k'_{off}). These rate increases are particularly large for 2FGlc- and 2FGal-glucosidase and must correspond to a significant decrease in the activation energy of the transglycosylation process compared with that of hydrolysis. This reduction in the activation barrier could have a number of origins: firstly, the fact that the sugar hydroxyl group is more nucleophilic than water, plus the provision of a suitable binding mode to place the nucleophile could accelerate the reaction enormously. Secondly, the enzyme may be using some of the binding energy derived from interaction of the transglycosylation acceptor with the aglycone sites to stabilize the transition state for glycosyl-enzyme bond cleavage. If this rate increase were due solely to increased nucleophilicity then it could be predicted that k_{trans} should be independent of acceptor structure, providing the nucleophilic group is the same. That is at saturating concentrations of acceptor, transglycosylation rates should be the same for all different acceptors. This is because the main difference between the different glucosyl acceptors used in Table 2-III is the aglycone and it is unlikely that this type of

structural difference will have any significant effect on the nucleophilicity of the hydroxyl group at C-4. Even with the limited data that is available here it can be seen that this is not the case. The value of k_{trans} for 1dβGlcϕ and 2FGlc-enzyme (5.3×10^{-3} min.$^{-1}$) is still five fold smaller than the rate constant for transglycosylation at 20 mM βGlcPNP (0.027 min.$^{-1}$) the latter value may be even larger at saturating concentrations. It appears then that pABG5 β-glucosidase is able to use binding energy derived form protein-ligand interactions at the aglycone site to stabilize the enzymic transition state. This use of binding energy can also be seen in the hydrolysis of normal glucosides, by comparing the V_{max} values given in Day and Withers (1985) for hydrolysis of βGlcPNP (31.6 μmol. min.$^{-1}$ mg^{-1}) and cellobiose (30.34 μmol. min.$^{-1}$ mg $^{-1}$). The leaving group ability (as based on pKa) differs by over nine orders of magnitude and yet the enzymic hydrolysis rates are almost the same. This model would therefore predict that 2-deoxy-2-fluoro cellobiose would also be a good inhibitor of pABG5 β-glucosidase.

β-Glucosidase ^{19}F-NMR Experiments.

The high stability of the 2-deoxy-2-fluoro-glycosylated pABG5 intermediates along with the availability of this β-glucosidase in large quantities provided an ideal opportunity to confirm some of the conclusions drawn from the kinetic experiments by using ^{19}F-NMR.

Enzyme samples for these experiments were prepared by concentrating the highest specific activity fractions obtained after the gel exclusion step in the purification procedure. Enzyme from this stage in its purification was used because of the daunting task of purifying the large amounts of protein required for these experiments on an essentially analytical FPLC column. However we were able to estimate from SDS-PAGE that these fractions were 85-90% pure, and the inherent insensitivity of NMR techniques makes it extremely unlikely that the results that were obtained are due to a contaminating enzyme. Samples were prepared in a 50% deuterated phosphate buffer (50 mM, pH 6.8) and final enzyme concentrations were determined using the 2,4-dinitrophenolate burst method.

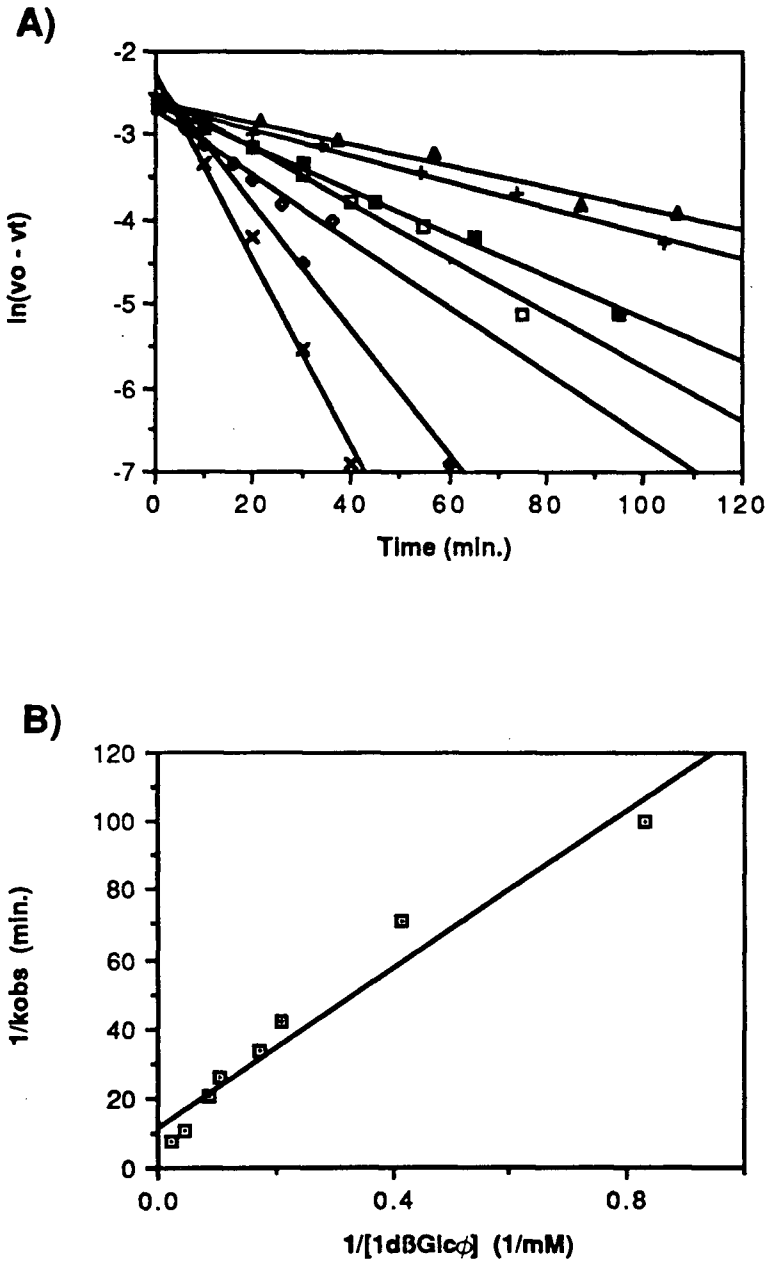


Figure 2-10. Reactivation of galactosylated pABG5 β-glucosidase by 1dβGlcϕ.

A) A semilogarithmic plot of activity versus time at 37°C, pH 6.8 in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. The following concentrations of 1dβGlcϕ were used (mM):❑ 5.74,◆ 11.48, ■ 4.79, ◊ 9.58, + 2.39,▲ 1.19, x 43.1. B) A double reciprocal plot of the following values obtained for k_{obs} at their respective inhibitor concentration: 0.03, 0.048, 0.024, 0.039, 0.014, 0.01, 0.137. These values were obtained from a non-linear least squares regression analysis.

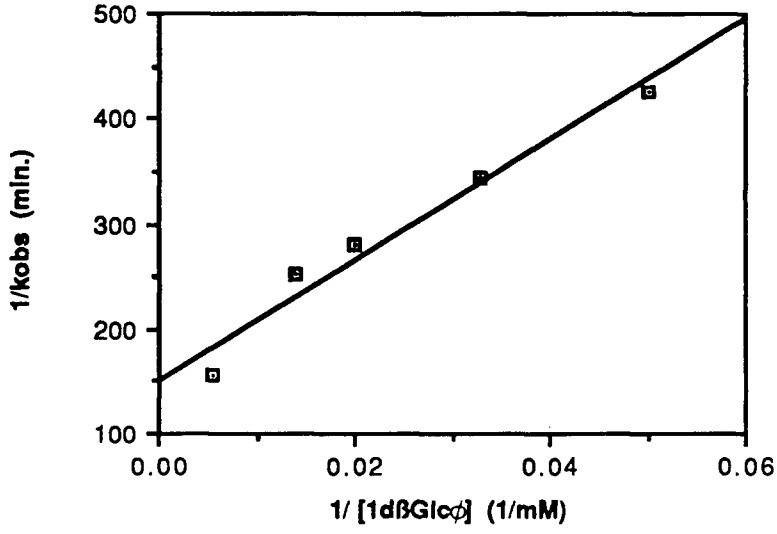


Figure 2-11. Reactivation of 2FMan-pABG5 β-glucosidase by 1dβGlcϕ.
Reaction conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. The following 1dβGlcϕ concentration (mM) gave the values of k_{obs} ($min.^{-1}$) presented in parentheses: 20.0, (0.0023); 30.0, (0.0029); 50.0, (0.0036); 70.0, (0.0040); 182.0, (0.0064).

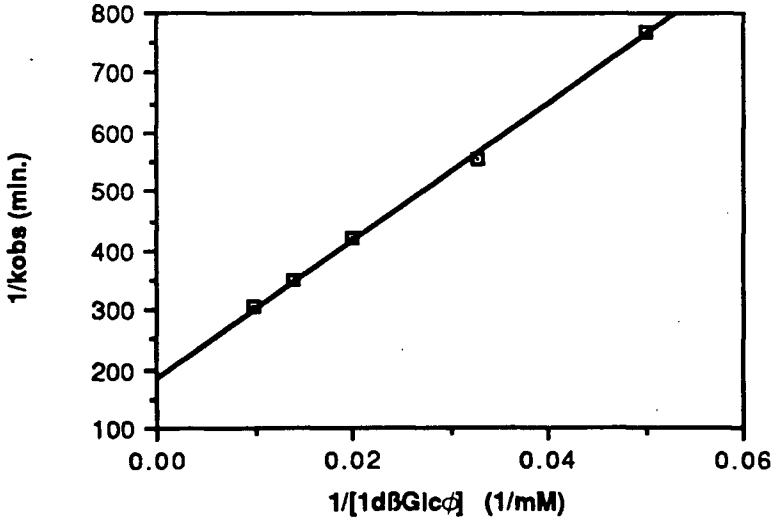


Figure 2-12. Reactivation of 2FGlc-pABG5 β-glucosidase by 1dβGlcϕ.
Reaction conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. The following 1dβGlcϕ concentration (mM) gave the values of k_{obs} ($min.^{-1}$) presented in parentheses: 20.0, (0.0013); 30.0, (0.0018); 50.0, (0.0024); 70.0, (0.0029); 100.0, (0.0033).

All chemical shifts are given relative to Freon ($CFCl_3$, δ = 0.00 ppm) and were measured from an internal standard of 6FαGlcP.(δ = 237.48 ppm) This compound was chosen as the chemical shift reference because of its high solubility in aqueous solvents, because its primary fluorine resonates well outside of the range expected for the inhibitors and because its α-phosphate ensures that it will not bind to the enzyme.

Upon addition of 2FβGlcF (1.05 mM) to 0.73mM pABG5 β-glucosidase, resonances at δ 121.4, 144.8 197.3 and 203.4 ppm were observed (Figure 2-13A). The signals at δ 144.8 and 203.4 ppm were assigned respectively to F-1 and F-2 of the free inhibitor. These assignments were made by comparison with a sample of this compound made up in the same buffer but lacking any enzyme. The sharp resonance at δ 121.39 ppm was assigned to inorganic fluoride, again by comparison with a buffered sample of sodium fluoride. The remaining resonance at 197.3 ppm was therefore assigned to the 2FGlc-enzyme intermediate. This is consistent with the comparatively large linewidth of this signal ($\Delta\nu_{1/2}$ = 130 Hz) and its chemical shift which is in a region characteristic of a fluorine attached to a non-anomeric secondary carbon on the sugar ring. Thus, in good agreement with the kinetic experiments it would appear that inhibition occurs by accumulation of all of the enzyme as a stable 2-deoxy-2-fluoro glucosyl-enzyme intermediate. A comparison of peak areas (taken from the proton coupled spectrum) for the glucosylated enzyme and released fluoride indicates that near stoichiometric (1 : 0.9) quantities of the intermediate are formed for every mole of fluoride produced. Again this results agrees well with the pre-steady state burst.

To examine the transglycosylation process 1dβGlcϕ (33.6 mM) and a further 3.2 equivalents of 2FβGlcF were added. Spectra were accumulated after 6, 24, 48 and 72 hours incubation at 30°C. The spectrum taken at 48 hours is shown in Figure 2-13B. Over the incubation period a slow decrease in intensity of the peaks due to the free inhibitor was observed with a concomitant increase in the fluoride peak and the appearance of a new resonance at δ 200.10 ppm. After 62 hours this reaction had essentially gone to completion.

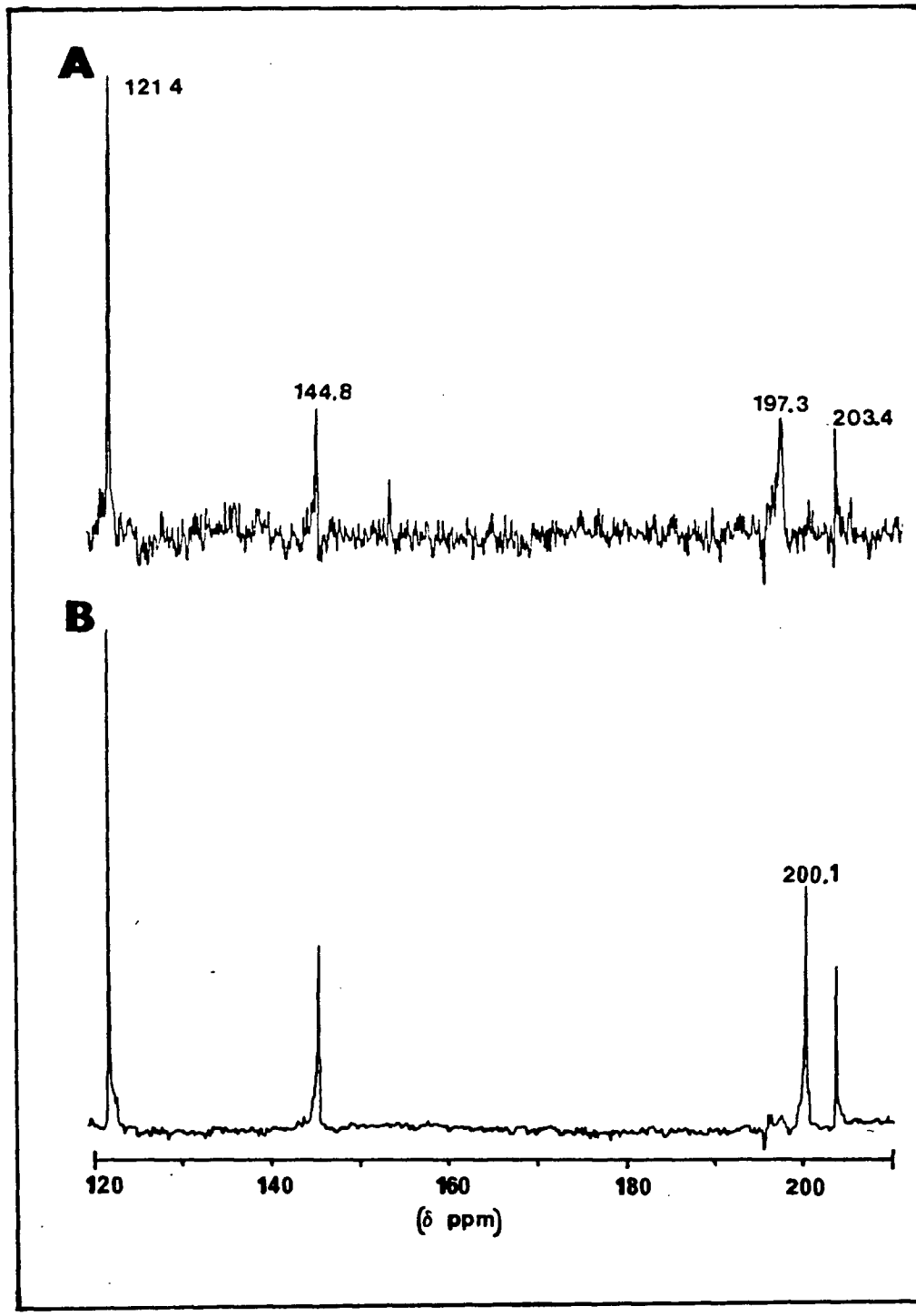


Figure 2-13. ^{19}F-NMR spectra of 2FGlc-pABG5 β-glucosidase.

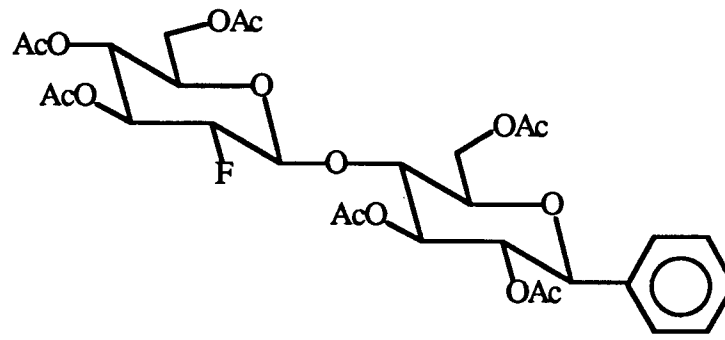
Proton decoupled spectra, Samples contained: A) 1.05 mM 2FβGlcF, 0.73 mM β-glucosidase in 50% deuterated 50 mM sodium phosphate buffer pH 6.8. Data was acquired at 20°C and signal averaged over 10,000 transients. B) 2.3 mM 2FβGlcF, 0.6 mM β-glucosidase, 35 mM β–D-glucosyl benzene in 50% deuterated 50 mM sodium phosphate buffer pH 6.8. Data was acquired at 20°C after 48 hours incubation at 30°C and signal averaged over 10,000 transients. All samples contained 1mM 6FαGlcP as an internal chemical shift reference.

The product and other low molecular weight components were separated from the protein by ultra-filtration with a Centricon microconcentrator. Desalting and a partial separation of the components contained in this mixture was achieved using gel exclusion chromatography on a column of Biogel P-2 eluted with deionized water. The column eluate was monitored at 254 nm to detect absorption by components containing a glucosyl benzene group. Separation between the fractions which containing fluorinated disaccharide (as located by ^{19}F-NMR) and the large excess of 1dGlcϕ was not very good since the two compounds coeluted approximately 17.5 hours after their introduction onto the column. However it was possible to obtain four fractions which had been enriched in the fluorinated product. Fractions containing U.V absorbing material and which eluted in the exclusion volume of the column (at 6.8 hours) and at 15 hours were also detected. However, as neither contained a fluorinated product (as evidenced by ^{19}F-NMR) both fractions were discarded.

Fluorinated product was separated from the remaining 1dβGlcϕ by acetylation with acetic anhydride in pyridine followed by chromatography on a small silica gel column. Approximately 20μg of the chromatographically pure (by thin layer chromatography) per-O-acetylated product was obtained in this way. ^{19}F-NMR (254 MHz, $CDCl_3$) of this compound showed a single resonance δ 199.28 ppm (dd, $J_{gem.}$ 50, $J_{vic.}$ 14 Hz) and 400 MHz proton NMR gave the following results: δ 7.28 (5H,), 5.37 (t, 1H, J 9.4 Hz), 5.29 (dt, 1H, J 8.9, 14 .6 Hz), 5.05 (t, 1H, J 9.6 Hz), 5.01 (t, 1H, J 9.7 Hz), 4.65 (dd, 1H, J 3.1, 7.8 Hz), 4.46 (AB multiplet, 2H, J 12.5, 4.3, 1.0 Hz), 4.39 (d, 1H, J 9.8 Hz), 4.16 (AB multiplet, 2H, J 12.3, 2.0, 4.2 Hz), 4.00 (t, 1H, J 9.6 Hz), 3.81 (m 1H), 3.71 (m, 1H), 2.09 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.79 (s, 3H).

Because of the extremely small amount of the pure product that was available it was not practicable to conduct the decoupling experiments necessary to produce unequivocal assignments for all of these resonances. However based on observed coupling constants and comparisons with the data obtained from the component monosaccharides it is reasonable to

assign the structure as, 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl-(1-4)-2,3,6-tri-O-acetyl-β–D-glucopyranosyl benzene, as follows:



3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl-(1-4)-2,3,6-tri-O-acetyl-β–D-glucopyranosyl benzene.

Resonances associated with six different acetate groups (δ 2.09 - 1.79 ppm) are observed, one of which resonates 0.2 ppm further upfield than the rest. This upfield shift of a single acetate group is also seen in the spectrum of per-O-acetylated β-D-glucopyranosyl benzene and is probably due to its situation just below the plane of the C-1 phenyl ring. Five protons resonating in the aromatic region of the spectrum (δ 7.28 ppm) are also observed. ^{19}F-NMR data is consistent both in chemical shift and ^{19}F-^{1}H coupling with a fluorine attached to a secondary carbon. The observed vicinal ^{19}F-^{1}H coupling of 14 Hz also allows assignment of the resonance seen at δ 5.29 ppm in the proton NMR spectrum as H-3 of the 2-deoxy-2-fluoro glucosyl residue.The magnitude of this coupling is typical of a cis-1,2 ^{19}F-^{1}H arrangement that would be expected in a sugar of the *gluco* configuration. The failure to observe a resonance possessing the characteristic geminal ^{19}F-^{1}H coupling of 50 Hz is somewhat puzzling, however it would be expected that due to its multiplicity (ddd, $J_{2\text{-}F}$ 50, $J_{2,1}$ 7.8, $J_{2,3}$ 8.9 Hz), the absolute intensity of this signal would be quite low, and consequently might not have been observed above baseline noise. Alternatively it simply might have been coincident with other resonances. However, the ^{19}F-NMR data leaves little doubt that such a proton does exist somewhere.

All of the remaining prerequisite number of secondary protons are observed (δ 5.37, 5.05, 5.01, 4.65, 4.39 and 4.00 ppm). The resonance at 4.00 ppm is at much higher field strength than any of the resonances observed in the spectra of the per-O-acetylated component monosaccharides which infers that this is the linking position in the disaccharide product, since it is not acetylated. Without decoupling experiments it is impossible to distinguish between a 1-4 and a 1-3 linked product, however the similarity in the chemical shift values of the two AB systems (δ 4.46 and 4.16 ppm) which are due to the primary C-5 hydroxymethyl groups does eliminate the possibility of a 1-6 linkage. The possibility of the 1-4 linkage is strongly supported by the structural requirements of the reactivating ligand (Table 2-III) as it was observed that galactosides were far less efficient at promoting reactivation than glucosides.

The anomeric configuration of the linkage can be determined from the resonance at 4.65 ppm.which is due to H-1 of the 2-deoxy-2-fluoro glucosyl residue The large coupling (7.8 Hz) is characteristic of a 1,2 transdiaxial arrangement of protons at C-1 and C-2 of a sugar possessing a β-linkage . This is corroborated by the second smaller coupling ($J_{1,F}$ 3.1 Hz) which is only seen in 2–deoxy-2-fluoro sugars of the β-configuration (Adamson et al., 1970). In those sugars which possess an α-anomeric linkage this coupling is less than 0.2 Hz and is not generally observed. The doublet at 4.39 ppm can be assigned to H-1 of the glucosyl benzene residue.

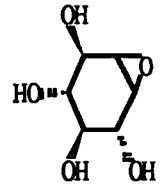
Further evidence for configuration of the anomeric linkage was obtained from the turnover product of 2FMan-pABG5 β-glucosidase and 1dβGlcϕ. Incubation of 1.52 mM 2FβMan F (resonances observed at δ 224.40, F-2 and δ 149.57 ppm, F-1) with pABG5 β-glucosidase (0.74 mM) resulted in the release of fluoride (121.03 ppm) and the appearance of a broad resonance due to 2FMan-enzyme (δ 201.00 ppm, $\Delta\upsilon_{1/2}$ = 145 Hz), see Figure 2-14A. Addition of 1dβGlcϕ at a concentration of 100 mM caused a time dependent depletion of resonances from the inhibitor with a concomitant increase in the signal from fluoride and a product at δ 221.24 ppm. After 24 hours this reaction had essentially gone to completion

(Figure 2-14B). Many workers have demonstrated that the ^{19}F-NMR chemical shift of 2-deoxy-2-fluoro mannosides show a marked dependence on the configuration of the neighbouring anomeric group (Phillips and Wray, 1971; Adamson et al., 1970). The difference commonly observed between α-mannosides (which generally resonate around δ 200-206 ppm) and β-mannosides is often as large as 18 - 20 ppm. A chemical shift of δ 221.24 ppm which was recorded for the enzymic transglycosylation product is consequently diagnostic of a β-linked disaccharide.

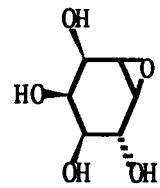
The Stereochemistry of the Glycosyl-enzyme Intermediate.

The reaction mechanism depicted for retaining glycosidases (Scheme 1-3) amounts to a classical double displacement mechanism, involving two steps proceeding with stereo-chemical inversion to give overall retention of configuration. This would imply that the enzyme-bound intermediate should posses the opposite anomeric configuration to the starting glycoside and there is a great deal of experimental evidence to support this, some of which is reviewed briefly below.

An enzyme carboxylate correctly placed to form an axial acylal with the glycoside has been identified in the X-ray crystal structure of bacteriophage T4 lysozyme (Anderson et al., 1981) as well as the hen's egg white enzyme (Imoto et al., 1972). Both of these enzymes are endo-glucosidases which hydrolyses substrates of the β-configuration. Evidence on many other glycosidases has been obtained by the use of affinity labels, most notably cyclitol epoxides.



D-gluco configuration



D-galacto configuration

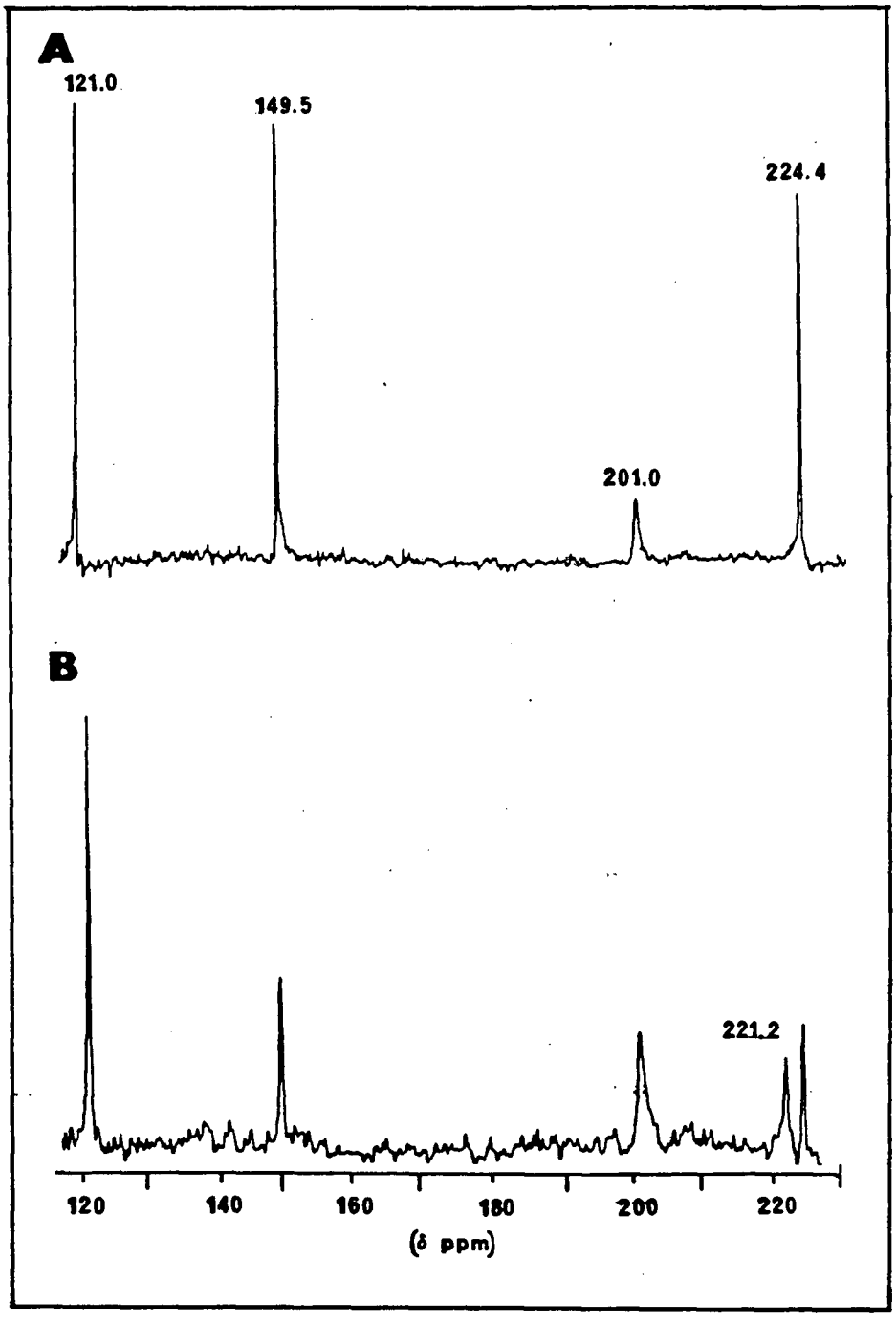
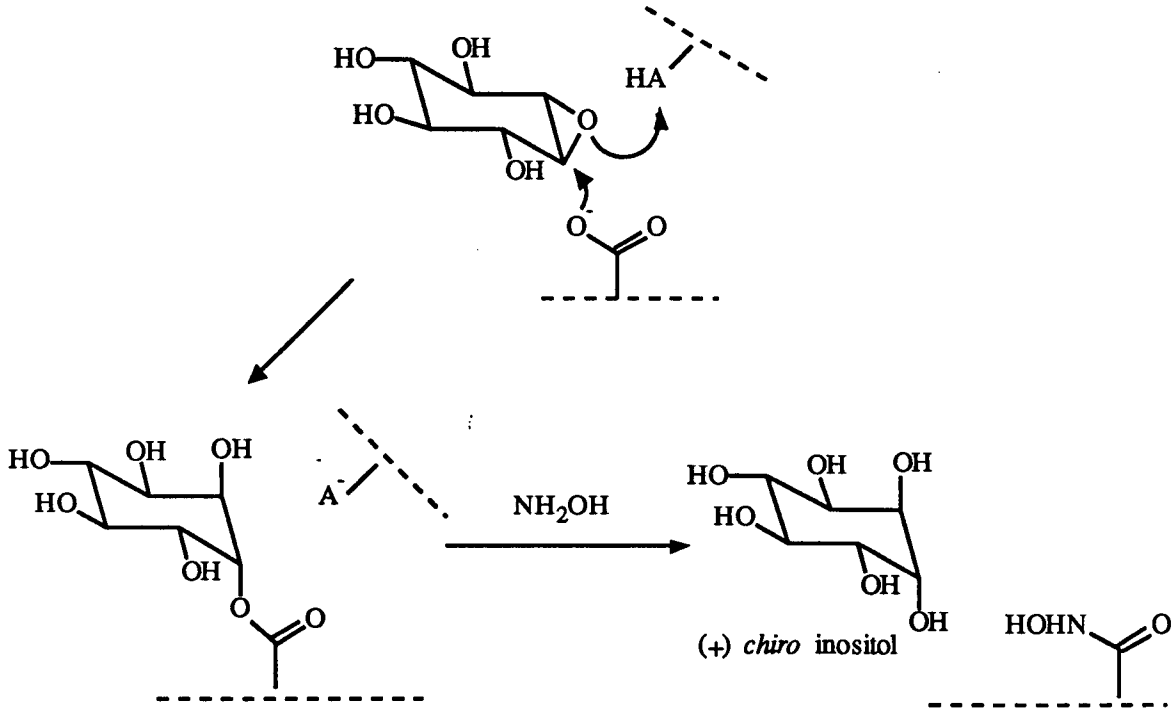


Figure 2-14. ^{19}F-NMR spectra of 2FMan-pABG5 β-glucosidase.

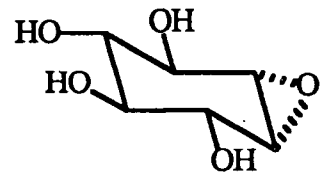
Proton decoupled spectra, Samples contained: A) β-glucosidase (0.74 mM), 2FβMan F (1.5 mM) in 50% deuterated phosphate buffer pH 6.8. Data acquisition was performed at 20°C and signal averaged over 10, 000 transients. B) 1.0 mM β-glucosidase, 1.5 mM 2FβManF and 100 mM β-D-glucosyl benzene Buffer and acquisition conditions as described above. Data was collected after 12 hours incubation at 30°C. All samples contained 1mM 6FαGlcP as an internal chemical shift reference.

An aspartic acid residue was labelled by the cyclitol epoxide possessing the *gluco* configuration in β-glucosidase A_3 from *A. Wentii* , (Bause and Legler, 1974) and the β-glucosidase isolated from bitter almonds (Legler and Harder, 1978). A glutamic acid in the *E. Coli* β-galactosidase has been identified in the same manner using the cyclitol epoxide of the *galacto* configuration (Herrchen and Legler, 1984). In each case the formation of an axial linkage between C-1 of the sugar and the enzyme carboxylate group was determined from the stereochemistry of the product released upon treating labelled enzyme with hydroxyl-amine. Only inositols of the (+) *chiro* configuration were produced, indicating an α-linkage in the inhibitor-enzyme complex.



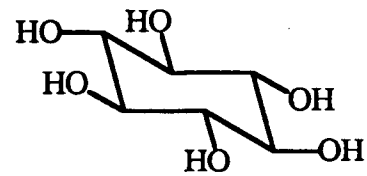
Scheme 2-8. Stereochemical determination of glucosyl-enzymes using cyclitol epoxides.

Although fewer studies have been done on retaining α-glycosidases, what evidence there is suggests the intermediacy of a β-glycosylated enzyme. Sucrose-isomaltase has two catalytically active subunits which hydrolyse maltose and aryl α-D-glucopyranosides. Both of these subunits are labelled by the D-isomer of conduritol epoxide B (Quaroni et al. 1974).



The D-isomer of conduritol epoxide B

In both cases an aspartic acid residue was labelled (Quaroni et al., 1976) and treatment of this enzyme with hydroxylamine released only *scyllo* inositol, thus implying a β-linkage in the normal glycosylated-enzyme complex.



scyllo inositol.

The evidence for the participation of a β-glucopyranosylated enzyme carboxylate group in catalysis by sucrose phosphorylase from *Pseudomonas saccharophilia* is also well established (Mieyal and Abeles, 1972; Voet and Abeles, 1970).

It had occurred to us that we should be able to establish the stereochemistry of our 2-deoxy-2-fluoro-glycosylated-enzyme intermediate directly from the ^{19}F-NMR data. Ideally this should be done using both ^{19}F-^{1}H coupling constants and chemical shift data. However the broad resonances which were observed for the glucosyl-enzyme intermediate would make it impossible to observe the small changes in coupling constants that would be necessary for this determination, but a careful analysis of the observed change in chemical shift should enable us to arrive at some conclusion based solely on this data.

The total observed change in ^{19}F-NMR chemical shift of the fluorine at C-2 of the inhibitor which occurs on reaction with the enzyme is probably derived from a combination of three different factors. Firstly, an electronic factor associated with the exchange of the fluorine at C-1 of the inhibitor for a ligand at the active site of the enzyme. Secondly, a factor associated with an environmental change occurring on removal of the inhibitor from free

solution to the enzyme's active site. Lastly, presumably a factor associated with the change in orientation of the group attached to C-1 of the glycoside. Ideally to arrive at a definitive conclusion this last effect should be as large as possible compared with the other two. An estimate of the change in chemical shift due to inversion of configuration at the anomeric centre can be obtained simply from the ^{19}F-NMR data of the anomerically equilibrated fluoro-sugar in free solution (Phillips and Wray, 1971). This data for 2FGlc would suggest that a shift of only 0.2 ppm might be expected if a change in the anomeric configuration of the 2-deoxy-2-fluoro glucosyl inhibitors occurred upon formation of the glucosyl-enzyme intermediate. Since a shift of ≈ 6 ppm was seen in this case, (Figure 2-13) it would appear that other factors (exchange of ligand and change of environment) are more important than a change in anomeric configuration in determining the chemical shift in 2FGlc-pABG5 β-glucosidase. However, a much larger change in chemical shift would be expected upon inversion of configuration at the anomeric centre of 2Fman (≈18 ppm), thus this would make a stereochemical determination based on ^{19}F-NMR chemical shift data much more reliable for 2FMan-pABG5 β-glucosidase. As we have already discussed pABG5 β-glucosidase has significant mannosidase activity and is readily inhibited by 2FβManF, and the large downfield shift seen upon formation of the 2FMan-intermediate (23.4 ppm change) in Figure 2-14 is clearly consistent with the exchange of an equatorial to axial ligand at C-1 of the sugar. To assess the contribution of environmental exchange to this shift and ensure that the large shift was not due to binding at the active site a sample of 2FMan-pABG5 β-glucosidase (0.74 mM) was denatured by overnight dialysis in 8 M urea and then spectra accumulated in the presence of this chaotropic agent. The results are shown in Figure 2-15 and indicate a small upfield shift (1.57 ppm) of the resonance due to the 2FMan-enzyme under these conditions. Also seen are resonances at δ 206.2 and δ 224.48 ppm which can assigned respectively to α- and β-2-deoxy-2-fluoro-D-mannose. The free sugars must be derived from the non-enzymic breakdown of the exposed intermediate, as the enzyme is certainly not active under these conditions.

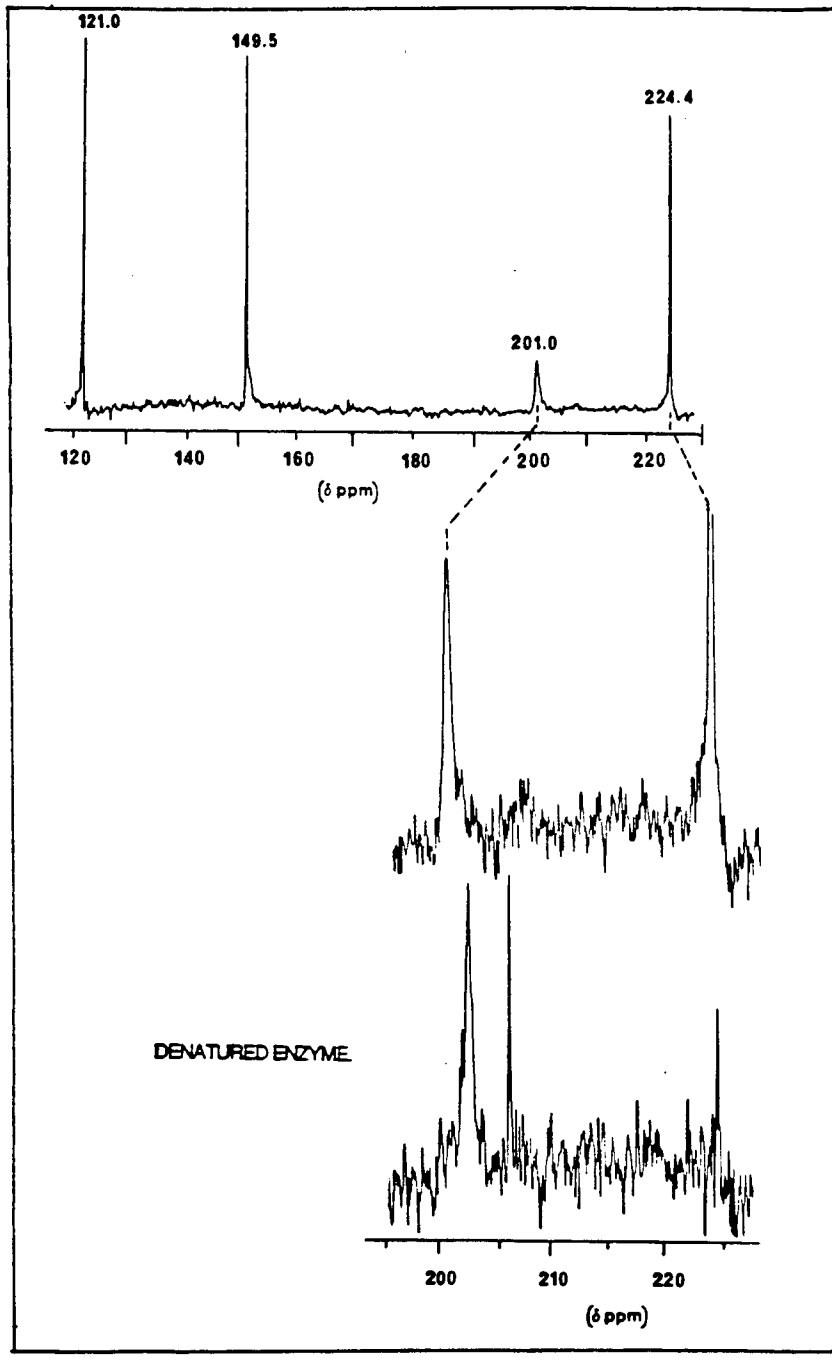


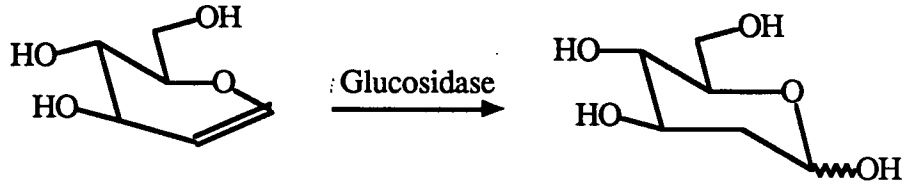
Figure 2-15. ^{19}F-NMR determination of the stereochemistry for 2FMan-pABG5 β-glucosidase

Proton decoupled spectra, Samples contained: A) Native enzyme; sample contained, β-glucosidase (0.74 mM), 2FβMan F (1.5 mM) in 50% deuterated phosphate buffer pH 6.8. Data acquisition was performed at 20°C and signal averaged over 10, 000 transients (^1H decoupled). B) Denatured enzyme, above sample after 24 hours dialysis (20°C) against 8M urea. Data acquisition as above signal averaged over 30, 000 transients. All samples contained 1mM 6FαGlcP as an internal chemical shift reference.

The size of the shift associated with exchange of ligand at C-1 cannot be analyzed conclusively, as we have no first hand knowledge as to the nature of the active site nucleophile in pABG5 β-glucosidase. However, as has already been discussed there is plenty of literature precedent for the participation of a carboxylate group in this function. On this basis the shift associated with the exchange of a the C-1 fluorine for an oxygen of a carboxylate should be no greater than 1 or 2 ppm . This is based on the observed chemical shifts of F-2 in the inhibitor (δ 224.39) and that of 2-deoxy-2-fluoro-β-D-mannose (under the same buffer conditions δ 224.10 ppm) and the observation made from Adamson et al, (1970) that only a 0.8 ppm (upfield) difference is seen between the F-2 resonances of 2-deoxy-2-fluoro β-D-mannose and its per-O-acetylated derivative. Thus the various contributions of these three factors to the observed shift of 23.4 ppm on formation of the 2FMan intermediate are ≈7 % derived from environmental exchange, at most 4 - 8 % associated with ligand exchange at C-1 and the remaining 85 - 89% being due to the formation of an α-linked 2FMan-intermediate. Thus 2FMan-pABG5 β-glucosidase would appear to an α-linked intermediate, a result which is in keeping with the other examples cited in the earlier discussion and in keeping with the generally accepted double displacement mechanism proposed for retaining β-glucosidase. The ^{19}F-NMR technique described above represents a very facile method of accomplishing this type of determination.

The 2-fluoro-D-glucal story.

Many glycosidases will catalyse the hydration of D-glycal to yield a 2-deoxyglycose as shown below for a glucosidase.



D-glucal

Scheme 2-9. Hydration of D-glucal.

The enzymic mechanism which has generally been accepted for this hydration reaction (Figure 2-16) is analogous to that of glycoside hydrolysis involving many of the same catalytic groups. However there is one major difference to this; in retaining glycosidases the glucal is protonated on the face opposite to that which would be expected from normal glycoside hydrolysis. That is D-glucal is protonated from above the β-face in α-glycosidases and from below the α-face in β-glucosidases. Largely through the efforts of E.J. Hehre and his group there are now many examples of this behaviour and it appears to be quite general. (for examples see Takahisa et al., 1986; Hehre et al. 1986 and Flowers and Sharon 1979). There is also significant evidence that the remainder of the reaction follows the normal pathway for glycoside hydrolysis, as products are released with the correct anomeric configuration. That is an α-glycose is released by a retaining α–glycosidase and the corresponding β-glycose will be released from a β-glycosidase. Roeser and Legler have also demonstrated that the same aspartate residue in β-glucosidase from *A. wentii* is labelled by D-glucal, p-nitrophenyl 2-deoxyglucopyranoside, (Roeser and Legler, 1981) and conduritol B epoxide (Roeser et al., 1979). In *(lac Z)* β-galactosidase from *E. coli* an active site carboxylate is also labelled by D-galactal (Kurz et al., 1981). So although the initial protonation step appears to differ significantly from that seen in normal glycoside hydrolysis the remainder of the mechanism is the same, involving the same active site residues and resulting in the same stereochemistry.

It was apparent that the reaction of a 2-fluoro-substituted glucal might in an analogous fashion provide an alternative route to the stable 2-deoxy-2-fluoro glucosyl-β-glucosidase, thus providing another class of glucosidase inactivators. However, as the properties (if any) of D-glucal as a substrate for pABG5 β-glucosidase were largely unknown, this was the initial subject of investigation.

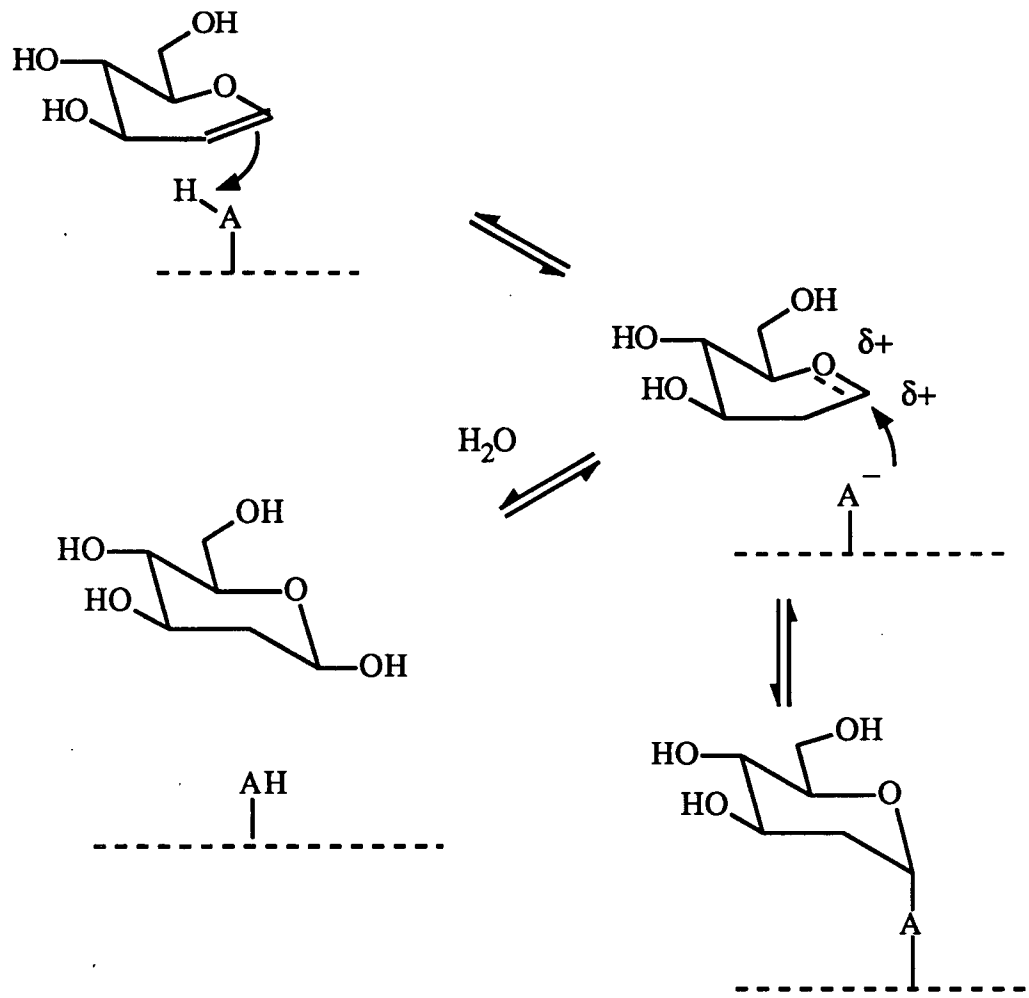
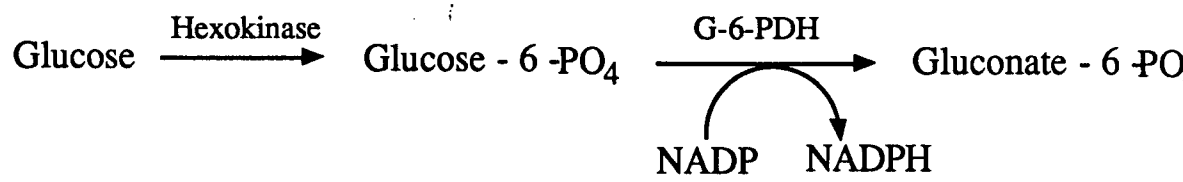


Figure 2-16. A reaction mechanism for hydration of D-glucal by a "retaining "β-glucosidase.

D-glucal as a Substrate for pABG5 β-Glucosidase.

A preliminary investigation along these lines had been conducted in this laboratory by G. Ohara (1987, Chemistry 449 thesis, University of British Columbia). She had found that D-glucal was quite an efficient competitive inhibitor of pABG5 β-glucosidase (K_i = 0.68 mM), but no time-dependent inactivation or substrate activity was apparent. However, the method used for detection of substrate activity (a direct U.V.absorption assay observing the double bond at 210 nm), under the conditions employed, could be quite insensitive. A re-

investigation was launched using a coupled enzyme system to detect the hydration production (2dGlc). This would hopefully provide the extra sensitivity necessary to detect substrate activity. The assay system was based on a hexokinase / glucose-6-phosphate dehydrogenase couple and was originally designed as a continuous assay system for glucose production.



Scheme 2-10. A hexokinase, glucose-6-phosphate dehydrogenase coupled assay for glucose.

The production of NADPH is detected spectrophotometrically by monitoring the absorption at 340 nm ($E = 6220\ M^{-1}\ cm^{-1}$). Compared with glucose, the production of NADPH by the coupled enzymes from 2dGlc was many fold slower, consequently the assay could not be used to monitor continuous production of the hydration product. The coupling enzymes had to be incubated with samples of 2dGlc for 1 hour at 30°C before a steady end-point measurement could be obtained. However under these conditions the assay provided reliable results and was sensitive in the 5 - 60 μM range (Figure 2-17) giving quantitative production of NADPH concomitant with phosphorylation and oxidation of 2dGlc. Initial rates of D-glucal hydration by pABG5 β-glucosidase at pH 6.8 and 37°C were determined by incubating the enzyme with eight different concentrations of substrate for 2 hours. At the end of this period the hydration reaction was stopped by heat denaturation of the enzyme (1 minute at 100°C) and the 2dGlc concentration measured using the coupled enzyme system. The results obtained in this manner are given in Figure 2-18. Values for K_m and k_{cat} were determined by extrapolating to saturating concentrations of substrate using the normal computer fitting procedure. D-glucal appeared to be quite a slow substrate $k_{cat} = 2.28 \pm 0.09\ min^{-1}$ and the value determined for K_m (0.85 ± 0.11 mM) was in good agreement with the previously determined (0.68 mM) K_i value.

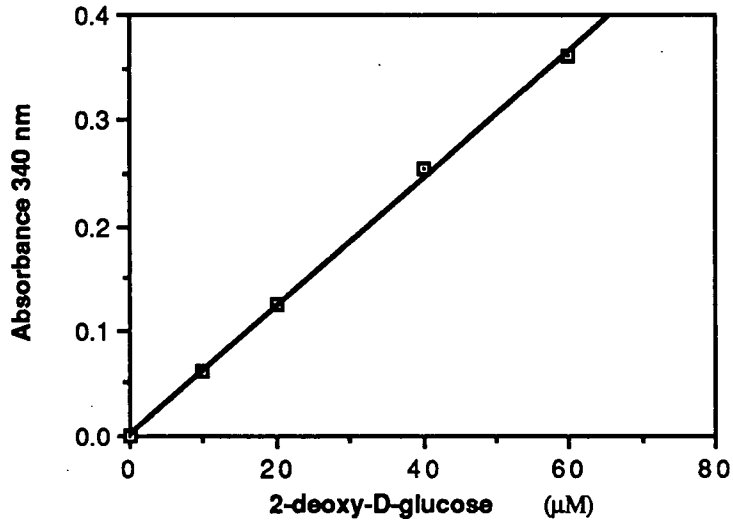


Figure 2-17. Standard curve for 2dGlc using the hexokinase / glucose-6-phosphate dehydrogenase coupled assay.

Reaction were run at pH 7.6 and 30°C in 100 mM triethanolamine buffer containing 25 U mL^{-1} hexokinase, 15 U mL^{-1} glucose-6-phosphate dehydrogenase,0.6 mM ATP, 0.35 mM NADPH and 1 mM magnesium chloride. Optical density reading were taken at 340 nm after 1 hour incubation.

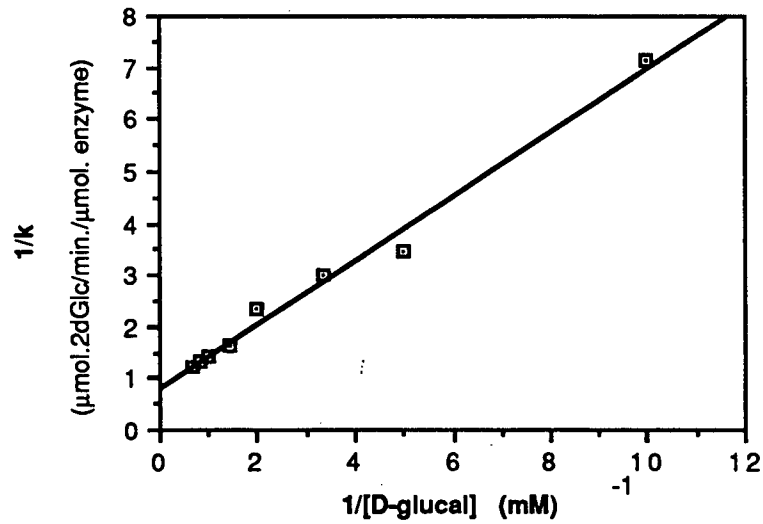


Figure 2-18. Determination of the kinetic constants for D-glucal hydration by pABG5 β-glucosidase.

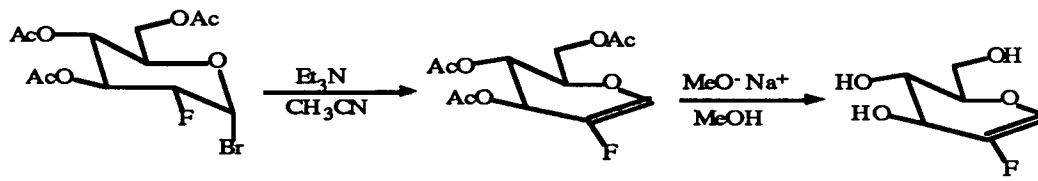
Reactions were conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. Reactions contained 0.114 nmol. of pABG5 β-glucosidase per assay and D-glucal at the following concentrations: 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 1.2, 1.5 mM. Reaction time was 1.5 hours.

The stereochemistry of the hydration reaction was determined by ^{1}H-NMR techniques from deuterium incorporation during turnover (Seiya et al., 1988). D-glucal was incubated

with the glucosidase at pH 6.8 and 30°C for 3 days in a fully deuterated buffer. After the incubation period was complete the reaction was quenched by lyophilization and the deuterium incorporation patterns analyzed by ^{1}H-NMR. The 400 MHz ^{1}H-NMR spectrum of the deuterated product is shown in Figure 2-19 C. Control reactions containing D-glucal but no enzyme (Figure 2-19 A) and 2dGlc with enzyme (Figure 2-19 B) provided assurance that the results were not due to non-enzymic hydration or from enzyme–catalysed exchange of the C-2 protons in the product. The resonances due to the geminal protons at C-2 are readily assigned on the basis of their high field resonance frequency and ^{1}H-^{1}H coupling constants. Thus the resonances seen in Figure 2-19 B at δ 1.46 ppm and δ 1.67 ppm are due to the axial protons at C-2 of β– and α-2-deoxyglucose, while the resonances at δ 2.03 and δ 2.25 ppm are assigned to the equatorial protons of the sugar. From the spectrum of the enzymic hydration product it is apparent that only the axial C-2 protons are present, hence 2-deoxy-[^{2}H-2e]-glucose must have been formed. This implies that the deuteron must have been added from below the α-face of the glucal, a result which is in complete agreement with those obtained by many other groups on many other glycosidases.

Synthesis and Testing of 2-Fuoro-D-Glucal.

Synthesis of 2-fluoro-D-glucal (2F-glucal) was accomplished by simple base catalysed elimination from the per-O-acetylated 2-deoxy-2-fluoro-α-glucopyranosyl bromide using triethylamine.



Scheme 2-11. Synthesis of 2F-glucal.

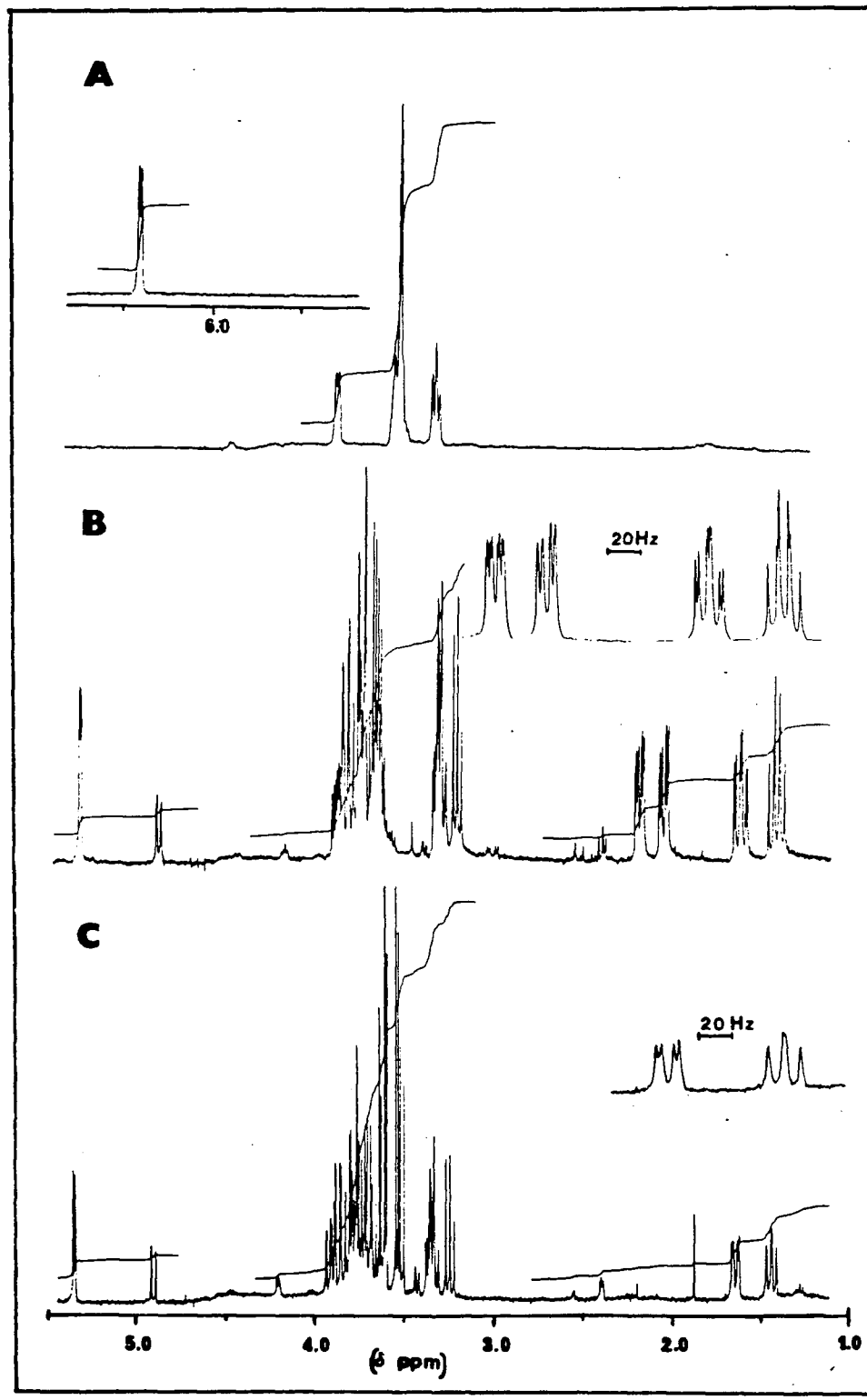


Figure 2-19. Determination of the stereochemical course of D-glucal hydration by pABG5 β-glucosidase.

The 400 MHz ^{1}H-NMR spectrum were recorded over a sweepwidth of 4,800 Hz using solvent suppression. All samples were in 50 mM sodium phosphate buffer pH 6.8 with an incubation time of 3 days. A) D-glucal no enzyme, signal averaged over 256 transients. B) 2dGlc with pABG5 β-glucosidase, signal averaged over 256 transients. C) D-glucal with pABG5 β-glucosidase, signal averaged over 324 transients.

The synthesis was completed by removal of the acetate blocking groups with catalytic amounts of sodium methoxide in methanol. Satisfactory spectral and analytical data were obtained from the intermediate and the final product.

The initial results from the testing of 2F-glucal as an inhibitor of pABG5 β-glucosidase appeared to suggest that it was indeed quite an effective inhibitor which operated in the same manner as the 2-deoxy-2-fluoro glycosyl fluorides (through accumulation of a stable covalent intermediate). However, as this work progressed it became apparent that the observed time dependent inhibition was due to a contaminant in the 2F-glucal preparation present at very low amounts (0.015%).

Incubation of pABG5 β-glucosidase with various concentrations of the "2F-glucal preparation" gave rise to an exponential decrease in activity with time, the rate constant for which was dependent on the concentration of inhibitor present in the mixture (Figure 2-20 A and 20 B). Extrapolation of the rate data to saturating concentrations of inhibitor gave the following values for k_{on} 0.092 ± 0.08 min.$^{-1}$ and K_d 34 ± 6 mM. In this case as the rate of covalent bond formation was relatively slow we were able to assess 2F-glucal as a reversible competitive inhibitor of hydrolysis of a normal substrate. As both K_d and the inhibition constant (K_i) for a competitive inhibitor are both true thermodynamic dissociation constants for the non-covalent 2F-glucal-enzyme complex these experiments should provide an independent check of the data obtained from irreversible inhibition experiments. Initial rates for βGlcPNP (at five different concentrations) and three different concentrations of 2-F-glucal were obtained and plotted using the standard double reciprocal plot (Figure 2-21 A). The inhibition pattern obtained (a series of convergent lines intersecting at the Y axis) is typical of a competitive inhibitor. A value of K_m apparent from each curve was obtained by fitting of the data to the non-linear form of the Michaelis-Menten equation using the normal computer aided procedure. A replot of apparent K_m versus inhibitor concentration was fitted using a linear least squares regression analysis to give a value of 24 ± 4.1 mM for K_i (Figure 2-21 B). This is in reasonable agreement with the value obtained for K_d (34 mM) from the

irreversible inhibition experiment. Similar values were obtained for both Ki and Kd in experiments performed on two separate preparations of inhibitor.

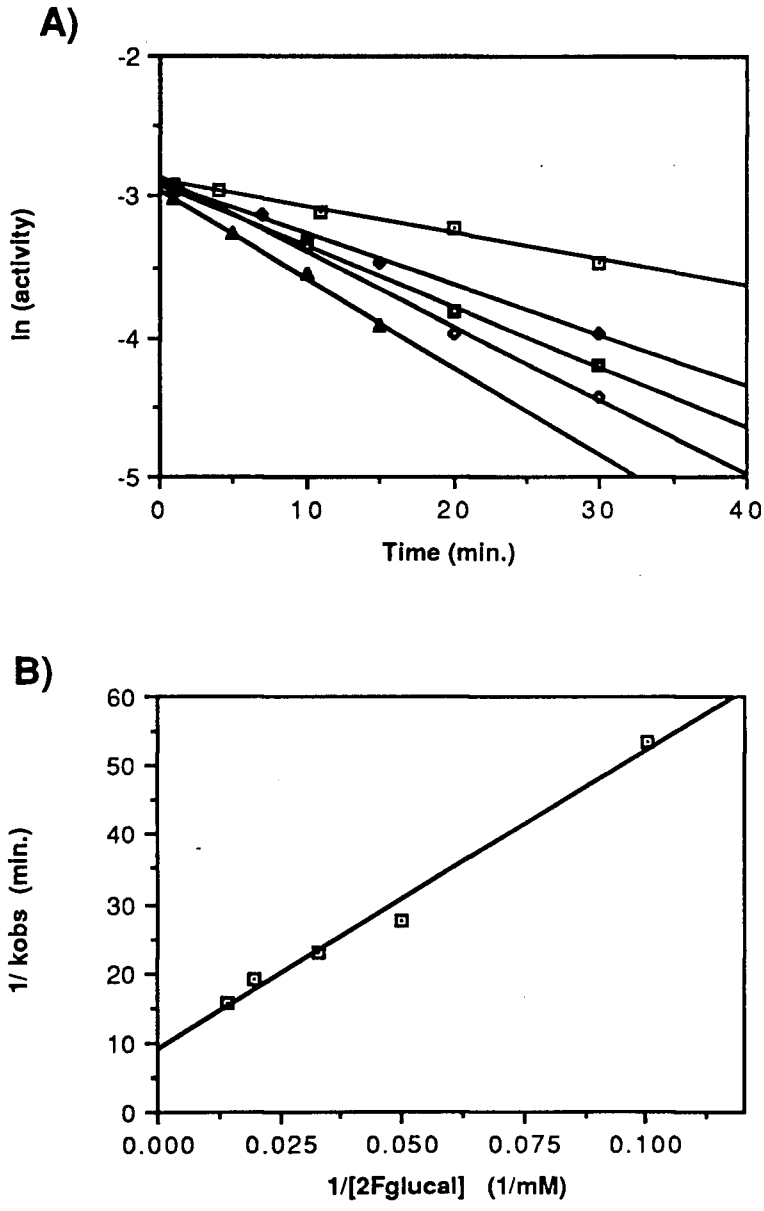


Figure 2-20. Inhibition of pABG5 β-glucosidase by "2F-glucal preparation".
A) A semi logarithmic plot of activity versus time at 37°C, pH 6.8 in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. The following inhibitor concentrations were used:□, 10.0 mM,◆ 20.0 mM, ■ 30.0 mM, ◊ 50.0 mM, ▲ 70.0 mM. B) A double reciprocal plot of the following values obtained for k_{obs} at their respective inhibitor concentration .019, 0.036, 0.044, 0.052, 0.063 min^{-1}. These values were obtained from a non linear least squares regression analysis.

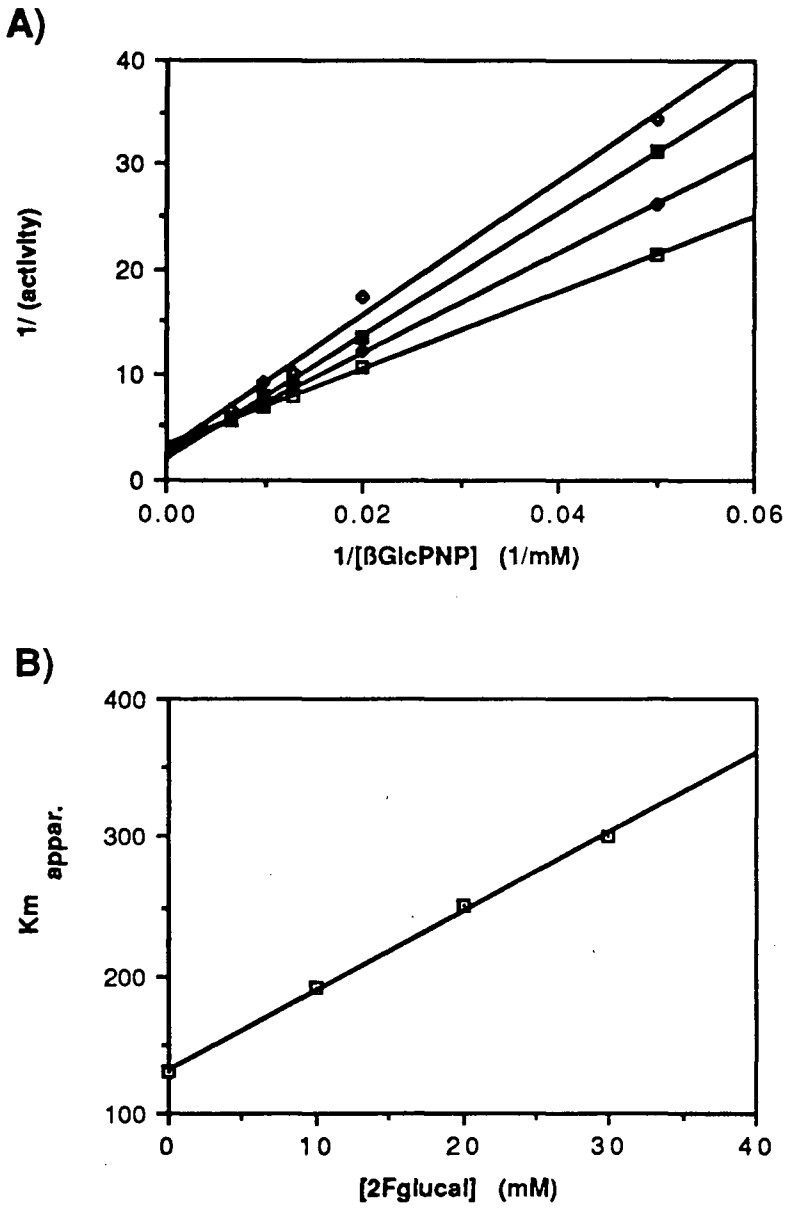


Figure 2-21. Reversible inhibition of pABG5 β-glucosidase by 2F-glucal.
Reactions were conducted at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. A) The following substrate (βGlcPNP) concentrations were used: 20, 50, 80, 100, 150 μM. The following concentrations of 2Fglucal were used: ❐, 0 mM; ◆, 10.0 mM; ■, 20.0 mM; ◊, 30.0 mM. B) A replot of $Km_{appar.}$ against 2Fglucal concentration, the following values of $Km_{appr.}$ were obtained: 131, 192, 252,301 μM.

Protection against inactivation by the "2F-glucal preparation" in the presence of a competitive ligand was also demonstrated. As previously, the competitive ligand used was βGlcS-iPr (Ki = 4 mM) and introduction of this into the inactivation mixture caused a marked decrease in the rate of irreversible inhibition (Figure 2-22). This provided further evidence that inactivation was occurring at the active site of the enzyme.

As was the case with the 2-deoxy-2-fluoro glycosyl fluorides we were able to demonstrate that inhibition was occurring by accumulation of a stable glycosylated-enzyme intermediate by its preparation and isolation. pABG5 β-glucosidase was inactivated at 37°C pH 6.8 with 93 mM "2F-glucal preparation", excess inhibitor removed by gel permeation chromatography and return of activity to the sample monitored in the presence or absence of 1dβGlcϕ. No spontaneous reactivation of the enzyme was observed (over a period of 2 hours), but on addition of 1dβGlcϕ return of activity followed an exponential time course the rate constant for which was dependent on the concentration of transglycosylation acceptor present in the reaction.

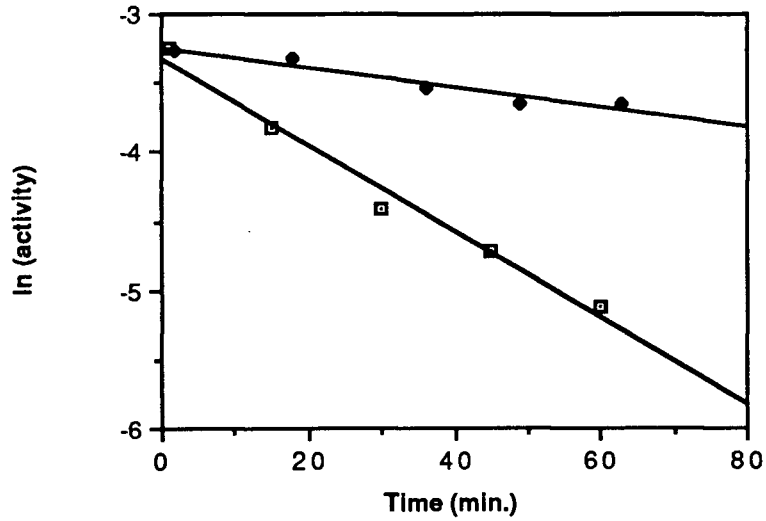
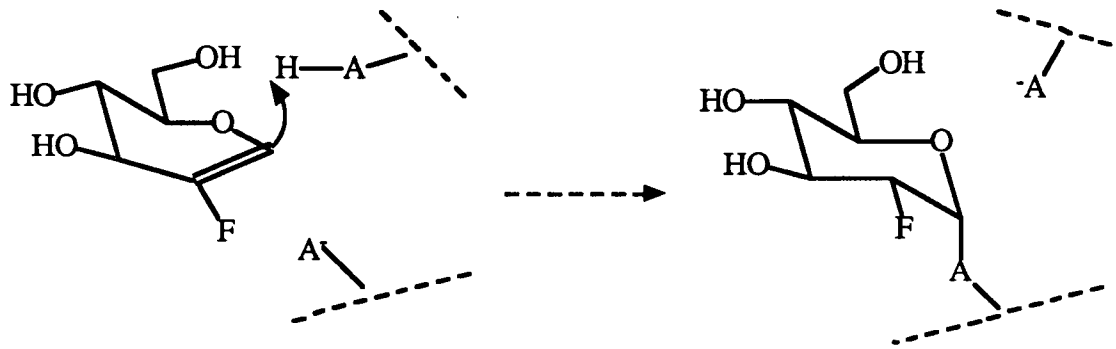


Figure 2-22. Protection against irreversible inactivation by a competitive ligand.

Inactivation of pABG5 β-glucosidase at pH 6.8 and 37°C in 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA. Inactivation at a single fixed concentration of 2Fglucal (19.0 mM) with the following concentrations of βGlcS-iPr: ❑ 0 mM, (k_{obs} = 0.033 min^{-1}), ◆ 20 mM, (k_{obs} = 0.007 $min.^{-1}$).

Extrapolation of the rate data to a saturating concentration of acceptor using the standard computer fitting technique yielded values of (4.5 ± 0.22) x 10^{-3} min.$^{-1}$ and 54 ± 5.7 mM for k_{trans} and K_d respectively. These values demonstrate that a 2FGlc-enzyme intermediate (k_{trans} = 5.3 x 10^{-3} min.$^{-1}$, K_S = 59 mM, very slow spontaneous reactivation) rather than a 2FMan-enzyme intermediate has been formed. This would indicate that the 2F-glucal had been protonated from the top face (β-face) of the double bond to give a 2-deoxy-2-fluoro *glucosyl* intermediate.



Scheme 2-12. A protonation scheme for 2F-glucal derived from kinetic experiments.

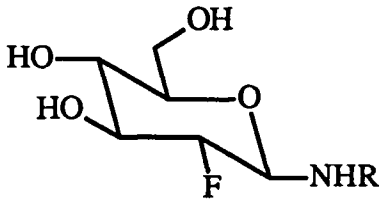
This protonation scheme is consistent with normal protonation of a glycosidic substrate in a "retaining" glycosidase, but is opposite to the direction expected for a substrate of this type on the basis of our studies with D-glucal. From the foregoing discussion on D-glucal, protonation from the bottom face would have been expected, which in the case of 2F-glucal would have produced a 2FMan- intermediate.

To try and resolve this conflict between the "2F-glucal" and the D-glucal results a further ^{19}F-NMR experiment was planned. The chemical shifts of both the glycosylated-enzyme intermediate and its turnover products with 1dβGlcϕ are characteristic of the type of intermediate formed. It was decided that because of the relatively slow inhibition and high dissociation constant of 2F-glucal it would be wise to perform the inactivation at high concentrations of the inhibitor and subsequently free the preparation from the excess before

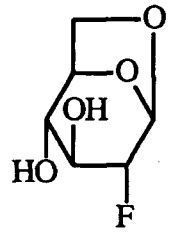
data acquisition. The inhibition reaction contained 0.4 μmol. of pABG5 β-glucosidase and 1.65 mmol. of "2F-glucal preparation" at pH 6.8 and its progress was followed using the standard techniques. Firstly it was noted that the inactivation rate was much slower than would have been anticipated from the kinetic experiments described earlier. Secondly the inhibition failed to go to completion even after 20 hours incubation. It seemed strange, to say the least, that a 4,000 fold excess of inhibitor should only inactivate 63% of the enzyme leading one to suspect that the inactivation was actually due to a small amount of a highly reactive inhibitory contaminant which was used up. To test this idea, the remaining 2F-glucal was separated from the enzyme using a Millipore CX-10 ultrafiltration membrane and concentrated by lyophilization. The organic soluble components from this mixture were extracted into ethyl acetate and purified by flash chromatography on silica gel as described in the original purification procedure for 2F-glucal in the Materials and Methods section. Inhibitor from this preparation was spectroscopically identical in every respect with the original sample of 2F-glucal, however it was found to be virtually non-inhibitory to pABG5 β-glucosidase.

Thus it would appear that the time dependent inhibition seen in the earlier experiments was due entirely to a contaminant in the preparation, and this only became apparent when large and near equivalent amounts of inhibitor and enzyme were required for the NMR sample. From the percentage inhibition observed in the preparation of this sample it was estimated that the contaminant composed approximately 0.015% of the "2F-glucal preparation", an amount well below the detection limits of most modern spectroscopic techniques. What makes this result even more remarkable is that the contaminant must have co-migrated with 2F-glucal on two chromatography columns during its preparation and that consistent kinetic data were obtained on two different batches of 2F-glucal prepared over eight months apart. Further the K_i and K_d values appeared to in reasonable agreement a result which is hard to explain as anything but bad luck. The identity of this inhibitor has not yet been discovered, but this should be pursued, as undoubtedly this compound is an excellent

inhibitor of β-glucosidase. Based on our knowledge of β-glucosidase and the structure of possible by-products which could be formed during the synthesis of 2F-glucal, the following compounds are suggestions for the structure of the inhibitory component in the 2F-glucal preparation.



2-deoxy-2-fluoro-β-glucosylamine



1,6-anhydro-2-deoxy-2-fluoro glucose

An important requirement of this inhibitor is that it must show a very high affinity for the enzyme, since based on the value determined for K_d in the irreversible inhibition experiments and the amount of inhibitor present in the 2F-glucal preparation (as calculated from the amount of enzyme inhibited in the preparation of the NMR sample) a K_d of ≈ 5 μM can be calculated. It should also be noted that this is a minimum estimate, as the 2F-glucal in the mixture, through competitive inhibition, would act to increase this value. An amine group at the anomeric centre might serve to provide the required high affinity, and such a compound (2-deoxy-2-fluoro β-glucosylamine) could have been formed from small amounts of contaminating primary amine present in the triethylamine used in the synthesis of 2F-glucal. A second possible structure is that of a 1,6-anhydro sugar, the reactive anhydro linkage would thus provide the good "leaving group" necessary to trap the enzyme as the covalent intermediate. Compounds of this type can be formed by treatment of glycosides with base, hence the compound 1,6-anhydro-2-deoxy-2-fluoro-β-D-glucose could have been formed from small amounts of residual per-O-acetylated glucopyranosyl bromide carried over from the elimination step in the synthesis of 2F-glucal. However, whether this unusual sugar structure would have fortuitously, resulted in the high binding affinity shown for pABG5 β-glucosidase remains untested.

A general survey of 2-deoxy-2-fluoro-glycosyl fluoride inhibition.

To conclude this section, I have included some discussion on a survey as to the general applicability of these 2-deoxy-2-fluoro glycosyl fluoride inhibitors to a series of the corresponding glycosidases. This work was carried out in this laboratory by K.Rupitz in conjunction with the work on pABG5 β-glucosidase and has been published (Withers et al, 1988). The inhibition experiments were performed on commercially available preparations of the enzymes under the appropriate reaction conditions.

The results of this survey are given in Table 2-V. It is possible to divide the results into three categories depending upon the type of inhibition kinetics (or lack thereof) that were observed; Group A: these enzymes are inactivated completely and in most cases extremely rapidly. Group B: complex inhibition patterns were observed which often did not cause complete inactivation of the enzyme. Group C: no inactivation was observed. It is possible to explain all three of these categories.using the kinetic scheme derived from the general reaction mechanism.

Group A.

This group contains three enzymes which like, pABG5 β-glucosidase, were all rapidly inactivated showing pseudo first order inactivation kinetics and giving well defined inactivation parameters. Inactivation of *E. coli* β-galactosidase was very rapid, the half life of inactivation at saturating concentrations of inhibitor being 3 seconds. Protection against irreversible inactivation was seen in all cases upon inclusion of a thio-glycopyranoside in the reaction mixture, demonstrating that inactivation was occurring at the active site. Thus these three enzymes all seem to fit into the same category as pABG5 β-glucosidase where the glycosylation rate is much greater than the deglycosylation rate and all of the enzyme rapidly accumulates as the covalent glycosylated enzyme complex.

Table 2-V. Inactivation of glycosidases by 2-deoxy-2-fluoro-glycosyl fluorides [a].

Enzyme	Inhibitor	Inactivation Kinetics
E. coli β-galactosidase	2FβGal F	Pseudo first order k_{on} = 13.2 min.$^{-1}$, K_d = 1.3 mM Protection by βGal S-iPr
A. oryzae β-galactosidase	2FβGal F	Pseudo first order k_{on} = 2.5min.$^{-1}$, K_d = 5.4 mM Protection by βGal S-iPr
A. niger β-galactosidase	2FβGal F	Pseudo first order k_{on} = 0.8min.$^{-1}$, K_d = 1.3 mM Protection by βGal S-iPr
Sweet Almond β-glucosidase	2FβGlc F	Complete Inactivation Complex Kinetics
Yeast α-glucosidase	2FαGlc F	Incomplete Inactivation Complex Kinetics
Bovine Liver β-galactosidase	2FβGal F	Incomplete Inactivation Complex Kinetics
A. niger α-galactosidase	2FαGal F	Incomplete Inactivation Complex Kinetics
Jack Bean α-mannosidase	2FαMan F	No inactivation
Almond α-mannosidase	2FαMan F	No inactivation
Coffee Bean α-galactosidase	2FαGal F	No inactivation

[a] Conditions as described in Withers et al., 1988.

Group B.

For this group of enzymes inactivation is seen, but according to complex kinetics. This situation might arise in several ways. One possibility is that the enzyme might be impure and contain several isozymes which inactivate at different rates. Such a situation might be expected in a number of these cases given the known propensity for glycosidases to exist in a number of isoenzymic forms (Legler, 1967). This is the most likely case for sweet almond β-

glucosidase as the commercial preparation is known to contain a number of isozymes (Weber and Fink, 1980) and no attempt was made to purify this preparation further.

Another possible cause of these inactivation kinetics could be a nearer equivalence of the rates for enzymic glycosylation and deglycosylation. Under these conditions it might be possible to produce a steady state concentration of the glycosylated-enzyme complex which only represents a fraction of the total enzyme. It can be seen from the derivation of the steady state rate expressions for the two step hydrolase that the steady state concentration of E-G is dependent on the ratio of k_{on} to k_{off}.

$$[E\text{-}G] = \frac{k_{on}}{k_{off}}[E \bullet GX]$$

Thus as k_{off} increases relative to k_{on} the steady state concentration of E-G will become less important in terms of the total enzyme. It can also be seen from steady state expressions (Equation 1) that the fraction of the total enzyme present as E-G will depend on the initial substrate concentration. A typical example of an inhibition time course for this group of enzymes is shown in Figure 2-23. The example given is that of yeast α-glucosidase. After an initial transient phase a steady state level of inhibition is seen which is dependent on the initial concentration of inhibitor present in the reaction.

It would be difficult to verify this hypothesis using the α-glucosidase as it would require both large amounts of enzyme and 2FαGlcF. We therefore considered that it might be possible to provide a reasonable model for this hypothesis on pABG5 β-glucosidase from a study of an inactivator with a relatively low rate of glycosyl-enzyme formation and a relatively high deglycosylation rate. Such a system could be furnished by inhibition of pABG5 β-glucosidase by 2FβGalF in the presence of 1dβGlcϕ. Under these conditions 1dβGlcϕ would act as both a competitive inhibitor (binding to E) and an acceptor in the transglycosylation reaction (binding to E-G).

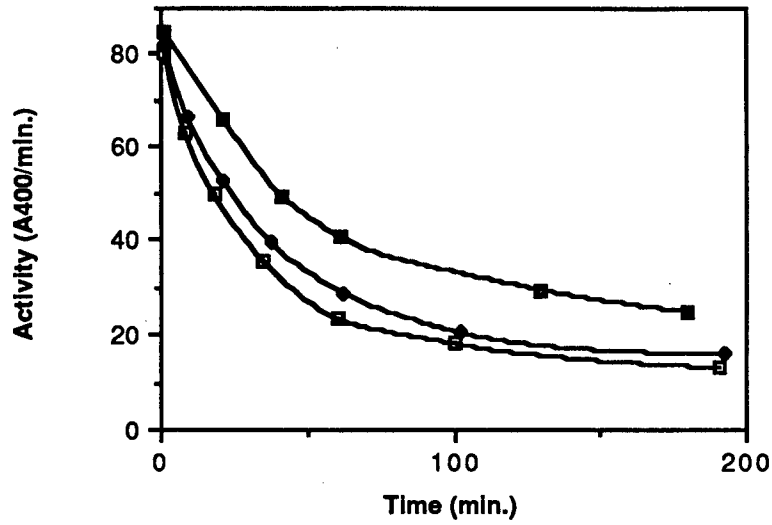
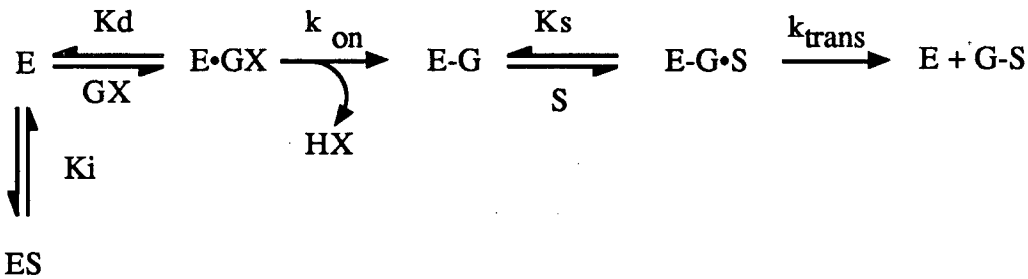


Figure 2-23. Time dependent inhibition of α-glucosidase (Type III) from yeast by 2FαGlcF. Reactions were carried out at pH 6.8 and 25°C in 50 mM sodium phosphate buffer containing 1mg mL^{-1} BSA. The following inhibitor concentrations are displayed in the graph: o 152 mM; u 95 mM; n 38 mM.



Scheme 2-13. A kinetic model for steady state fractional inhibition.

Thus the 1dβGlcϕ has two complementary roles, to increase the transglycosylation rate and via competitive inhibition of the initial glycosylation reaction, to decrease the rate of E-G formation. Both of these functions will act to reduce the steady state concentration of E-G. From Scheme 2-13 it is possible to derive an expression which allows prediction of the steady state fraction of E-G from the individual rate constants determined previously.

$$[E\bullet GX] = \frac{[E]\,[GX]}{K_d} \qquad [E\bullet S] = \frac{[E]\,[S]}{K_i} \qquad [E\text{-}G\bullet S] = \frac{[E\text{-}G]\,[S]}{K_s}$$

At the steady state

$$k_{on}\,[E\bullet GX] = k_{trans}\,[E\text{-}G\bullet S]$$

Substituting for [E•GX] and [E-G•S]

$$\frac{k_{on}}{K_d}[E]\,[GX] = \frac{k_{trans}}{K_s}[E\text{-}G]\,[S]$$

$$\frac{k_{on}\,K_s}{K_d\,k_{trans}}\,\frac{[E]\,[GX]}{[S]} = [E\text{-}G]$$

Also at the steady state

$$[E\text{-}G\bullet S] = \frac{[E\text{-}G]\,[S]}{K_s}$$

substituting for [E-G]

$$[E\text{-}G\bullet S] = \frac{k_{on}}{k_{trans}\,K_d}\,[GX]$$

At the steady state the fraction of the total enzyme which is present as E-G will be given by

$$\frac{[E\text{-}G]}{[E_o]} = \frac{[E\text{-}G]}{[E] + [ES] + [E.GX] + [E\text{-}G] + [E\text{-}G\bullet S]}$$

Substituting for the concentration terms and dividing by [E]

$$\frac{[E\text{-}G]}{[E_o]} = \frac{\dfrac{k_{on}\,K_s\,[GX]}{K_d\,k_{trans}\,[S]}}{1 + \dfrac{[S]}{K_i} + \dfrac{[GX]}{K_d} + \dfrac{k_{on}\,K_s\,[GX]}{K_d\,k_{trans}\,[S]} + \dfrac{k_{on}\,[GX]}{k_{trans}\,K_d}}$$

$$= \frac{k_{on}\,K_s\,[GX]}{K_d\,k_{trans}[S]\left(1 + \dfrac{[S]}{K_i}\right) + (k_{on} + k_{trans})[GX][S] + k_{on}\,K_s\,[GX]}$$

From this expression it can be seen that when [S] is not present then inhibition will go to completion (that is [E-G] / $[E_o]$ = 1) and also that the magnitude of this fraction will be dependent on both [S] and [GX].

The experimental results are presented in Figure 2-24 and a comparison of the experimentally observed values for the fraction [E-G] / $[E_o]$ with the values calculated from the above expression in Table 2-VI. The agreement between theory and experiment is quite good. While model reactions can never prove a particular reaction mechanism they do provide the basis for further experimentation and further work is planned with group B enzymes to try and verify this hypothesis.

Group C.

No inactivation is observed in this class of enzymes. This would be expected if $k_{on} < k_{off}$ or if k_{on} is zero, since in neither case would the intermediate accumulate. Experiments performed in this laboratory by C. Armstrong with α-glucosidase from yeast suggest that rate limiting glycosylation is, at least in some cases, a reasonable explanation. The compounds 2dαGlcPNP, 2FαGlcPNP and 2FαGlcDNP gave no time dependent inhibition of this enzyme, but all were found to act as slow substrates and at different rates.

Absence of inactivation would also be expected if the inhibitor binds extremely poorly. In addition, any glycosidase which does not catalyse hydrolysis according to the general mechanism depicted in Scheme 3-1 would not be expected to succumb to these inhibitors. The most probable examples would be glycosidases which catalyse glycoside hydrolysis with inversion of configuration, since the presumed mechanism for these enzymes does not involve a glycosyl-enzyme intermediate. Unfortunately, since the stereochemistry of hydrolysis has not been determined for all of the enzymes in question, this possible reason for the lack of inhibition remains untested.

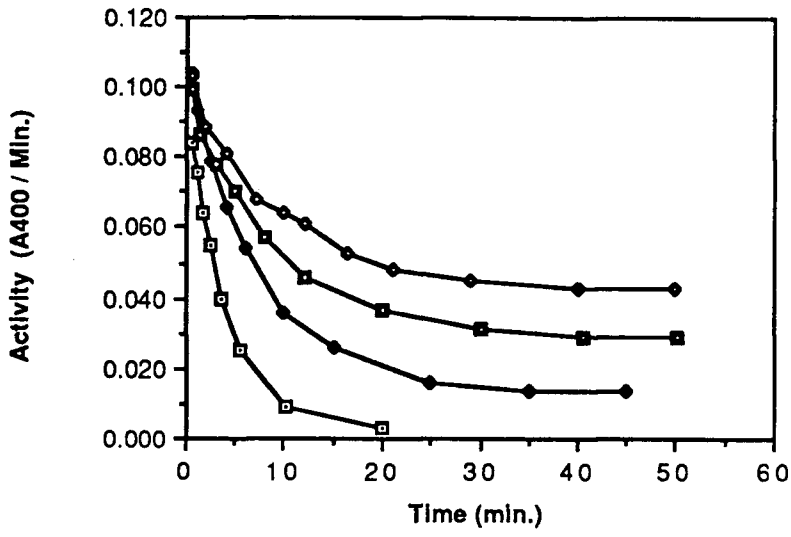


Figure 2-24. Steady State inhibition of pABG5 β-glucosidase by 2FβGalF and 1dβGlcΦ.

Reaction were conducted under the following conditions: In 50 mM sodium phosphate buffer at pH6.8 and 37°C. The buffer also contained 1 mg mL^{-1} BSA and enzyme activity was determined by dilution of a small aliquots into 1 mL of 3.0 mM βGalPNP at 37°C containing 1mg mL^{-1} BSA. The following concentrations of 1dβGlcϕ were used: ❑, 0 mM;◆, 3.83 mM; ■, 7.66 mM; ◊, 11.49 mM.

Table 2-VI. A comparison of experimentally observed [a] and calculated values [b] of [E-G]/ [E_o]

2FβGal F (mM)	1dGlc ϕ (mM)	$\frac{[E\text{-}G]}{[E_o]}$ Observed	$\frac{[E\text{-}G]}{[E_o]}$ Calculated
0.258	3.83	0.86	0.88
0.258	7.66	0.72	0.73
0.258	11.49	0.59	0.58
0.258	0	1.0	1.0
0.258	19.1	0.42	0.41
0.516	19.1	0.57	0.55
1.032	19.1	0.70	0.72

[a] Calculated from $\frac{\text{initial activity - steady state activity}}{\text{initial activity}}$. [b] Calculated using the following kinetic constants: K_d = 2.58 mM, K_i = 3.4 mM, K_s = 69 mM, k_{on} = 3.6 $min.^{-1}$, k_{trans} = 0.355 $min.^{-1}$.

CONCLUSION

It was postulated the compounds such as 2-deoxy-2-fluoro glycosides which bear a reactive leaving group would be mechanism-based inhibitors of "retaining" glycosidases because they should allow accumulation of a stable intermediate. These compounds were found to be effective inhibitors of a number of different glycosidases. The active site-directed nature of these inhibitors was demonstrated by showing "protection" against time-dependent inhibition in the presence of normal substrates (competitive ligands) and by showing that the effectiveness of these compounds as inhibitors closely resembled the specificity requirements of the enzymes for its natural substrates. Therefore these inhibitors are as specific as they can possibly be. Further, it was shown that inhibition involved the formation of a 1:1 enzyme-inhibitor complex, thus eliminating the possibility that inhibition was caused by non-specific alkylation reactions.

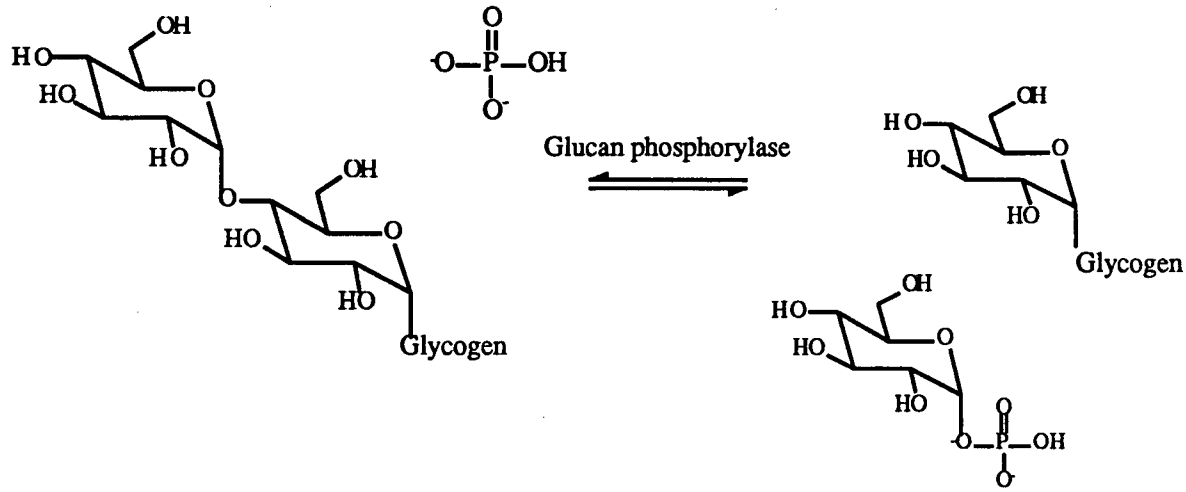
Retaining glycosidases conform to the general kinetic mechanism of a two-step hydrolase, the rate expression for which is dominated by the rate constants for the formation and decomposition of a glycosyl-enzyme intermediate. By using the techniques of pre-steady state kinetics it was found that the rates of hydrolysis of the glycosyl-intermediate formed by reaction of enzyme and inhibitor were 3 to 5 orders of magnitude slower than the rate of its formation. Thus, the enzyme is inhibited by its rapid accumulation as this stable intermediate. The glycosyl-enzyme intermediate was isolated at room temperature and characterized by kinetic and ^{19}F-NMR techniques and was found to be catalytically competent in that it could participate in the normal reactions expected for this type of intermediate; hydrolysis and transglycosylation. From a simple ^{19}F-NMR experiment it was demonstrated directly for the first time that the glycosyl-residue was covalently linked to the enzyme via an axial (α-) linkage, as would have been predicted from the double displacement mechanism proposed by Koshland and in keeping with the results obtained from other studies.

CHAPTER 3

Introduction.Glycogen Phosphorylase

Physiological Function and Regulation.

α-Glucan phosphorylases are found in organisms as phylogenetically diverse as bacteria and man, but they all catalyse a common reaction, the phosphorolysis of α-1,4-linked glucans.



Scheme 3-1. The reaction catalysed by α-glucan phosphorylase.

The equilibrium constant for the phosphorolysis reaction is 0.28 at pH 6.8 the equilibrium lying in favour of glycogen synthesis (Cori and Cori, 1940). However, under physiological conditions (high phosphate and low αGlcP concentrations) the reaction proceeds in the direction of glycogen degradation (Lazner et al., 1960).

The main physiological function of mammalian glycogen phosphorylase is to provide the tissue with a tightly regulated supply of phosphorylated glucose. This is liberated as required from a store of α–1,4–linked glucans, which in both animals and plants is the most common way of storing glucose. The catalytic and regulatory characteristics of individual enzymes will depend largely on the function of its host tissue. In muscle, this function is associated with the energy requirements of contraction and in the liver with maintenance of blood sugar levels. In tissues such as muscle which can go through a wide range of activity states in a very short period of time, glycogen phosphorylase must be responsive to the quickly changing metabolic needs. This has been accomplished by the evolution of a very complex control system, which involves both allosteric and covalent forms of regulation.

Glycogen phosphorylase exists in two forms; firstly, phosphorylase *b*, which is found in resting muscle. This form of the enzyme is normally inactive (existing predominantly in the inactive T state) and is subject to control by allosteric activators such as adenosine monophosphate (AMP), glycogen and high levels of inorganic phosphate. Phosphorylase *b* can also be inhibited by allosteric inhibitors such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), glucose and glucose-6-phosphate. This form of the enzyme requires AMP for activity.

The second form of the enzyme is known as phosphorylase *a*. Here the enzyme has been covalently modified by attachment of a phosphate residue to serine 14 (Fischer and Krebs, 1955) and exists predominantly as the active R-state enzyme. This form of the enzyme has largely escaped the control of allosteric activators, addition of AMP will only provide a small increase in activity (Morgan and Parmeggiani, 1964). However, like phosphorylase *b* this form of the enzyme is still inhibited by glucose.

Phosphorylase *b* is converted to the more active phosphorylase *a* by a specific protein kinase. The kinase is a calcium dependent enzyme and is under the control of the central nervous system. Augmenting this nervous control is a hormonally induced system known as the glycogen cascade (Krebs and Beavo, 1979; Cohen, 1978).

Structural Studies.

Both phosphorylase *a* and *b* can exist as tetramers or dimers of identical subunits in solution (Madsen and Cori, 1956; Madsen and Cori, 1955), the dimer-tetramer equilibrium depending on the activation state of the enzyme (Madsen and Cori, 1957). *In vitro*, in the absence of glycogen and other effectors, phosphorylase *a* is a tetramer and phosphorylase *b* a dimer (Dombradi, 1981). Activation of phosphorylase *b* by AMP shifts the dimer-tetramer equilibrium in favour of the tetramer. However, for both phosphorylase *a* and *b*, addition of glycogen dissociates all tetrameric species and it is likely to be the dimeric form which is of physiological importance.

The full amino acid sequence of the phosphorylase monomer has been determined (Titani et al., 1977) and found to have molecular weight of 97,432 (Johnson et al., 1987). Extensive crystallographic studies have been carried out on both phosphorylase *a* and *b*. In both cases moderately high resolution structures are available, 2.1 Å for phosphorylase *a* (Sprang and Fletterick, 1979; Sprang et al., 1982; Withers et al., 1982a) and 2.0 Å for phosphorylase *b* (Jenkins et al., 1981; Sansom et al., 1984; Johnson et al., 1987). The monomeric subunit is composed of two domains, the N-terminal domain extending from residues 1 to 489 and the C-terminal domain from residues 490 to 842. Each domain consists of approximately 25% β-sheet and 45% α-helix; the core of the domain containing most of the β-sheet which is surrounded by α-helical segments. Each subunit in the dimer is intimately associated with its partner, mainly through contacts within the N-terminal domains (see Figure 3-1).

All of the primary ligand binding sites have been located within the crystal structure. The phosphate residue attached to serine 14 can be seen near the AMP (activator) binding site. It has also been shown, mainly through studies with phosphorylase *b*, that ATP, ADP and glucose-6-phosphate also bind at this site (Lorek et al., 1980). The glycogen storage site is situated on the N-terminal domain. Both glycogen and smaller oligosaccharides such as maltopentaose bind strongly to this site while only weak interactions are observed with the active site. It has been estimated that the dissociation constant for maltoheptaose at the catalytic site is 20 fold greater than for the storage site (Kasvinsky et al., 1978). *In vivo* this glycogen binding site is thought to act as an anchor point, firmly affixing the enzyme to the glycogen molecule, and hence providing the enzyme with a high local concentration of the glycogen chain ends. Prior occupation of the oligosaccharide binding site has been shown to be an obligatory part of the kinetic mechanism (Kasvinsky et al., 1978).

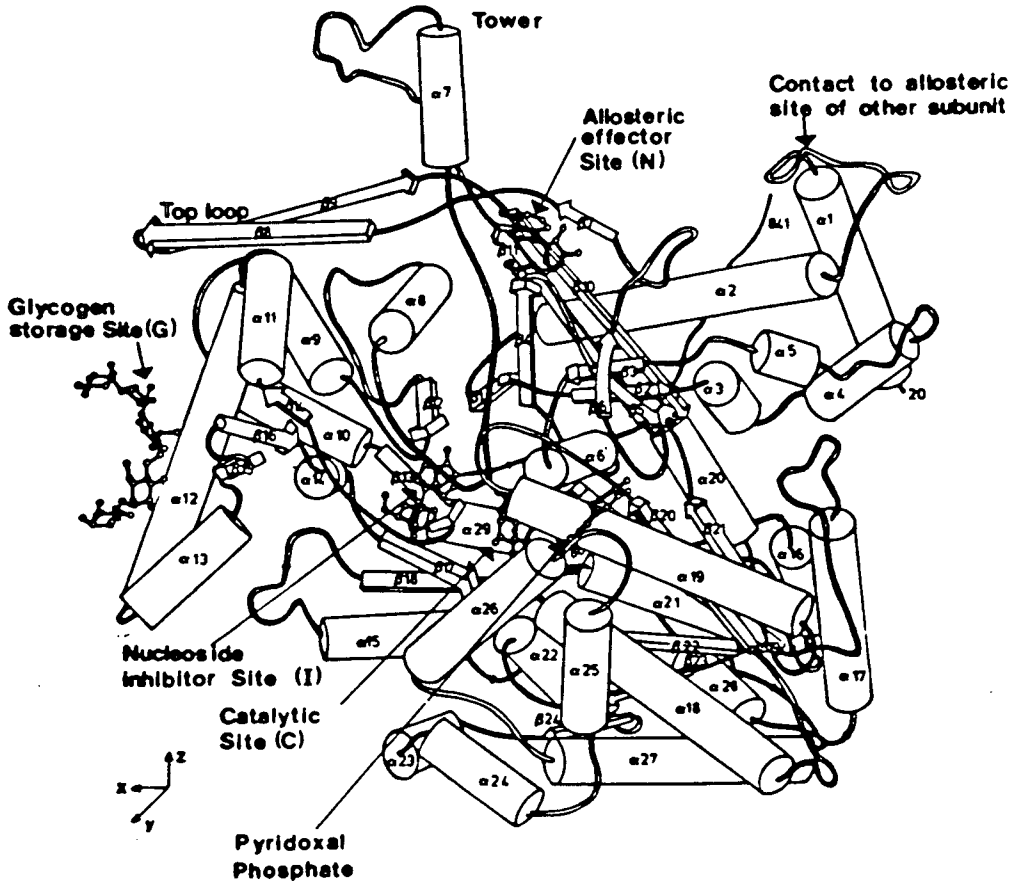


Figure 3-1. The three dimensional structure of glycogen phosphorylase.

A) A barrel and arrow representation of the phosphorylase b monomer, showing all of the primary ligand binding sites. Johnson et al., 1987.

A second inhibitor site (a purine binding site) is situated at the boundaries of the N- and C-terminal domains at the entrance to the active site cleft. This site appears to be specific for binding bicyclic and tricyclic aromatic molecules (for example caffeine), but as yet, its exact physiological function is not understood.

Glucose is found to bind in a high specificity pocket (which also comprises a part of the active site) in the N-terminal domain, with its anomeric hydroxyl group projecting into a cavity between the two domains. The glucopyranosyl moiety of αGlcP appears to bind in the same pocket with its phosphate group bound into a subsite directly adjacent. The remainder of the active site is composed of the cofactor subsite, where a molecule of pyridoxal 5'-phosphate is bound covalently by formation of a Schiffs base to lysine 680. The cofactor is buried in a nonpolar pocket some 15Å from the surface of the enzyme. Its 5'-phosphate group is positioned in a polar subsite which orients it towards the phosphate subsite of the substrate binding pocket.

The glucose binding site in both phosphorylase *a* and phosphorylase *b* has been characterized extensively both by X-ray crystallography (Sprang et al., 1982) and in a study which probed the specificity of the binding site by use of a wide variety of deoxy and deoxyfluoro sugars (Street et al., 1986). Substitution of a hydroxyl group by fluorine has a number of interesting consequences for potential ligand-protein interactions. The smaller size of a C-F group when compared to a C-OH group makes this a sterically conservative modification and therefore removes the possibility of unfavorable steric interactions between the modified ligand and binding site. While a hydroxyl group is capable of acting as both a donor and acceptor in hydrogen bonding interactions, its deoxyfluoro replacement cannot possibly act as a donor but can act as an acceptor, albeit weakly (Murray-Rust et al., 1983). Replacement of a hydroxyl group by hydrogen (deoxy sugars) of course removes any possibility of significant hydrogen bonding interactions between that position of the ligand and the protein.

It has often been suggested that hydrogen bonding interactions which occur between a protein and a bound ligand do not contribute significantly to the overall energetics of the binding process, since hydrogen bonds which are formed between the solvent and the solvated ligand are simply exchanged for hydrogen bonds between the ligand and the protein (see scheme below).

$$E\text{–}H\text{-}OH_2 + H\text{–}O\text{–}H\text{--}A\text{–}L \longleftrightarrow [E\text{–}H\text{--}A\text{–}L] + H\text{–}O\text{–}H\text{-}OH_2$$

Where E-H is a enzyme bound hydrogen bond donor and A-L is a hydrogen bond acceptor on the ligand.

Since there are two hydrogen bonds on each side of the equation, both number and types of hydrogen bonds are conserved in the binding process. However, it need not be the case that this process is energetically neutral, since the hydrogen bond geometries may be quite different. in the two cases. Thus the binding energy expressed results partially from enthalpic differences caused by these different hydrogen bonding geometries and partially from the increase in entropy associated with water release from the active site and the solvation shell of the ligand, into bulk water.

Removal of a hydrogen bonding group from the ligand (L) need not lead to the loss of the full energy of the hydrogen bond since in this case no hydrogen bond would exist between the modified ligand and water before binding, as shown in the following scheme.

$$E\text{–}H\text{-}OH_2 + H\text{–}O\text{–}H + L \longleftrightarrow [EH\ L] + H\text{–}O\text{-}H\text{-}OH_2$$

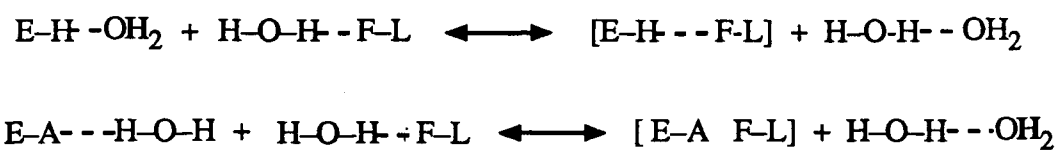
Thus any energetic differences described by these two equations will not be equal to the full hydrogen bond energy but to the differences in strengths of hydrogen bonds formed with the protein and with water. This could, however, be quite significant when comparing hydrogen bonds in an evolved enzyme-ligand complex with those formed between a relatively mobile water molecule and a protein residue.

By comparison of the dissociation constants for the modified and natural ligand a value for the free energy of binding of the substituent can be determined; $\Delta G_{bind} = - RT \ln [K_d/ K_{do}]$ where K_d and K_{do} are the dissociation constants for the modified and natural

ligand. This value has been attributed, as discussed above, to the difference in strengths of the individual hydrogen bonding interactions which occur between the ligand and the enzyme at the position of modification, that is the contribution of this substituent to the overall ΔG_{bind} (Fersht et al., 1985; Street et al., 1986). A later publication by Fersht (1988) shows that this interpretation is an over-simplification and in reality there are contributions from other factors. These arise from a value derived from the free energy of reorganization of the protein to accommodate these modifications and a term derived from the free energy difference between the normal and modified systems. However, these factors tend to be small when the substituent is involved in a neutral-neutral hydrogen bonding interaction and under these circumstances the value of ΔG_{bind} provides, to a close approximation, the difference in hydrogen bonding strengths of the substituent in the enzyme-ligand complex and with water. For substituents which participate in a neutral-charge interaction, under most circumstances the value of ΔG_{bind} is an overestimation of actual contribution of hydrogen bonding to the overall binding energy .

Two types of modifications to the ligand were used to map the hydrogen bonding interactions in the phosphorylase *b*-glucose complex, these were; deoxygenation (OH to H) and deoxyfluorination (OH to F). The former will result in the complete removal of hydrogen bonds, leaving a space in the molecule which might accommodate a water molecule in the enzyme-ligand complex, but which is probably too small. Fluorination results in very little size difference, certainly not permitting coincident binding of water, but is more complex since the fluorine could be involved in a hydrogen bond, but only as a hydrogen bond acceptor.

Thus two situations could be obtained with deoxyfluoro ligands, In one case the enzyme donates a hydrogen bond at that position, which the fluorine can accept. In the other case the enzyme accepts a hydrogen bond at that position and thus cannot interact favourably with the fluorine.



Since the fluorine can still hydrogen bond as an acceptor with water, there is a net loss of a hydrogen bond upon formation of the enzyme-ligand complex. In this case a considerable loss in affinity should result.and in such a situation the deoxy sugar might have greater affinity than the deoxyfluoro sugar.

Thus, by systematically preparing deoxy and deoxyfluoro analogues of each position on the glucose molecule and measuring their binding affinities to the phosphorylase *b* glucose site, Street et al. (1986) were able to define hydrogen bonding polarities and measure relative strengths of hydrogen bonds in the enzyme-glucose complex. It was found that the glucose binding site achieved a high degree of specificity through a complex network of hydrogen bonds and steric constraints which prevented the binding of unwanted epimeric sugars. These results agreed well with those obtained from an X-ray crystallographic study and are summarized in Figure 3-2 and Table 3-I. It was also noticed that the hydrogen bonding energies fell into two distinct ranges 0.5 - 1.5 kcal mol $^{-1}$ and 1.5 - 4 kcal mol $^{-1}$. By comparison with the recently refined crystallographic data the authors proposed that energetic contributions within the lower range arose from interactions between neutral-neutral hydrogen bonding partners while interactions in the higher range came from neutral-charged pairs. These conclusions were essentially identical to those reached by Fersht et al., (1985) using the complementary technique of site directed mutagenesis on tyrosyl t-RNA-synthetase.

<u>The Catalytic Mechanism: Analogies with the Reaction Mechanism of Glycosidases.</u>

The reaction catalysed by α-glucan phosphorylase is in fact a glucosyl transfer; glucose being transferred from phosphate to the 4-hydroxyl group of the terminal residue of the oligosaccharide acceptor. Thus there are many analogies which can be drawn between the

reaction mechanism of α-glucan phosphorylase and the transfer processes which are often catalysed by glycosidases.

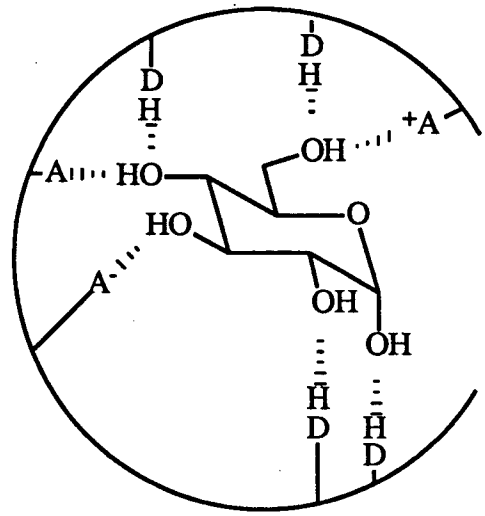


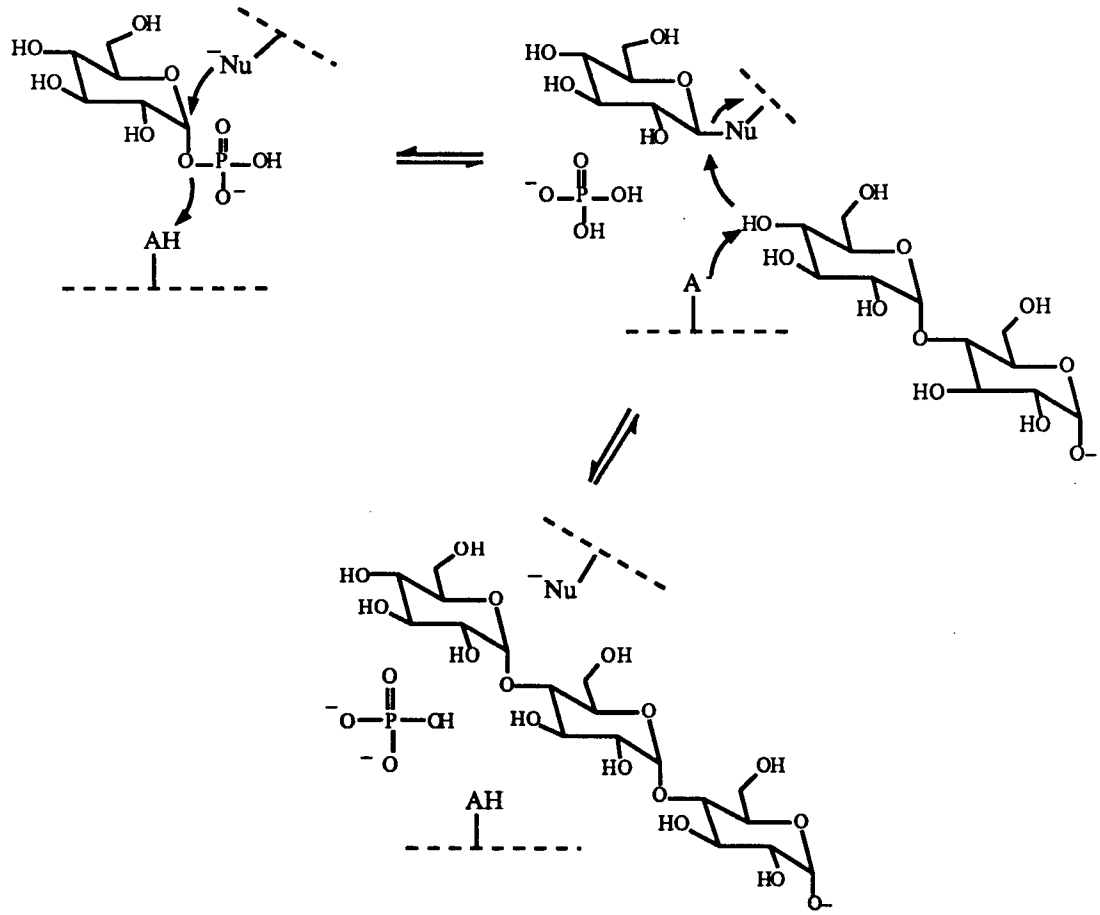
Figure 3-2. A schematic representation of the hydrogen bonding network in the glucose-phosphorylase b complex.

Table 3-I. Hydrogen bonding interactions in the glucose-phosphorylase a complex, as determined from X-ray crystallography.

Sugar hydroxyl	Protein residue, atom involved (donor or acceptor)	Distance (Å)	Angle oxygen
1-OH	Leu 136 N (D)	3.4	121
2-OH	Asn 284 Nδ2 (D)	2.9	106
	Tyr 573 OH (D/A)	3.3	146
3-OH	Glu 672 Oε2 (A)	3.1	119
	Ser 674 N (D)	3.1	126
4-OH	Asn 484 Oδ1 (A)	3.3	160
	Gly 675 N (D)	2.9	147
6-OH	His 377 Nδ1 (D)	2.7	147
	Asn 484Oδ1 (A)	2.8	131

Data from Street et al., (1986).

The general catalytic mechanism for α-glucan phosphorylase proposed long since (Cohn, 1949) (Scheme 3-2) includes many of the features of the glycosidase reaction mechanism; the involvement of a covalent glucosyl-enzyme intermediate, or a stabilized ion pair, acid catalysis to assist in departure of the aglycone (sugar or phosphate) and general base catalysis to assist attack from the incoming nucleophile. For dissaccharide phosphorylases such as sucrose phosphorylase, these analogies with the glycosidase reaction mechanism are easily observed, particularly since, Voet and Abeles (1970) were able to demonstrate conclusively the intermediacy of a covalently linked β-glucopyranosylated-enzyme. However, for polysaccharide phosphorylases such as glycogen phosphorylase most of these analogies are implied rather than demonstrated. Indeed a mechanism which does not involve a glucosyl-enzyme-intermediate has recently been proposed (Klein et al., 1986).



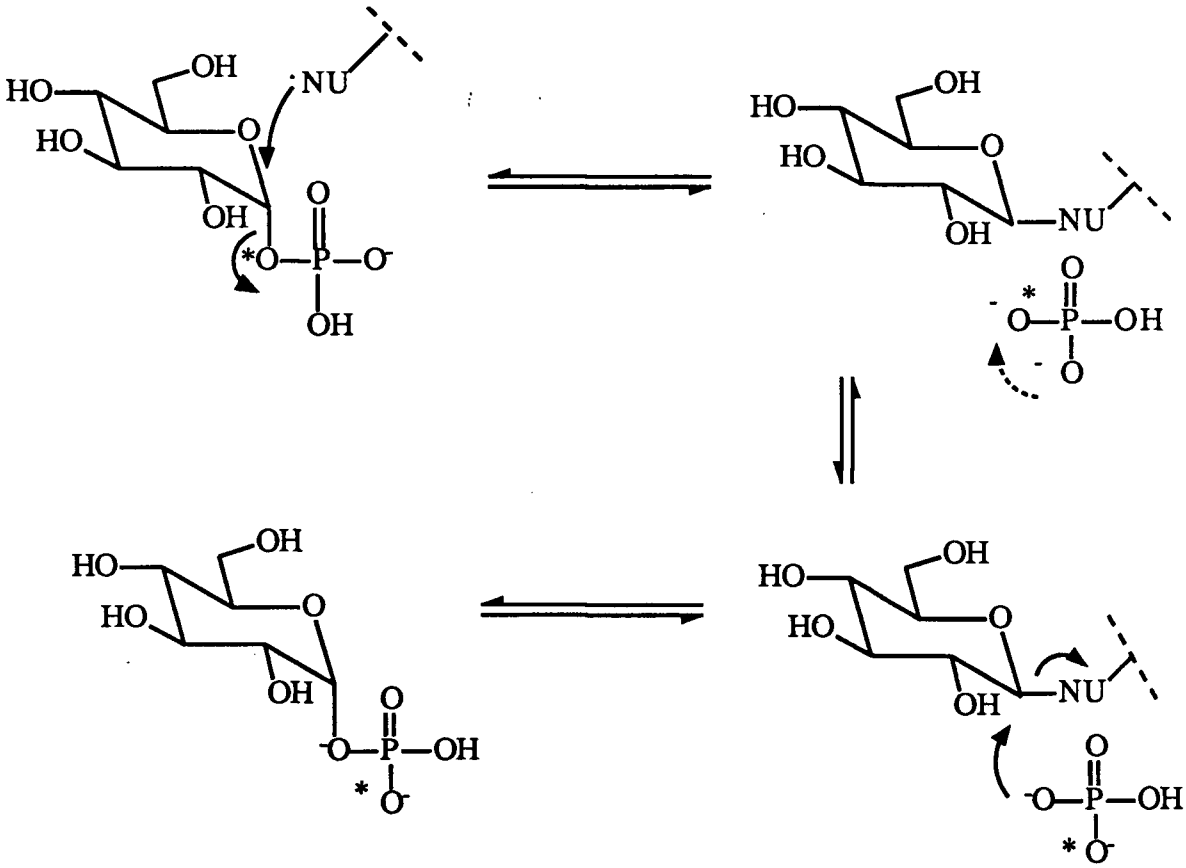
Scheme 3-2. A general mechanism for α-glucan phosphorylase.

Glycogen phosphorylase catalyses its reaction with retention of configuration at the anomeric centre, thus it is quite possible that the reaction does proceed through an enzyme intermediate of some sort. However, the evidence for such an intermediate can at best be considered unconvincing. An intermediate has never been isolated or trapped, although Klein et al., have provided preliminary evidence for a covalent intermediate in the reaction of D-glucal with potato phosphorylase and by analogy with sucrose phosphorylase have suggested that a carboxylate might be involved (Klein et al., 1981). These authors were able to demonstrate that in the presence of labelled D-glucal, arsenate (which can act as a substrate replacing phosphate) and a pseudo substrate, a cyclodextrin, α-glucan phosphorylase from potato was quantitatively labelled by D-glucal. The cyclodextrin is a cyclic oligosaccharide, which means of course that it does not possess a free 4-hydroxyl group and cannot therefore act as a glucosyl acceptor. On separation of the labelled enzyme and subsequent incubation with normal oligosaccharide the labelled glucosyl residue was transferred to the oligosaccharide acceptor.

The need for the presence of the cyclodextrin (or oligosaccharide) can be explained in terms of the kinetic mechanism of α-glucan phosphorylase which has been determined as a random rapid equilibrium bi bi, with the rate determining step being the interconversion of the ternary enzyme-substrate complex (Maddaiah and Madsen, 1966; Chao et al., 1969; Engers et al., 1970a; 1970b; Gold et al., 1970). From this mechanism it follows that no bond cleavage occurs, or at least no product is released until, the ternary enzyme-substrate complex is fully formed.

A number of workers have tried to detect the presence of an intermediate by looking for molecular exchange or positional isotope exchange (see Scheme 3-3).Cohn and Cori (1948) have tried to detect an intermediate by looking for molecular exchange between ^{32}P-labelled phosphate and αGlcP in the absence of a second substrate. However as might have been predicted from the kinetic model this is unlikely to occur at a significant rate in the absence of a productive ternary enzyme-substrate complex and indeed no exchange was

observed. Likewise, attempts to observe positional isotope exchange between bridging and non-bridging oxygens in αGlcP in the absence of a second substrate were unsuccessful (Gold and Osber, 1972). In this case some positional isotope exchange was observed with phosphorylase *a* in the absence of glycogen, but at only 0.3% of the rate expected from catalysis. Greater success was achieved using potato phosphorylase, where, by using a cyclodextrin as a "dead end" second substrate a non-productive ternary enzyme-pseudo substrate complex could be formed and isotope exchange was observed at similar rates to those expected from catalysis (Kokesh and Kakuda, 1977). This experiment provides the best evidence to date for the participation of a glucosyl intermediate in reactions catalysed by α-glucan phosphorylase.



Scheme 3-3. Isotopic exchange between bridging and non-bridging oxygens via an enzyme intermediate.

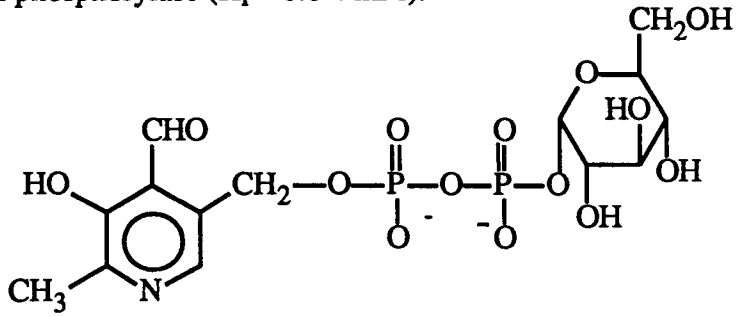
Evidence for the involvement of an oxocarbonium ion-like transition state is also rather inconclusive. Two studies, both of which determined kinetic isotope effects (KIE's) have provided conflicting results. Tu et al., (1971) prepared both the C-1 deuterio and protio αGlcP, and using these compounds they observed an secondary α-deuterium KIE (k_H / k_D ratio of 1.1) with phosphorylase *b*. These results were interpreted as being consistent with a considerable increase in sp^2 character on going to the transition state. Firsov et al., (1974) obtained a result more in keeping with a S_N2 like transition state (k_H / k_D or $k_H / k_T = 1.0$). However this could also be due to some other step being rate determining, such as a rate limiting conformational change of the ternary enzyme-substrate complex. The difference in these two results might be explained in terms of the different methodology used by these two different groups. Tu et al. simply compared rates of glycogen synthesis from the C-1 protio and C-1 deuterio derivatives of αGlcP, while Firsov et al. used doubly labelled compounds which enabled direct competition between the different C-1 isotopomers to be monitored in a single experiment. Given the tendency of glycogen phosphorylase towards erratic kinetic behaviour and the sensitivity to many different types of inhibitors, the direct competition method will provide the more reliable data.

The suggestion that a protein conformational change may in some way be rate determining is likely in the light of recent evidence provided by a ^{19}F-NMR study of phosphorylase *b* containing a modified cofactor 6-fluoropyridoxal phosphate (Chang and Graves, 1985; Chang et al., 1986). This ^{19}F-NMR data suggested different environments for the cofactor in the enzyme-αGlcP-glycogen complex and enzyme-phosphate-glycogen complex and it was suggested that catalysis occurred in two steps; 1) a conformational interconversion. 2) Chemical steps of bond breaking and bond making. Further, the rate of this enzyme ($k_{cat} \approx 100\ s^{-1}$) is similar to that of many enzymes for which the rate determining step is known to be a conformational change.

Within the context of the reaction mechanism of glycosidases, evidence for the oxocarbonium ion-like structure of the transition state was advanced from both KIE's and in

the form of tight binding "transition state analogues". For glycogen phosphorylase we have already seen that the results obtained from KIE's can be interpreted in a number of different ways and as might have been expected evidence obtained from potential transition state analogues is also suitably vague. Glucono-1,5-lactone is a reasonable inhibitor (K_i = 1 mM) (Tu et al., 1971; Gold et al., 1971), but when compared to the binding of glucose (K_i = 2 mM) this can hardly be considered a "tight binding" transition state analogue. However the study of Gold et al., (1971) demonstrated that the affinity of glucono-1,5-lactone was 40-fold greater for the enzyme-glycogen phosphate complex (K_i = 0.025 mM). This increase in affinity might be considered to arise as a consequence of providing the other half of the transition state structure (the phosphate), which then allows the enzyme-gluconolactone-phosphate complex to adopt a truly "transition state-like" conformation. Other compounds which have proven to be powerful inhibitors of glycosidase like nojirimycin were found to be virtually non-inhibitory towards glycogen phosphorylase (Ariki and Fukui, 1977). The compound gluconohydroximino-1,5-lactone-N-phenyl urethane also a good inhibitor of β-glucosidase, is a reasonable inhibitor of glycogen phosphorylase *b* (K_i = 0. 6 mM), but was found to promote the inactive T-state form of the enzyme (Johnson, L.N. personal communication). This can hardly be described as a desirable attribute in a transition state analogue.

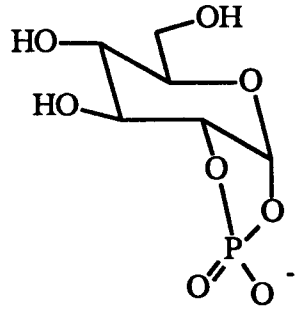
There are a number of other compounds which bind tightly to glycogen phosphorylase. Withers (1985) found pyridoxal diphospho-α-D-glucose to be a potent R-state inhibitor of glycogen phosphorylase (K_i = 0.0 4 mM).



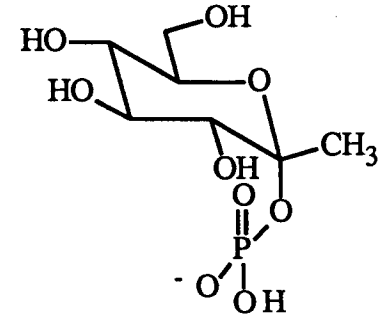
pyridoxal (5')-diphospho 1-α-D-glucose (PLPPαGlc)

Because the inhibitor bears a free aldehyde group there is a possibility that the tight binding of this inhibitor is a result of covalent imine formation with an amino acid residue on the protein. This was demonstrated not to be the case, since upon reduction of the pyridoxal-aldehyde functionality, the resulting compound retained most of its inhibitory properties. It was also demonstrated that binding of this compound to phosphorylase *b* promoted the active R-state conformation of this enzyme. The strength of the interactions between this inhibitor and enzyme are probably not related to any resemblance of this molecule to the enzymic transition state, but due to fortuitous interactions at the active site. Since the active site is known to contain a large number of basic groups (Withers et al., 1982a), such a compound bearing an extra phosphate would be capable of additional attractive electrostatic and hydrogen bonding interactions, plus hydrophobic interactions with the aromatic ring of the enzyme bound pyridoxal cofactor. Indeed, this type of aromatic stacking interaction has recently been seen from the X-ray crystallographic examination of the inhibitor uridine(5')diphospho(1)-α-D-glucose-phosphorylase *b* complex (Oikonomakos et al.,1988) and also in the gluconohydroximino-1,5-lactone-N-phenyl urethane-phosphorylase *b* (L.N. Johnson, personal communication).

There are two compounds glucose-1,2-cyclic phosphate (Hu and Gold, 1978; Kokesh et al., 1977; Withers et al.,1981a) and heptulose 2-phosphate (Klein et al,1984) that have been considered to be transition state analogs by virtue of the position that their phosphate groups occupy in the active site.



Glucose-1,2-cyclic phosphate



Heptulose 2-phosphate

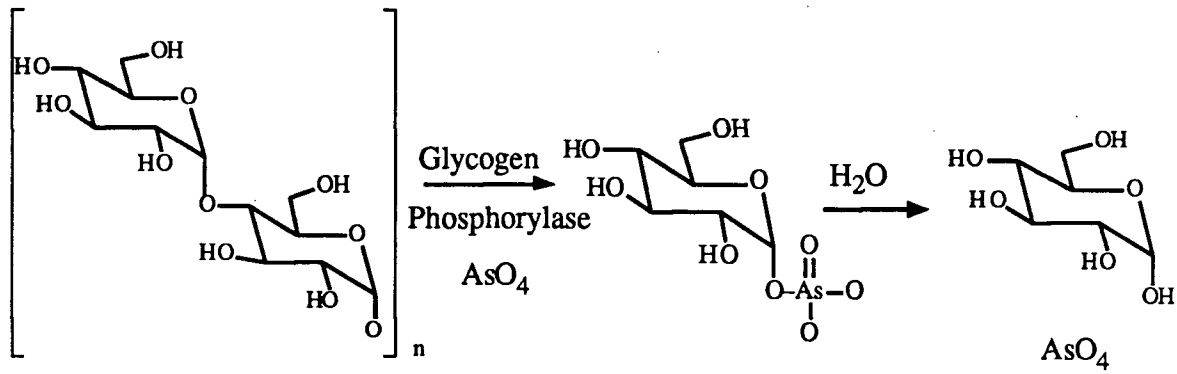
Both of these compounds have preferred conformations with the phosphate group oriented under the C-2 hydroxyl group of the sugar ring, as opposed to αGlcP which preferentially orients its phosphate group away from the ring (trans to C-2) (O'Connor et al.,1979). Indeed, heptulose 2-phosphate has been observed by X-ray crystallography to adopt this conformation while bound to the active site of glycogen phosphorylase *b* (McLaughlin et al., 1984). Both of these compounds are also potent inhibitors of glycogen phosphorylase (glucose-1,2-cyclic phosphate, K_i = 0.2 mM), heptulose 2-phosphate (K_i = 0.014 mM). In contrast to this the enzyme's natural substrate αGlcP which does not readily adopt this "under the ring" conformation of its phosphate group binds to phosphorylase *b* comparatively poorly (K_m = 2 mM). Heptulose 2-phosphate is one of the tightest binding inhibitors known for phosphorylase and it has been suggested that the unusual conformation adopted by its phosphate group is close to that adopted by the phosphate group of the normal substrate during catalysis.

Evidence for an Essential Acid Catalyst .

Continuing the analogies with the properties of glycosidase-catalysed reactions, it may be remembered that a number of compounds known as glycosylic compounds (D-glucal, heptenitol, and glucosyl fluoride) were efficient substrates for glycosidases. These compounds are also substrates for glycogen phosphorylase, although they also require the addition of one or more cosubstrates (inorganic phosphate or arsenate, oligosaccharide acceptor) before catalysis is observed.

D-glucal is a particularly efficient substrate for a number of α-glucan phosphorylases (Klein et al., 1982), the maximal rate of utilization of this substrate being 20 to 30% of the rate of glycogen synthesis with αGlcP. Upon incubation of α-glucan phosphorylase with D-glucal, inorganic phosphate and an oligosaccharide acceptor, the products are a 2-deoxyglucosylated-polysaccharide and 2dαGlcP, the latter product probably being derived

from the normal degradation of the modified polysaccharide. If phosphate is replaced by arsenate then the ultimate product is 2dGlc. Arsenate can take the place of phosphate in many phosphorylase-catalysed reactions and is often used as an alternate substrate in glycogen degradation. Under these conditions the initial reaction product is presumably the hydrolytically labile 1-arsenate ester of glucose, which then hydrolyses rapidly under the assay conditions to give glucose and arsenate. As these two compounds are not substrates in the direction of glycogen synthesis, the degradation of glycogen in the presence of arsenate is essentially irreversible.



Scheme 3-4. The arsenolysis of oligosaccharide by α-glucan phosphorylases.

Thus the production of 2dGlc from D-glucal by phosphorylase in the presence of arsenate is analogous to the reaction in the presence of phosphate, except that the degradation of the 2-deoxyglucosylated-polysaccharide is driven by the irreversible hydrolysis of the 1-arsenate ester of 2dGlc.

The stereochemistry of the protonation step in the D-glucal reaction catalysed by glycogen phosphorylase *b* has been probed by deuterium incorporation (Klein et al., 1982). For this enzyme utilization of D-glucal in deuterated buffers resulted in the production of 2-deoxy sugars with the deuteron incorporated into the equatorial position of C-2, thus protonation occurs from below the α-face of the sugar.

Heptenitol, which like D-glucal has a reactive double bond, but one which is exocyclic to the ring, is also a substrate for glycogen phosphorylase. However reaction of

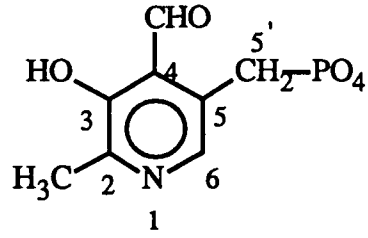
this compound in the presence of inorganic phosphate produces heptulose 2-phosphate as the only product, and no heptulosyl acceptor-saccharides are formed. Indeed the presence of saccharide acceptor is not even required for reaction to take place (Klein et al., 1986). As mentioned earlier heptulose 2-phosphate is also a potent competitive inhibitor of glycogen phosphorylase and after production of small amounts of this product further turnover is effectively blocked. The production of heptulose 2-phosphate is thus known as a "dead end" reaction. Given that this reaction also does not require the presence of any saccharide acceptor, heptenitol is classified as a substrate exclusively for the degradative pathway.

The compound αGlcF is also a substrate for α-glucan phosphorylase, although it is the poorest of these alternate substrates. As with D-glucal incubation of αGlcF with enzyme, phosphate and saccharide acceptor results in the transfer of a glucosyl residue from the substrate to acceptor (Palm et al., 1983), but at exceedingly slow rates.

The Pyridoxal 5'-Phosphate Cofactor.

Glycogen phosphorylase contains one molecule of the cofactor pyridoxal-5'-phosphate (PLP) per enzyme monomer (Baranowski et al., 1957). This cofactor is essential for activity (Illingworth et al., 1958). Its role in catalysis however, is unique amongst PLP containing enzymes, in that the aldehyde group of the cofactor is not required for catalytic activity. This was demonstrated by Krebs and his coworkers (Fischer et al., 1958), who found that under mildly acidic conditions the imine linkage between the cofactor and the enzyme could be reduced while retaining most of the enzyme's activity. Many groups have since demonstrated, by means of replacing PLP with a variety of structural analogues that the 5'-phosphate group on the cofactor is essential for activity of the enzyme. For reviews of this work see Graves and Wang, (1972) and Shaltiel et al., (1969). A small selection of these analogues and their effects are presented in Table 3-II.

Table 3-II . Reconstitution of Apophosphorylase b with analogues of PLP.



Pyridoxal Analogue	Modified Position	Modification	% Reactivation	Evidence for Binding
None	–	–	0	–
Pyridoxal 5'-phosphate	–	–	100	+
[a] N-methyl PLP	1	$-CH_3$	0	-
[b] N-Oxide PLP	1	–N–O	25*	+
[a] 2-Nor PLP	2	–H	65	+
[a] 3-O-methyl PLP	3	$-OCH_3$	25	+
[c] 3-O-methyl N-oxide PLP	3 & 1	$-OCH_3$ & N-O	0	+
[a]Pyridoxamine 5'-phosphate	4	$-CH_2NH_2$	0	-
[a]4-deoxypyridoxine phosphate	4	$-CH_3$	0	-
[a] Lys 679 linkage reduced	4	$-CH_2-NH_2$	60	-
[d] 4-vinyl PLP	4	$-C=CH_2$	7	+
[e] Pridoxal	5	$-CH_2OH$	0	+
[f] Pyridoxal + Phosphite	5	HPO_3	24	+
[f]Pyridoxal + Fluorophosphate	5	$HFPO_3$	12	+
[g] Deoxypyridoxalyl methane phosphonic acid	5	$-CH_2PO_3$	25	+
[a] Pyridoxal 5'-sulphate	5	$-CH_2OSO_2$	0	+
[h] Pyridoxal 5'-fluorophosphate	5	$-CH_2OPFO_2$	0	+
[c] Pyridoxal methyl ester	5	$-CH_2PO_3OMe$	0	+
[a] 6-Methyl PLP	6	$-CH_3$	8	+
[i] 6-Fluoro PLP	6	–F	28	+

* Some of the bound derivative revert to the natural coenzyme. Table from Madsen and Withers (1984). [a] Shaltiel et al. 1969: [b] Fisher et al., 1970 and Pfeuffer et al., 1972: [c] Pfeuffer et al., 1972: [d] Graves and Wang, 1972 and Graves et al., 1978: [e] Fisher et al., 1958 and Illingworth et al., 1958: [f] Parrish et al., 1977; Chang et al., 1983: [g] Vidgoff et al., 1974: [h] Klein et al., 1982 and Withers et al., 1982a: [i] Chang and Graves, 1984.

From the data in this table it can be seen that with the exception of the 1- and 5-positions of the cofactor, modifications are tolerated to varying extents, as evidenced by the ability of the analogue to both bind and restore a measure of activity to apophosphorylase *b*. On this basis it is tempting to suggest that the nitrogen (N-1) of PLP plays some role in catalysis. However, this is rendered less likely from results obtained with 6-fluoro PLP, since this analogue both binds to, and restores some activity to apophosphorylase *b*, but the pKa of the pyridine is reduced by over 6 units upon fluorination.

Removal of the 5'-phosphate from PLP completely destroys the ability of the cofactor to restore activity to apophosphorylase, though it still binds to the enzyme. This suggests that the 5'-phosphate plays an important role in catalysis or may be important in maintaining the active structure of phosphorylase. The studies of Graves and his colleagues have served to focus attention on these proposals (Parrish et al., 1977; Chang et al., 1983). They noted that apophosphorylase *b* which had been reconstituted with pyridoxal (PL-phosphorylase) could to be reactivated to a considerable extent (12 - 30%) by addition of phosphate or the phosphate analogues, phosphite or fluorophosphate.

They suggested that the phosphate and phosphate analogues activated the enzyme by occupying the vacant phosphate binding site adjacent to the pyridoxal ring and fulfilling the role of the missing cofactor-phosphate. This proposal has been supported by recent crystallographic evidence obtained from crystalline PL-phosphorylase *b* (Oikonomakos et al., 1987) These results have shown that the phosphite does indeed bind in a site directly adjacent to the PL cofactor, but that it is translated by ≈1Å from the site normally occupied by the covalently bound phosphate of PLP.

Evidence for Direct Phosphate-Phosphate Interaction During Catalysis.

Evidence that direct phosphate-phosphate interaction between the substrate and cofactor is essential for catalysis has been gathered from both X-ray crystallographic and solution studies.

Crystallographic studies on phosphorylase *a* and the inhibitor glucose-1,2-cyclic phosphate indicated that the glucopyranose ring of the inhibitor bound in the same binding site as did glucose (with a slight translation) and the cyclic phosphate group was oriented towards the phosphate group of PLP (Withers et al., 1982a). In this crystalline form of the enzyme, a phosphorus–phosphorus distance of ≈ 6.8 Å was measured. The inter-phosphorus distance is decreased to 4.8 Å in the phosphorylase *b*-heptulose 2-phosphate complex (McLaughlin et al., 1984) and in this structure it is possible that hydrogen bonding interactions could be formed between the two phosphate groups. However it must be remembered that both the crystalline forms of phosphorylase *a* and phosphorylase *b* are of the largely inactive T-state enzymes, and that the structural changes which occur upon introduction of R-state effectors are undoubtedly attenuated by crystal packing forces and at best represent only a qualitative picture of a small part of the T- to R-state transition. Much larger structural changes occur if both αGlcP and oligosaccharide are introduced into the crystal; so large that cracking of the crystal often results.

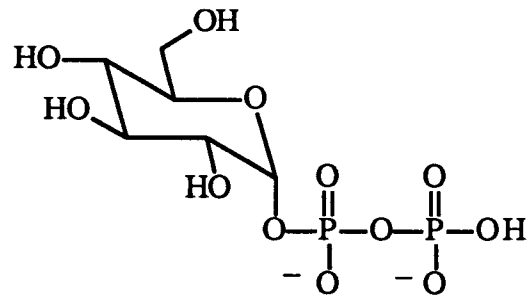
Because of the difficulties associated with obtaining a crystal structure of the fully active R-state enzyme, perhaps the strongest evidence for direct phosphate-phosphate interactions during catalysis has come from solution studies. Using PL-phosphorylase *b* Parrish et al., (1977) were able to demonstrate that pyrophosphate was a good inhibitor of phosphite activation. They demonstrated that stoichiometric amounts of pyrophosphate were incorporated into the PL-phosphorylase and that this inhibitor was competitive with both αGlcP and phosphite. This led these authors to suggest that there existed two phosphate binding sites which were adjacent to each other in the active enzyme (one in which the 5'-phosphate of PLP bound and the other for the substrate). This idea was latter extended by Withers and his colleagues (Withers et al., 1982a) who used a series of alkane diphosphonates with different numbers of bridging methylene groups to probe the interphosphate distance in PL-phosphorylase *b*. Methylene, ethylene and propylene diphosphonates were all inhibitory to the phosphite-mediated activation of this enzyme, and

were found to be competitive with both phosphite and αGlcP, demonstrating that these inhibitors were behaving in the same manner as pyrophosphate. The most effective inhibitor of the three diphosphonates was found to be the methylene diphosphonate and assuming that this compound bound to the enzyme in its normal extended conformation a distance of ≈3 Å was calculated to separate the two phosphate binding sites in the active enzyme.

Further evidence that phosphate-phosphate interaction is important for both allosteric activation and/or catalytic activity has been obtained from a number of different cofactor/substrate analogues. The first of these analogues was the compound pyridoxal 5'-diphospho α-D-glucose (PLPPαGlc, see page 15), which contains all of the potential participants in the catalytic reaction, but covalently linked. When added to apophosphorylase this compound readily produces an enzyme which will slowly catalyse the cleavage of the glycosidic bond to yield enzyme containing covalently bound pyridoxal 5'-pyrophosphate. The rate of this reaction is very slow ($t_{1/2} > 2$ days) but is greatly speeded up by introduction of oligosaccharide into the reaction mixture ($t_{1/2} < 30$ min.) (Withers et al., 1981b). Fukui and his colleagues, later demonstrated that the glucosyl residue of PLPPαGlc was transferred to glycogen (Tagaya and Fukui, 1984; Tagaki et al., 1982) in this reaction.

Withers et al. (1982c) have demonstrated that occupation of both the phosphate sites is also important for the allosteric activation of glycogen phosphorylase. Apophosphorylase *b* reconstituted with pyridoxal 5'-pyrophosphate was found to be locked in the active R-state conformation normally adopted by phosphorylase *b* only in the presence of substrates and nucleotide activators. This was evidenced by the observation that this covalently activated form of the enzyme bound R-state effectors such as AMP with high affinity, while T-state effectors such as glucose and caffeine did not bind at all.

A compound which covalently links three out of the four potential participants in the phosphorylase catalysed reaction, α-glucose 1-pyrophosphate is also a substrate for PL-glycogen phosphorylase (Klein et al., 1984).



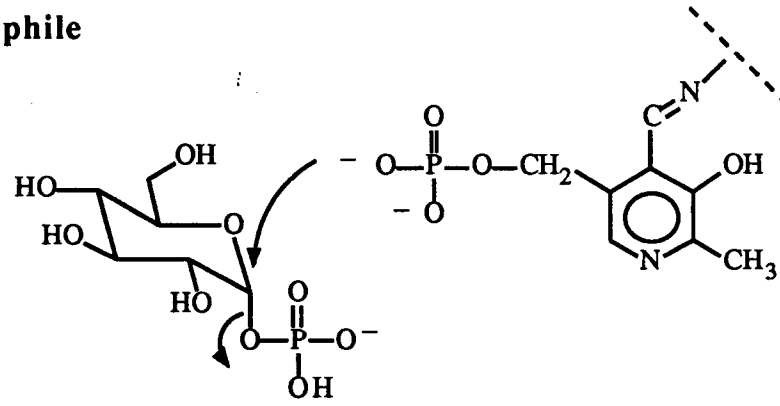
α-glucose 1-pyrophosphate

As would be expected, by analogy with the PLPPαGlc reaction, incubation of the above compound with PL-phosphorylase *b* in the presence of AMP and glycogen produces pyrophosphate, with concomitant transfer of the glucosyl residue to the polysaccharide-acceptor. The activity of this compound along with the preceding analogs serves to suggest that while occupation of the adjacent phosphate binding sites is important for catalytic activity, the covalent linkage between phosphate and the enzyme-bound cofactor is not essential.

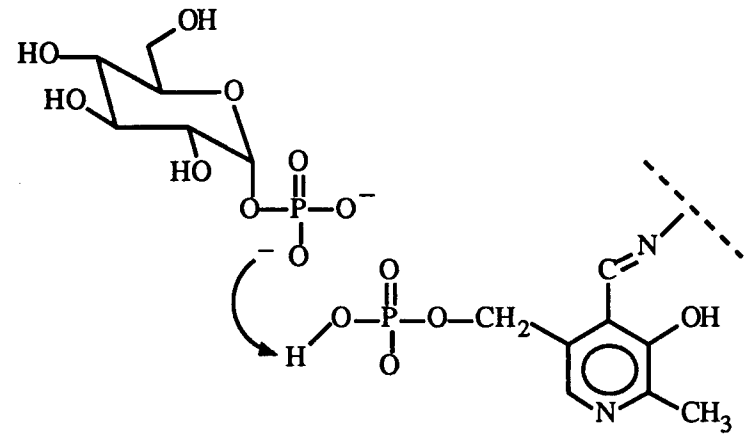
A Possible Role for PLP in Catalysis.

A potential role for the cofactor 5'-phosphate in a glucosyl transfer reaction is not immediately obvious and it is this question which has dominated mechanistic research on this system over the last 20 years. From the preceding discussion it is evident that any catalytic mechanism must include a number of features; the close proximity of phosphate groups of cofactor and substrate, general acid/base catalysis (as would be required by the reaction of the glycosylic substrates) and possibly, the intermediacy of a β-glucosylated-enzyme. In keeping with these requirements the function of the cofactor has variously been proposed as a nucleophile (Johnson et al., 1980) an acid or base catalyst (Pfeuffer et al., 1972; Klein et al., 1982; Klein et al. 1984) or an electrophilic catalyst (Withers et al., 1981a,1981b; Takagi et al., 1982; Tagaya and Fukui, 1984). The respective roles of the pyridoxal cofactor in these different mechanisms are outlined in Figure 3-3. All of the proposals above require the

Nucleophile



Acid Catalyst



Electrophilic Catalyst

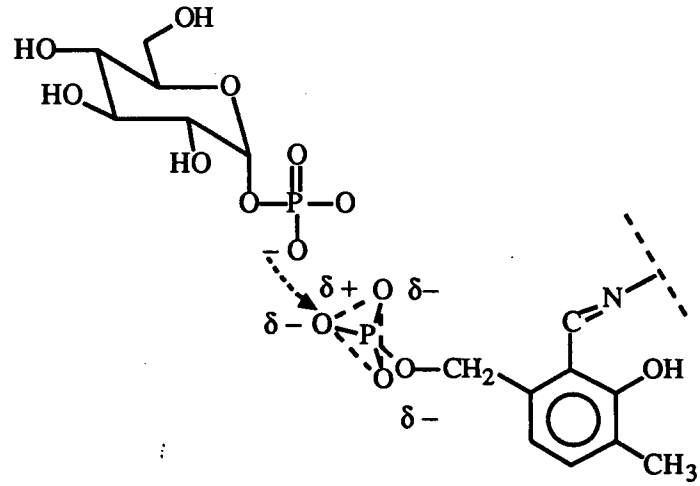
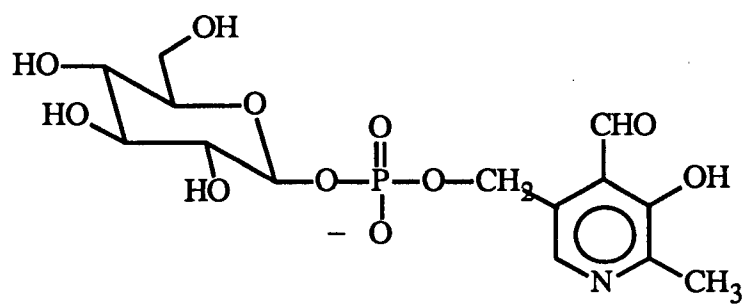


Figure 3-3 . Possible catalytic roles of pyridoxal phosphate.

coenzyme and substrate phosphates to be directly interacting or at least the participation of the PLP in some role. The different evidence in favour of each proposal will be outlined in the subsequent sections.

PLP as a Nucleophile.

The role of the PLP as a nucleophilic catalyst was proposed largely because a protein functional group which might act as a nucleophile could not be readily identified from X-ray crystallographic studies. It was proposed that the binding mode for αGlcP which was evident from X-ray crystallography was in fact a non-productive binding mode, and that productive binding would place the cofactor phosphate above the glucopyranose ring of the substrate. However, this mechanism now seems less likely as β-glucopyranosyl phosphate, a compound which might bear a structural resemblance to the intermediate arising from the backside attack of PLP on αGlcP, and might be expected to act as a transition state analogue, is not a substrate nor does it bind to PL-glycogen phosphorylase (Withers et al., 1982a). In agreement with this latter observation apophosphorylase when reconstituted with pyridoxal 5'-phospho β-glucose, which is an intermediate in this particular catalytic mechanism, does not transfer a glucosyl moiety to glycogen (Takagi, et al., 1981).

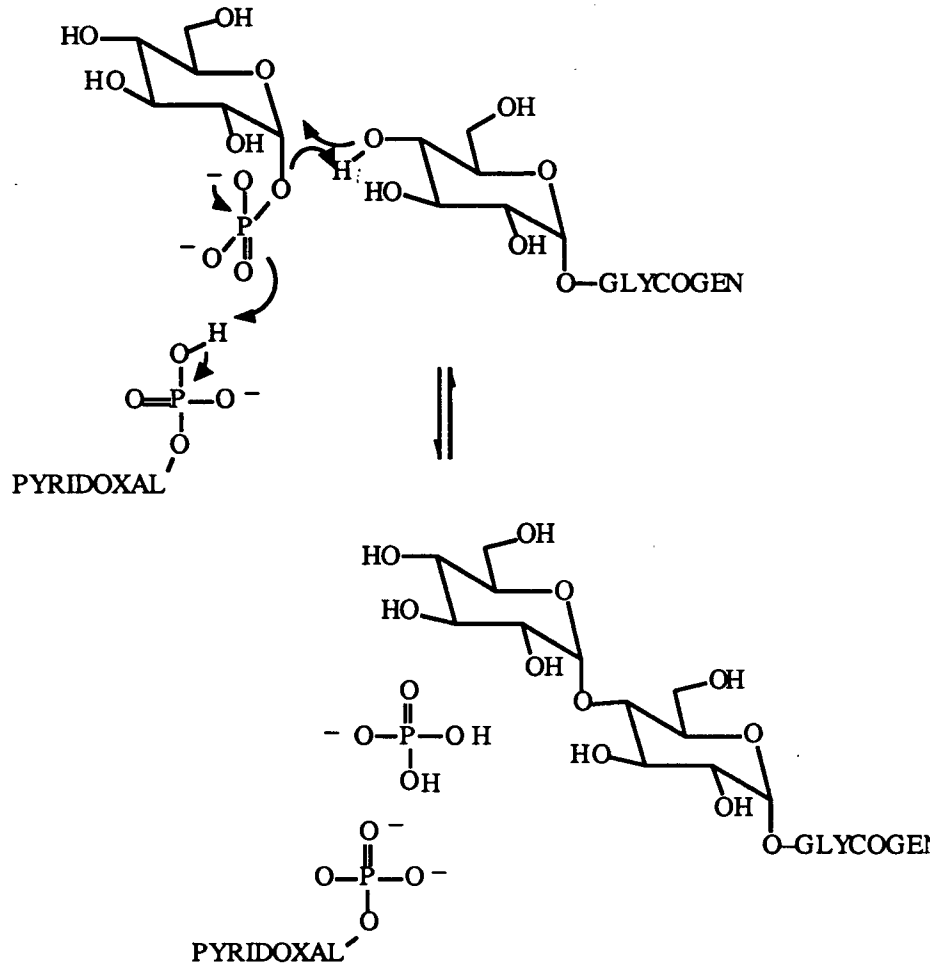


Pyridoxal 5'-phospho β-glucose

PLP as an Acid/Base Catalyst.

The interacting phosphates hypothesis led to a proposal that a direct proton transfer could occur between the phosphates of the cofactor and the substrate as shown in Figure 3-3. The bound substrate would therefore be protonated by the cofactor phosphate making it a better leaving group and thus labilizing the glycosidic bond. In the reverse reaction, the cofactor phosphate protonates the bound inorganic phosphate substrate which in turn protonates the glycosidic bond between the glucose moieties of the oligosaccharide, thus effecting acid catalysis of bond cleavage. The substrate phosphate would therefore be involved in a proton transfer relay. Early mechanisms proposed by Helmreich and Klein involved an acid/base catalytic role of the PLP and the intermediacy of a glucosyl-enzyme. These mechanisms and others are reviewed by Helmreich and Klein (1980), and Klein, Im and Helmreich (1984). More recently in the absence of evidence for such an intermediate, Klein et al. (1986) have proposed a concerted mechanism (see Scheme 3-5).

The role of PLP as the essential acid catalyst would be in concurrence with some of the data obtained from the glycosylic substrates, particularly as the substrate D-glucal was protonated by a group situated below the plane of the sugar ring, a position probably occupied by inorganic phosphate which is postulated to participate in the "proton relay system". However this evidence does not exclude other protein functional groups from functioning in the role of acid catalyst, and by analogy with *E . coli* β-galactosidase where a tyrosine is a good candidate for the acid catalyst, it is tempting to place tyrosine 572 in this role. This group has been identified from X-ray crystallography as being in approximately the right position to act as an acid catalyst and is seen in the three dimensional structure hydrogen bonded to the hydroxyl group at C-2 of the ligand. Circumstantial evidence for a tyrosine in this role has recently been provided by Fukui and his coworkers (Horinishi et al., 1988), who have suggested that a group of pK 8.2 is situated near the C-2 hydroxyl group of the ligand. This pK value is well within the range that might be expected for a tyrosine residue within a protein.



Scheme 3-5. The acid catalysed reaction proposed by Klein et al., 1986.

Further evidence which is suggestive of the acid catalytic function of PLP has also been gathered from the rates of utilization of D-glucal in the presence of different phosphate analogues. It was found that phosphate and arsenate were the only anionic cosubstrates which would stimulate the utilization of D-glucal. Fluorophosphate and phosphite were found to be ineffective in this role. Based on this evidence it was postulated that fluorophosphate, which has a low pKa_2 value (4.8) could not function in the proton relay because it could not be protonated within the pH ranges which phosphorylase is active. However, it should be noted that phosphite (pKa_2 = 6.6) was also inactive and that neither of these phosphate analogues can act as a replacement for phosphate in the normal catalytic reaction of phosphorylase.

Much of the evidence which originally led to the proposal of PLP as the essential acid catalyst was derived from ^{31}P-NMR experiments (Hoerl et al., 1979; Palm et al., 1979; ; Klein et al., 1979). The use of ^{31}P-NMR would appear in many ways to be an ideal method of studying the coenzyme phosphate during catalysis, since the phosphate chemical shifts are sensitive to both ionization state and environment. Observation of the ^{31}P-NMR resonance of the bound cofactor phosphate in the presence of various effectors and substrates should therefore provide useful information regarding its ionization state and hence its mechanistic roles.

Early studies on the phosphorylase system involved comparisons of chemical shift values for the enzyme bound cofactor with those obtained from a model Schiffs base (pyridoxal phosphate with ε-aminocaproate) to assign the ionization state of the bound cofactor (Feldman and Hull 1977). The frequency of the ^{31}P-NMR resonance for the model compound was observed to shift to lower field strengths upon deprotonation. Accordingly, the high-field resonance observed for the enzyme-bound PLP (δ = 0 ppm relative to 85% phosphoric acid) was assigned an ionization state equivalent to that of a monoanion. The resonance from more activated forms of phosphorylase which were obtained by addition of nucleotide activator and/or substrates such as arsenate, was observed down-field from the resonance for the inactive T-state enzyme. By analogy with the model Schiffs base it was assigned the ionization state of a dianion. Activation thus appears to be accompanied by deprotonation of the enzyme-bound PLP. These results are also consistent with the effect of the T-state promoters glucose and caffeine upon the ^{31}P-NMR resonance of PLP in AMP-activated phosphorylase *b* (Withers et al., 1981a).

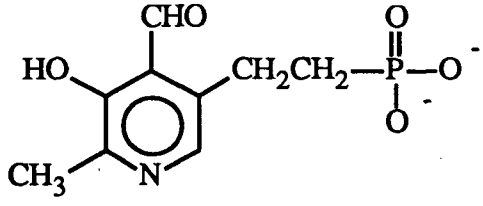
Experiments using ^{31}P-NMR and the non-regulatory α-glucan phosphorylase from potato have been interpreted as supporting the role of PLP as the essential acid catalyst (Klein and Helmreich, 1979). The ^{31}P-NMR spectrum of potato phosphorylase shows a single resonance at low-field presumably from the dianionic form of the enzyme bound PLP. Binding of glucose shifts the resonances upfield, suggesting some degree of protonation,

further addition of arsenate caused a small down-field shift. Addition of oligosaccharide to potato phosphorylase caused the appearance of an additional broad resonance at high-field. Higher concentrations of oligosaccharide caused the apparent collapse of these two PLP resonances to a single exchange-averaged signal of intermediate chemical shift. Non-productive oligosaccharides such as cyclodextrin which bind to the enzyme but do not have a free hydroxyl at the 1- or the 4-position do not affect the ^{31}P-NMR spectrum. These results were interpreted in terms of the binding of the oligosaccharide effecting a protonation of the PLP; the proton probably deriving from the oligosaccharide. Very similar results were observed upon addition of the tight-binding inhibitor heptulose 2-phosphate to potato phosphorylase (Klein et al., 1984). A significant upfield shift and broadening of the ^{31}P-NMR resonance due to PLP was observed upon addition of heptulose 2-phosphate to potato phosphorylase. The resonance due to the heptulose 2-phosphate shifted downfield also with a similar increase in the linewidth of the signal. Again these results were interpreted as indicating an interaction between the two phosphate groups, most likely in the form of a rapid protonation-deprotonation equilibrium.

However, in any interpretation of ^{31}P-NMR studies it should be realized that the present understanding of factors which determine chemical shifts in ^{31}P-NMR is very limited. This problem is discussed at length in a review by Cohn and Rao (1979), who point out that as well as ionization state, any distortion of the oxygen-phosphorus bond angle is also a major determining factor in ^{31}P-NMR chemical shifts. An example which is cited by these authors is that of alkaline phosphatase, an enzyme that utilizes a covalently phosphorylated serine residue within its catalytic pathway. The ^{31}P-NMR resonance of this phosphoryl serine is at very high-field strengths (- 8.5 ppm), a resonance position which cannot be simply attributed to the ionization state of the phosphate (the dianion of serine phosphate would resonate about 0 ppm). In this case the abnormal resonance position could be accounted for by a distortion of the O–P-O bond angle of 2° away from its normal tetrahedral geometry (Cohn and Rao 1979). This idea was based upon the work of

Gorenstein and his colleagues (Gorenstein, 1975; Gorenstein and Kar, 1975) who demonstrated that a simple empirical relationship existed between ^{31}P-NMR chemical shift values and the phosphorus-oxygen bond angle (as determined from X-ray crystallography) for a wide variety of phosphate.esters.

Within the context of ^{31}P-NMR experiments performed with glycogen phosphorylase a number of problems of interpretation become apparent when one considers the following evidence. Addition of glucose to nucleotide-activated phosphorylase *b* causes an upfield shift of the PLP resonance from the supposed dianionic form to the monoprotonated from (Withers et al., 1979). An equivalent experiment performed with pyridoxal 5'-deoxymethylenephosphonate-reconstituted phosphorylase a resulted in a similar upfield shift (Hoerl et al., 1979). However, since the ^{31}P-NMR response of this phosphonate derivative to changing pH is known to be *opposite* to that of PLP itself (Schnackerz and Feldman, 1980) the simplest interpretation of this experiment would require deprotonation of the cofactor upon glucose addition. Such a reversal of effects would seem very unlikely and cannot be accounted for simply by invoking the different pKa values that would be expected from these different cofactors. Addition of heptulose 2-phosphate to phosphorylase *b* reconstituted with this modified cofactor also elicits this puzzling reverse response (Klein et al. 1984). This casts doubt upon the validity of the previous interpretations of observed shifts in terms of ionization state and makes one consider distortion of the cofactor phosphate as a more reasonable explanation.

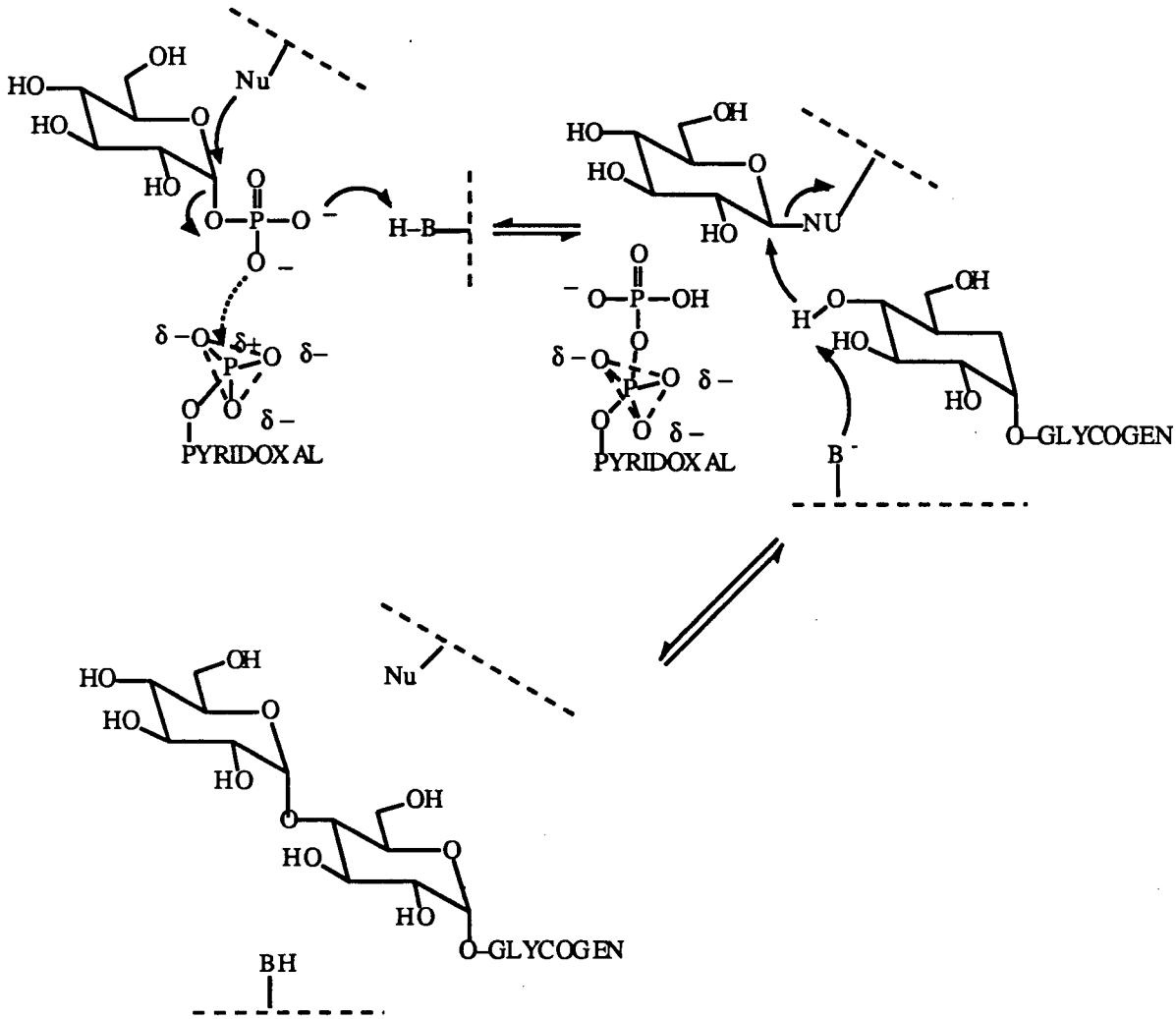


Pyridoxal 5'-deoxymethylene phosphonate

There is a body of evidence that strongly suggests that the pyridoxal phosphate cofactor cannot be involved in any protonation or deprotonation processes at all. The pyridoxal-reconstituted phosphorylase *b* (PL-phosphorylase *b*) is activated by both phosphite and fluorophosphate and the relatively low value of pKa_2 (4.8) for fluorophosphate would almost certainly exclude this phosphate analogue from participation in the proton shuttle mechanism. Furthermore, ^{19}F-NMR data of fluorophosphate bound to PL-phosphorylase indicated that this phosphate analogue remained in its dianionic state throughout catalysis (Chang et al., 1983). In a subsequent pH/activity study of PL-phosphorylase *b* activated with fluorophosphate or phosphite Withers et al., (1982c) demonstrated that although these phosphate analogues have very different pKa's (fluorophosphate pKa = 4.8, phosphite pKa = 6.6) the pH optima for the pyridoxal-enzyme activated with these analogues were virtually identical. These results were interpreted as meaning that the phosphate analogues (and hence the natural pyridoxal 5'-phosphate) cofactor could not be acting as the essential acid catalyst.

PLP as an electrophilic catalyst.

The function of PLP as an electrophilic catalyst is depicted in Scheme 3-6 below and has recently been reviewed by Madsen and Withers (1984a; 1984b). In this mechanism binding of substrate αGlcP results in tight co-ordination of the coenzyme phosphate by basic amino acid side chains, and distortion of the phosphate towards a trigonal bipyramidal configuration with an empty apical position oriented towards the phosphate of the substrate. Such a distorted phosphate would be an electrophilic species and could effect a partial withdrawal of electron density from the substrate phosphate, thus labilizing the glycosidic bond. This formation of a pseudo pyrophosphate bond (not a full bond) would be analogous to an abortive phosphate transfer. This mechanism has an attractive analogy in the normal phosphoryl-transfer reactions catalysed by kinases and phosphatases.



Scheme 3-6. The electrophilic mechanism for glycogen phosphorylase.

The general reaction mechanism of these enzymes is commonly thought to involve a transition state of a pentacoordinate-trigonal-bipyramidal phosphate species; a transition state structure which has obvious similarities to that of the transition state structure proposed by the electrophilic mechanism. Further, there is similarity between the active site complex proposed by the electrophilic mechanism (and that actually present in PLPPGlc reconstituted apo-phosphorylase) and uridine diphosphoglucose, the natural substrate of glycogen synthase, involved in glycogen synthesis, perhaps suggesting a common structural requirement in catalysis of glucosyl transfer to and from phosphates.

The electrophilic mechanism has also gained considerable support from ^{31}P-NMR experiments. Withers et al., (1981) showed that in the presence of maltopentaose, nucleotide activator and glucose-1,2-cyclic phosphate the cofactor 5'-phosphate resonates in the position of the monoanion but is considerably broadened. This shift was interpreted as representing *either* protonation of the PLP or the tighter binding of the dianion with possible distortion of the normal tetrahedral geometry of the phosphorus. The assignment of the ^{31}P-NMR resonances as a monoanion in T-state phosphorylase and as a more tightly constrained and possibly dianionic species in the nucleotide-activated enzyme has recently been supported by a study which measured the spin-spin and spin lattice relaxation rates of the two enzymic forms (Withers et al., 1985).

The compound pyridoxal 5'-diphospho 1-α–glucose which was discussed earlier in this section bears a strong structural resemblance to the expected transition state of the electrophilic mechanism. The observation that this compound does indeed bind to apophosphorylase and in doing so produces a catalytically competent enzyme (albeit only for a single catalytic cycle) can be taken as supporting evidence for the electrophilic mechanism. The substrate activity of the related compound α-glucose 1-pyrophosphate, with PL-phosphorylase has been been taken as evidence disputing the validity of the electrophilic mechanism (Klein et al., 1984). They argued that without the constraints of the covalent bond between the cofactor and phosphate it is unlikely that the enzyme would be able to distort the terminal phosphate into the trigonal bipyramidal geometry required by the electrophilic mechanism.

If the trigonal bipyramidal geometry of the distorted phosphate proposed in the electrophilic mechanism is a structure on the catalytic pathway, then it would be expected that compounds which resemble this structure might be efficient inhibitors of PL-phosphorylase. A number of oxyanions of the early transition metals (vanadium, tungsten and molybdenum) adopt a trigonal bipyramidal configuration and their inhibitory effect on PL-phosphorylase has been investigated. Anions of vanadate, molybdate and tungstate were all found to be

good inhibitors of PL-phosphorylase (Soman et al., 1983) as required by this mechanism. However interpretation of the results obtained from this type of study are clouded by the propensity of these oxyanions to exist in oligomeric forms in free solution. In keeping with this, vanadate was observed to be competitive with both substrate αGlcP and activating phosphite suggesting that the apparently tight binding of these oxyanions might be a result of interactions with both the cofactor phosphate and substrate binding sites. Indeed by using vanadium-NMR Soman et al., were able to show that decavanadate was the major oligomer interacting with the protein and that these formed a stable complex with a protein functional group, probably an arginine residue. Hence it is unlikely the apparent tight binding of these compounds is a result solely of a resemblance to a transition state structure. An earlier study Chang et al. (1983) demonstrated that molybdate bound to the cofactor phosphate site in PL-phosphorylase approximately 13 times tighter than phosphate itself. This suggested that this site could readily accommodate a compound with trigonal-bipyramidal geometry but it is arguable that only a 13 fold increase in affinity is not really indicative of this oxyanion binding as a transition state analogue. When molybdate was used as a transition state analogue in phosphatases, it bound to these enzyme $10^3 - 10^4$ times more tightly than phosphate itself. The reaction mechanism for this class of enzyme is thought to involve a pentacoordinate trigonal-bipyramidal phosphate species in the transition state.

The Aims of This Study.

The structure of glycogen phosphorylase in the inactive T-state conformation and its interaction with glucose are well understood from X-ray crystallography and our study with deoxy and deoxyfluoro analogues of glucose. Unfortunately, relatively little information is available concerning the structure of the R-state enzyme and its interactions with substrate since neither phosphorylase a or b have been successfully crystallized in this conformation. Some structural information has been obtained by diffusion of substrates or substrate analogues into the crystal and subsequent collection of diffraction data (Withers et al. 1982;

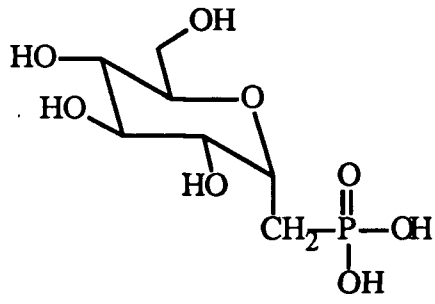
McLaughlin et al., 1984; Oikonomakos, et al. 1987). However, such data are of relatively poor quality and any conformational changes which are observed are certainly attenuated by crystal packing forces.

Kinetic studies with substrate analogues have not been much more successful since, with the exception of glucosyl fluoride (Palm et al., 1983), D-glucal (Klein et al. , 1982) or the related heptenitol (Klein et al., 1984), nothing except αGlc P has been shown to act as a substrate for the muscle enzyme, indicating the high specificity of this enzyme for its substrate. Useful information may, however, be obtained through more conservative modifications of the substrate producing analogues which might then be accepted by the enzyme. This should then help to answer important questions regarding the mechanism of the enzyme and whether the T-state glucose subsite remains intact during the T to R state transition.

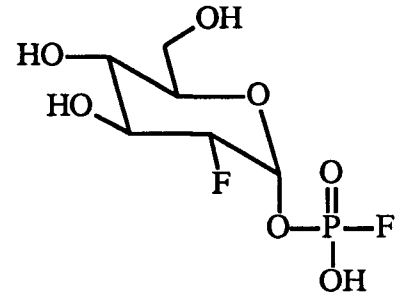
The study on the specificity of the glucose binding site (Street et al., 1986) has therefore been extended by synthesis and kinetic characterization of a series of deoxy and deoxyfluoro derivatives of αGlcP. The well defined stereochemistry offered by this carbohydrate system has provided insights into the substrate specificity of glycogen phosphorylase *b* and demonstrated the importance of hydrogen bonding interactions to stabilization of the enzymic transition state. Through the substituent effects of these modified substrates, and by comparison to a model reaction, their acid catalysed hydrolysis, supportive evidence has been gained for an oxocarbonium ion-like transition state in the enzymic reaction.

Lastly we have prepared a series of analogues of αGlcP designed to probe the specificity of the substrate's phosphate binding site by means of both kinetic and NMR techniques .These compounds include (1-deoxy-α-glucopyranosyl) methylphosphonate (1dαGlcCP), 2-deoxy-2-fluoro-α-glucopyranosyl phosphofluoridate (2FαGlcPF) and α-mannopyranosyl phosphofluoridate (αManPF). The derivative 1dαGlcCP also allowed us to probe this binding site using ^{31}P-NMR, since the resonance frequency of this phosphonic

acid derivative is well downfield of the ^{31}P-NMR signal from the bound PLP and is also dependent on its own ionization state. This along with a similar study which utilizes the pH dependence of the ^{19}F-NMR signal from 2FαGlcP has allowed us to determine the ionization state of the bound substrate.



(1-deoxy-α-glucopyranosyl) methylphosphonate (1dαGlcCP)



2-deoxy-2-fluoro-α-glucopyranosyl phosphofluoridate (2FαGlcPF)

CHAPTER 4.

Mechanistic Studies of Glycogen phosphorylase.

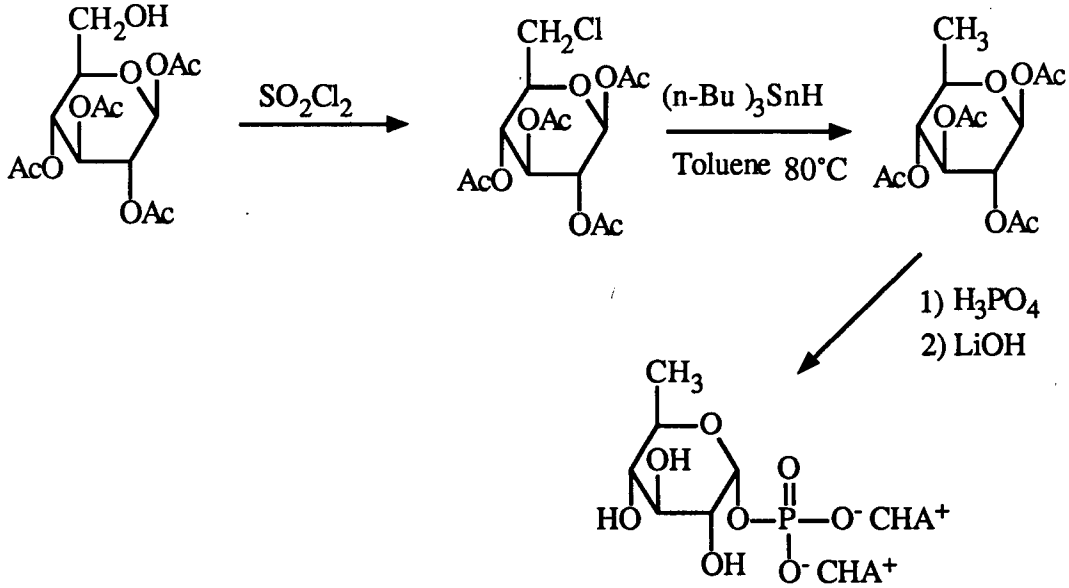
Results and Discussion.

Synthesis of α-Glucose 1-phosphate Analogues..

Synthesis of the monodeoxy analogues of αGlcP presented few problems and all of the synthetic routes utilized the same basic strategy, which was to prepare the appropriately protected chlorodeoxy sugar followed by reduction with tributyltin hydride (Arita et al.,1982).This reduction step proceeded in all cases with yields greater than 85%. The chlorodeoxy derivatives were prepared in good to moderate yields (55 - 80%) by treating the appropriately protected sugar with sulfuryl chloride in pyridine. Through a variety of different manipulations these deoxy sugars were then converted to their β-per-O-acyl derivatives which were subsequently converted to the 1-phosphate ester by the method of MacDonald (1972).The sugar phosphates were isolated and purified as their bis cyclohexylammonium salts (CHA^+).

6-deoxy-α-D-glucopyranosyl phosphate.

The following synthetic route was used in the preparation of 6-deoxy-α-D-glucopyranosyl phosphate.

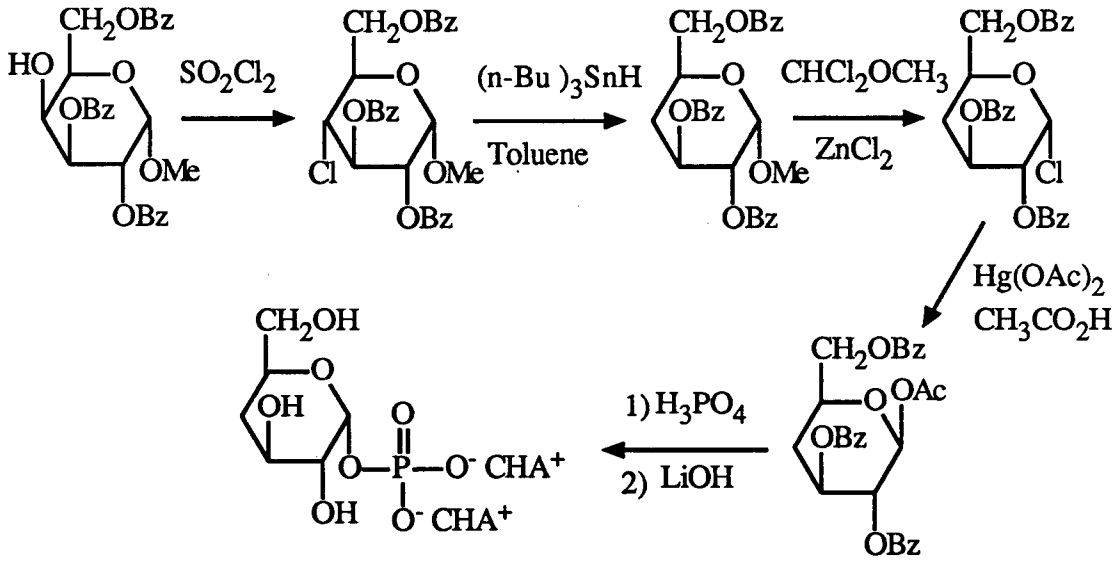


Scheme 4-1. The synthetic route used for preparation of 6-deoxy-α–D-glucopyranosyl phosphate.

The β-per-O-acetylated derivative of 6-deoxyglucose has been prepared before by the sequence used here (Arita, et al., 1982). This route represents a very fast and efficient method of preparing the target molecule, giving an overall yield of 18%.

4-Deoxy-α-D-xylohexopyranosyl phosphate..

The original synthesis of the per-O-acetylated derivative of 4-deoxy-D-xylohexopyranose (Lopes and Taylor, 1979) prepared this sugar in 6 steps starting from methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside. The synthetic route presented here (which also starts with the same compound) represents both a significant shortening of the procedure as well as a considerable increase in overall yield, the per-O-acyl derivative of 4-deoxy-D-xylohexopyranose being obtained in an overall yield of 57%.



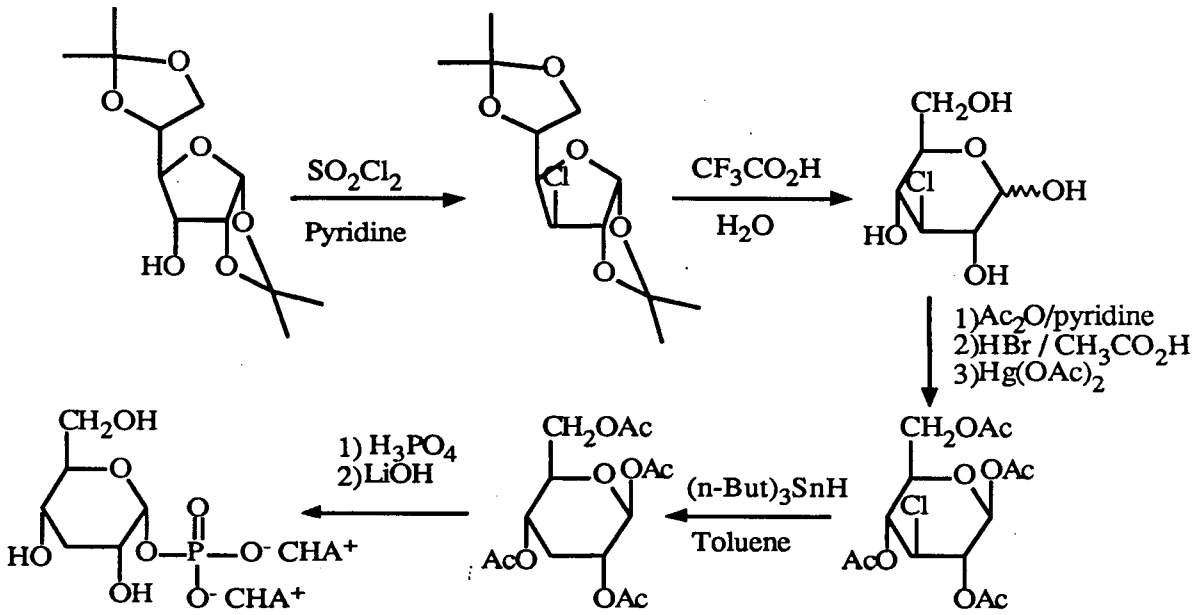
Scheme 4-2. The synthetic route for preparation of 4-deoxy-α-D-xylohexopyranosyl phosphate.

Although the methods of halogenation and reduction used here represent an improvement over the methods used by Lopes and Taylor the most significant advantage of this route comes from the direct conversion of the methyl per-O-acyl-4-deoxy-α-D-xylohexopyranoside to its α-chloride using 1,1-dichloromethyl methyl ether and zinc chloride (Gross et al.,

1978). This direct conversion removes the necessity for hydrolysis of the methyl glycoside under aqueous acidic conditions. Consequently steps requiring removal and re-introduction of the acyl blocking groups are also no longer needed. The 1-phosphate ester was obtained in an overall yield of 13%.

3-deoxy-α-D-ribohexopyranosyl phosphate.

The synthetic route for 3-deoxy-α-D-ribohexopyranosyl phosphate is presented in Scheme 4-3.



Scheme 4-3. The synthetic route for 3-deoxy-α-D-ribohexopyranosyl phosphate.

In early attempts at synthesis of this compound, reduction of 3-deoxy-3-chloro-1,2 : 5,6-di-O-isopropylidene-α-D-glucofuranose with tributyltin hydride, followed by removal of the blocking groups in aqueous acid, gave 3-deoxyribohexose in good yield. However, the propensity of 3-deoxyribohexose to exist in a relatively high percentage of its furanose forms in free solution (≈ 23%, Pfeffer et al., 1980) caused a number of problems. Re-acetylation of this mixture in acetic anhydride / pyridine produces a mixture of the acetylated pyranose and furanose forms of the sugar, however the acetylated furanose-sugars are essentially useless

for the remainder of the synthesis. This problem was overcome by leaving the reduction step until relatively late in the synthesis, since 3-deoxy-3-chloro suger does not suffer from these problems and acetylation of 3-deoxy-3-chloro-D-glucose yielded > 95% of the desired pyranose forms. The desired 1-phosphate ester was obtained in an overall yield of 8.0%

The compound (1-deoxy-α-glucopyranosyl) methanephosphonate was synthesized according to the procedure of Nicotra et al. (1982; Nicotra et al., 1984) except that iododemercuriation was performed instead of the published bromodemercuriation step. This modification to the procedure greatly increased the overall yield because it stopped the unwanted removal of the benzyl blocking groups during this step. The entire procedure including modifications is detailed in the Materials and Methods Section.

Kinetic Experiments with Glycogen Phosphorylase.

Our initial studies with glycogen phosphorylase and the deoxy and deoxyfluoro analogues of glucose 1-phosphate appeared to show that none of them acted as substrates (Street I.P., 1985 M.Sc. Thesis University of British Columbia). This prompted a more intensive search for turnover of these substrates, using high concentrations of enzyme and ^{19}F-NMR to observe the production of fluorinated products. Some examples of the spectra that were obtained are given in Figure 4-1. Over a period of time the resonance due to the substrate slowly decreased in intensity, with a concomitant appearance of a signal due to a single fluorinated product. The product was clearly not a simple hydrolysis product (corresponding to the deoxyfluoro glucose), as evidenced from its chemical shift and the absence of a pair of resonances which would have been expected for an α-, β-mixture of the anomerically equilibrated sugar. Also, in control experiments which did not contain AMP and/or glycogen, no product was formed. Futher, in subsequent experiments fluorinated glycogen derivatives were isolated and characterized by ^{19}F- and ^{1}H-NMR (Withers S.G.and Rupitz K.R. unpublished results). Hence the reaction being observed is certainly catalysed by glycogen phosphorylase.

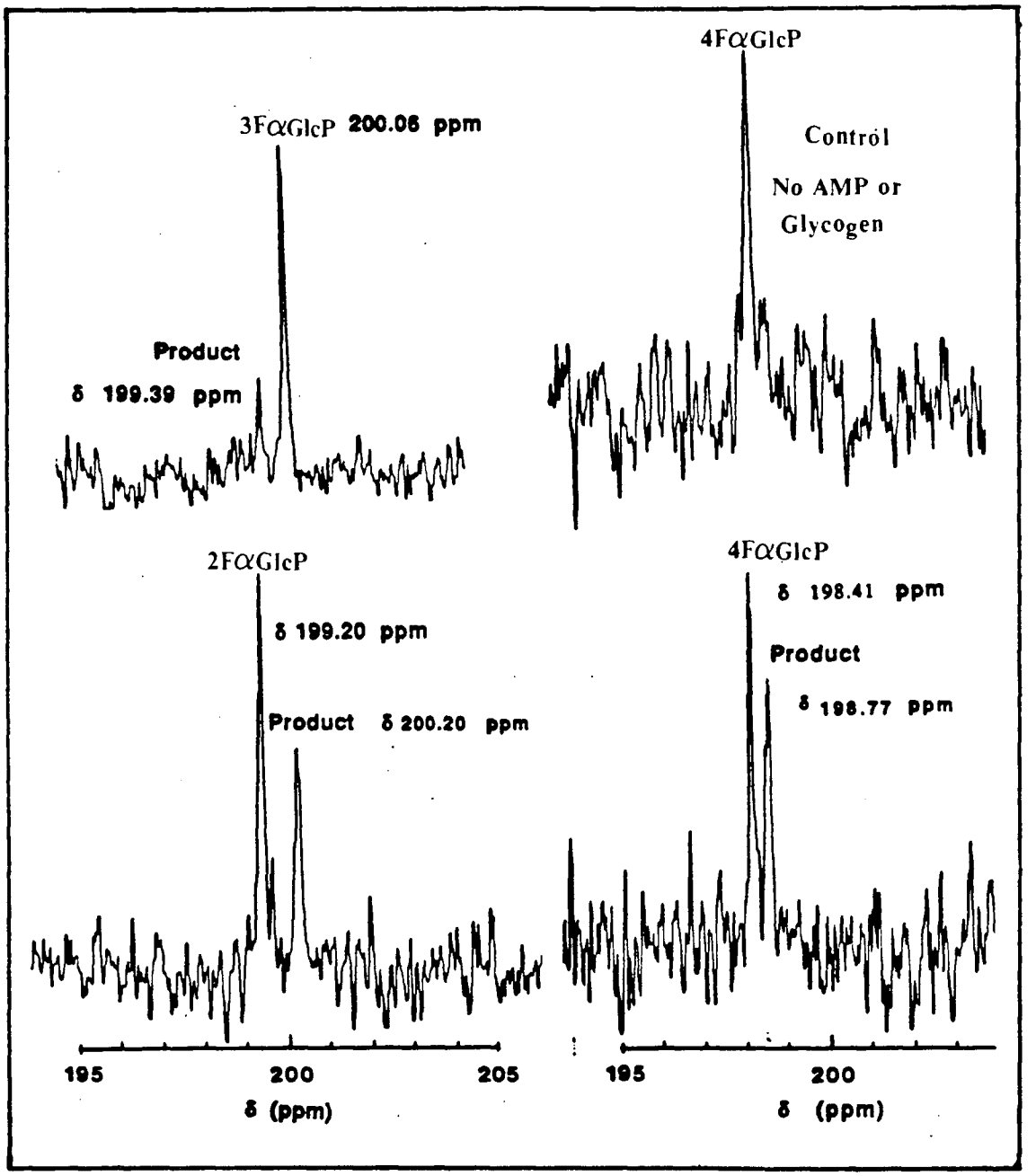
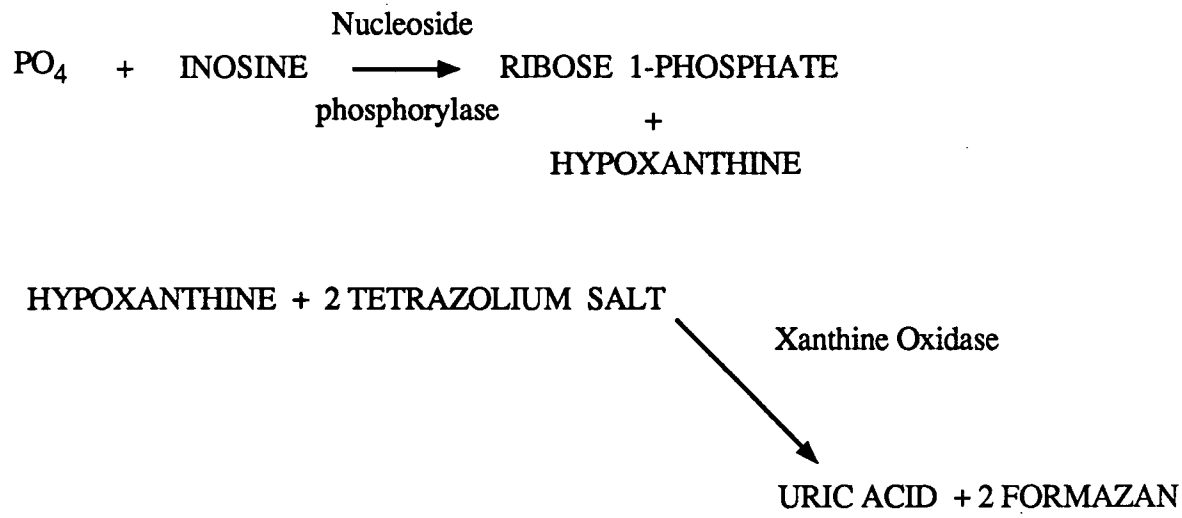


Figure 4-1. ^{19}F-NMR spectra of fluorinated glycogen derivatives produced by glycogen phosphorylase.

Reaction mixtures (0.7 mL) contained 5.0 mM substrate, 0.5% glycogen, 1mM AMP, 12.6 mg of glycogen phosphorylase b and 0.2mL of deuterated water. All reactions were conducted at pH 6.8 in a buffer containing 50 mM triethanolamine, 100 mM potassium chloride, 1mM EDTA and 1mM DTT. For 2FαGlcP the reaction was run under similar conditions except that 4.5 mg of phosphorylase a and 36 mM maltopentaose was used in place of phosphorylase b and glycogen. Reaction mixtures were incubated for 12 - 48 hours at 30°C prior to data acquisition.

Since ^{19}F-NMR spectroscopy could not be conveniently used for kinetic studies an alternative assay procedure based on phosphate release was sought. Two different assay procedures were found to possess the necessary sensitivity; a coupled enzyme system (nucleoside phosphorylase / xanthine oxidase, (Fossati, 1985; De Groot et al., 1985) and an ascorbate / molybdate assay (Baginski, 1967). Both of these assay systems had the added advantage that they could be used to measure inorganic phosphate in the presence of acid labile phosphate esters. This was essential since the deoxy α-glucose 1-phosphates are considerably more acid sensitive than the parent sugar phosphate and quickly hydrolyse under the aqueous acidic conditions employed in the standard Fiske Subarrow assay.

The coupled enzyme system is based on the series of reactions shown in Scheme 4-4 and relies on the reduction of a tetrazolium salt (2-p-iodophenyl-3-p-nitrophenyltetrazolium chloride) by xanthine oxidase with concomitant oxidation of hypoxanthine produced by the phosphorolytic cleavage of inosine catalysed by nucleoside phosphorylase. The reduction product of the tetrazolium salt is a coloured formazan the concentration of which can be determined spectrophotometrically. Under the conditions described by De Groot et al., the coupled assay was sensitive in the range 5-75 μM and gave reproducible results. However, this system could not be run as a continuous assay to monitor the reaction of glycogen phosphorylase since inosine is a good inhibitor of phosphorylase. The assay system was also found to be incompatible with dithiothreitol (DTT) a necessary component in the phosphorylase reaction buffer. Attempts to use the coupled enzyme system as a stopped assay for glycogen phosphorylase did not yield much greater success.Upon introduction of the phosphorylase reaction mixture into buffer containing the coupled enzymes the assay became unreliable, due largely to high and unreproducible background readings. Thus, this enzymic system was abandoned in favour of the less sensitive but more reliable ascorbate / molybdate assay.



Scheme 4-4. An assay for inorganic phosphate (De Groot et al., 1985).

Substrate analogues were assayed at high enzyme concentrations (up to 1 mg mL^{-1}) for long time periods (up to 28 hours) in the presence of 1mM AMP and 1% glycogen at pH 6.8 and 30°C. Control experiments with similar samples (without the substrate analogues) assayed over this time period showed negligible loss in enzyme activity. Once again, omission of either AMP or glycogen from these reaction mixtures resulted in no phosphate being released. This experiment, in conjunction with the similar results obtained from the ^{19}F-NMR experiments discussed earlier, essentially proves that the phosphate-producing reaction is catalysed by glycogen phosphorylase. Michaelis-Menten parameters determined in this way are presented in Table 4-I and Figures 4-2 to 4-8. Problems caused by substrate inhibition were encountered during determination of the kinetic parameters for 6FαGlcP (Figure 4-5). The values for this compound which are presented in Table 4-I were determined by extrapolation from data taken at lower substrate concentrations, where kinetic behaviour was still linear. Unfortunately data could not be obtained for 2dαGlcP as this very hydrolytically unstable sugar could not be prepared at sufficiently high purity to obtain reliable kinetic results. However, experiments involving D-glucal as a substrate for glycogen phosphorylase *b*, clearly demonstrate that 2dαGlcP is a reasonable substrate for this enzyme (Klein et al.,

1982). The maximal rates for utilization of D-glucal by α-glucan phosphorylases, in the presence of inorganic phosphate are between 20 - 30 % of the rate of glycogen synthesis from αGlcP.

Table 4-I. Kinetic parameters for deoxy and deoxyfluoro substrate analogues with glycogen phosphorylase b.[a]

Compound	k_{cat} ($min.^{-1}$)	K_m (mM)	k_{cat} / K_m ($min.^{-1}$ mM^{-1})	[b]$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})
αGlcP	8.77×10^{3}	4.8	1827.1	-
2FαGlcP	$(2.7 \pm 0.2) \times 10^{-2}$	2.6±0.4	0.010	7.3
3FαGlcP	0.54 ± 0.03	25±3	0.022	6.8
4FαGlcP	0.65 ± 0.03	1.8±0.2	0.36	5.1
6FαGlcP	0.24 ± 0.01	97± 2	2.5×10^{-3}	8.1
3dαGlcP	1.8 ± 0.1	31±3	0.058	6.3
4dαGlcP	175 ± 19.3	45±7	3.9	3.7
6dαGlcP	7.8 ± 1.1	70±14	0.11	5.9

[a] Reaction conducted at pH 6.8 and 30°C in 20 mM glycerophosphate buffer containing the following enzyme concentrations and incubation time: αGlc P, 4.3 μg mL^{-1}, 5 min.; 2FαGlcP, 993 μg mL^{-1}, 1680 min.; 3FαGlcP, 615μg mL^{-1}, 650 min.; 4FαGlcP, 615μg mL^{-1}, 254 min.; 6FαGlcP, 1042μg mL^{-1}, 965 min.; 3dαGlcP, 269μg mL^{-1}, 215 min.; 4dαGlcP, 50.4μg mL^{-1}, 17 min.; 6dGlcP, 504μg mL^{-1}, 60 min. [b] $\Delta\Delta G^{\ddagger} = -RT \ln \frac{(k_{cat} / K_m)}{(k_{cat} / K_m)_o}$. Where (k_{cat} / K_m) is the value for the analogue and $(k_{cat} / K_m)_o$ is the value for αGlcP.

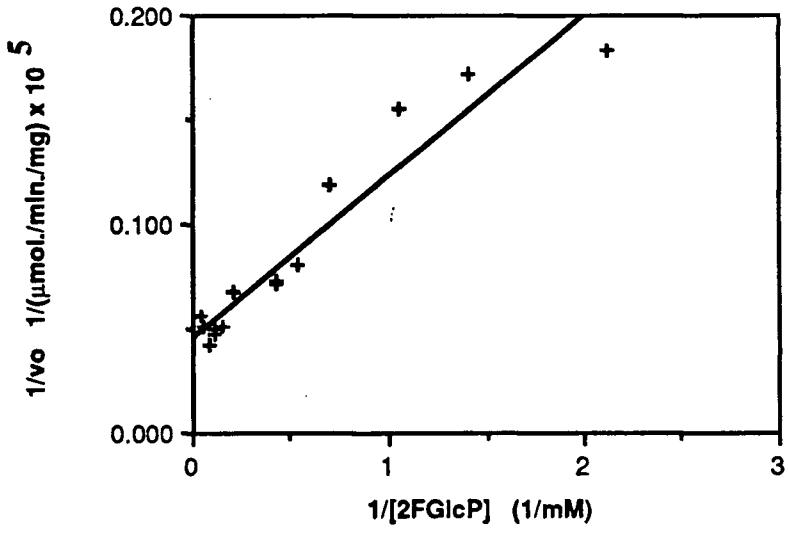


Figure 4-2. Determination of kinetic parameters for 2FαGlc P with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 0.47, 0.71, 0.94, 1.42, 1.89, 2.37, 4.74, 6.63, 9.47, 11.84, 16.57, 23.67.

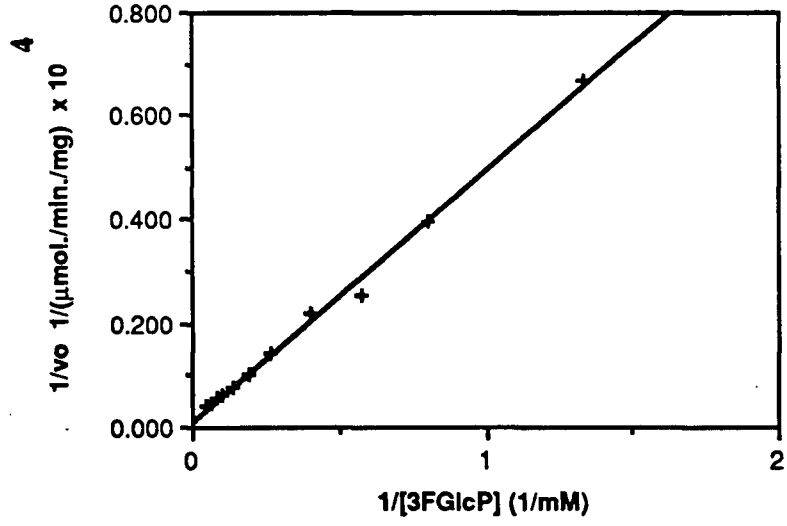


Figure 4-3. Determination of kinetic parameters for 3FαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 0.75, 1.25, 1.75, 2.50, 3.75, 5.25, 7.50, 10.0, 12.0, 16.0, 20.0.

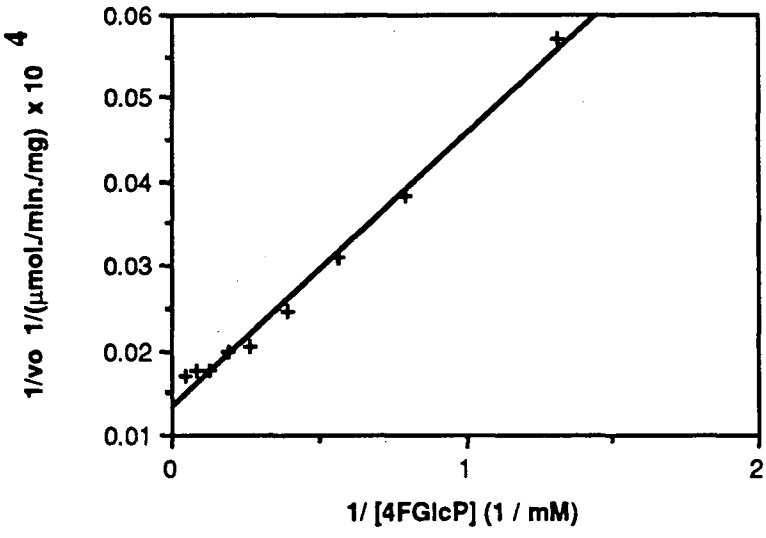


Figure 4-4. Determination of Kinetic parameters for 4FαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 0.76, 1.26, 1.77, 2.53, 3.79, 5.30, 7.58, 12.12, 20.20.

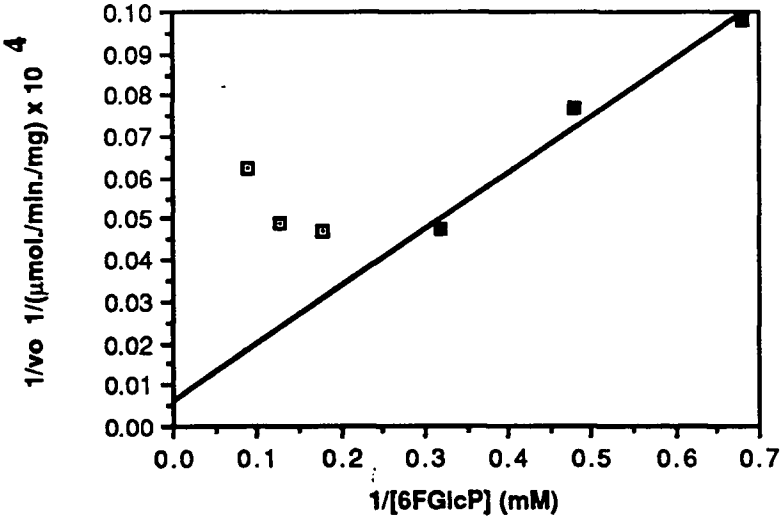


Figure 2-5 . Determination of Kinetic parameters for 6FαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 10.53, 14.29, 20.0, 33.3, 50.0.

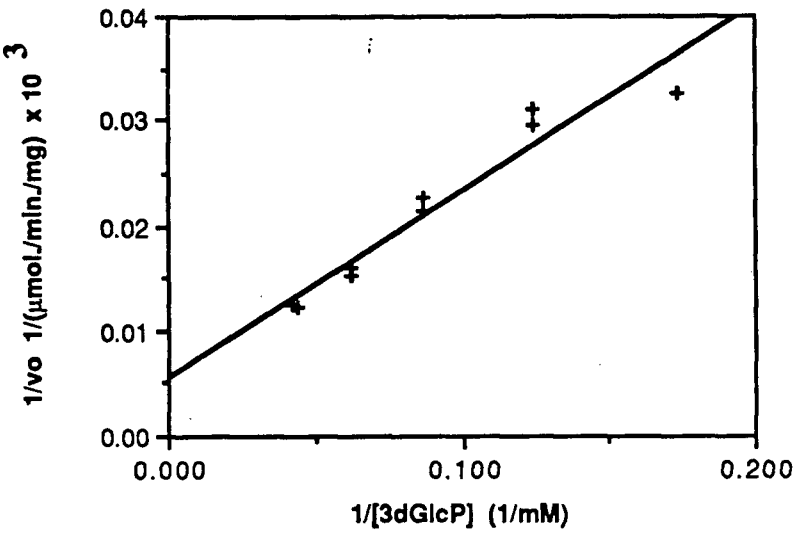


Figure 2-6. Determination of kinetic parameters for 3dαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 5.76, 8.07, 11.52, 16.14, 23.99, 23.06.

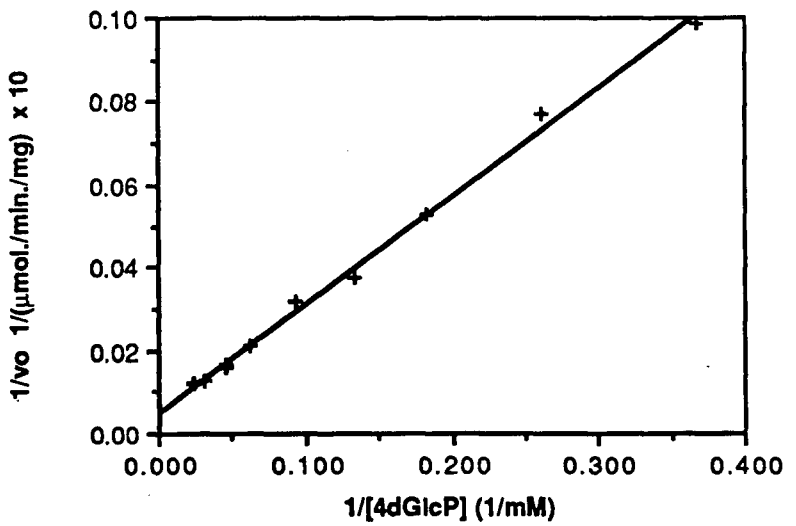


Figure 2-7. Determination of Kinetic parameters for 4dαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 2.72, 3.83, 5.45, 7.53, 10.75, 16.13, 21.93, 32.25, 43.0.

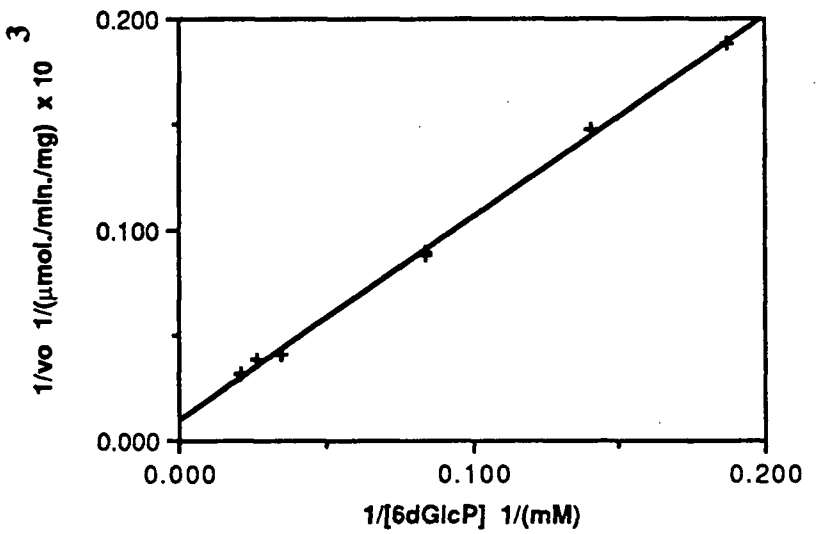


Figure 2-8. Determination of the kinetic parameters for 6dαGlcP with phosphorylase b.

Reaction conditions are given in Table 4-I. The following substrate concentrations were used (mM): 5.33, 7.11, 11.85, 28.47, 37.96, 47.45.

Probes of the substrate's phosphate binding site..

Much of the evidence which has been advanced in support of the function of PLP as the essential acid/base catalyst has been provided by ^{31}P-NMR studies on the non-regulatory α-glucan phosphorylases. From these experiments the changes in chemical shifts and linewidths observed for the resonances of the PLP, substrates and effectors were interpreted in terms of the various ligands participating in a rapid proton-exchange. This interpretation of the NMR-data assumes that a detectable portion of the ground-state enzyme-substrate complex is protonated by, or at least in rapid proton exchange with the acid catalytic group. Therefore, substrate analogues which provide a sensitive probe for pH changes which occur upon binding to the active site would yield useful information about the protonation events which occur in the active site complex. The compounds 1dαGlcCP and 2FαGlcP provide such "active site pH probes" since for both compounds the chemical shift of their respective NMR-nuclei show a characteristic pH dependence. The compound 2FαGlcP is a particularly

useful probe, due to the high sensitivity of ^{19}F-NMR, and since early NMR experiments performed on the binding of 2FGlc to phosphorylase *b* (I.P.Street, unpublished data) showed that shifts due to environmental factors were likely to be unimportant in determining the chemical shift of the bound species. Further, while the ^{19}F-NMR chemical shift is sensitive to ionization state of the adjacent phosphate ester, it should be relatively insensitive to factors such as distortion of phosphorus-oxygen bond angle; a factor which might affect the ^{31}P-NMR chemical shift of the 1dαGlcCP-phosphorylase complex.

Compounds which are "locked" into a particular ionization state, for example the compound 2FαGlcPF can only exist as a monoanionic species at physiological pH values, provide useful information about the selectivity of the phosphate binding pocket with regards to ionization state of the substrate. A study which utilizes both the NMR properties and the kinetic properties of a series of these substrate analogues to probe the ionization state of the substrate in the phosphorylase *b*-substrate complex is detailed below.

Kinetic probes of specificity in the phosphate binding pocket.

The compounds 1dαGlcCP, 2FαGlcPF, αManPF and 2FαGlcP were tested as reversible inhibitors of glycogen phosphorylase *b* and the results are presented in Table 4-II and Figures 4-9 to 4-11. The value of the inhibition constant for the compounds 1dαGlcCP and αManPF were determined by measuring initial reaction rates at five different concentrations of αGlcP in the absence of inhibitor and at four inhibitor concentrations The exact reaction conditions are given in Table 4-II and the legends to Figures 4-9 to 4-11. The standard Fiske SubbaRow phosphate assay was used to determine the amount of inorganic phosphate released during reaction. Inhibition type was determined from standard double reciprocal plots (Figure 4-9 and 4-10) and the values of K_i determined using the computer aided procedures detailed in the Materials and Methods Section. The inhibition constant for 2FαGlcP had been determined previously (Street I.P. 1985, M.Sc Thesis, University of British Columbia) under essentially identical conditions. Unfortunately, due to the very small amounts of 2FαGlcPF that were

available, this compound could not be subjected to a full kinetic analysis. The value presented in Table 4-II was obtained from an experiment performed at a single concentration of substrate (αGlcP) and six different inhibitor concentrations. A value for the inhibition constant was obtained by plotting the data as described by Loeffler et al., (1979) (See Figure 4-11). Loeffler et al., have determined that inhibition constants obtained in this manner are usually accurate to within 10% of the values obtained from the full kinetic analysis.

Table 4-II. Reversible inhibition of glycogen phosphorylase by substrate analogues [a].

Compound	K_i (mM)	Inhibition Type
1dαGlcCP	0.7	Competitive
[b] 2FαGlcP	2.0	Competitive
[c] 2FαGlcPF	6	-
αManPF	16	Competitive
[d] αManP	35	Competitive

[a] Reaction conditions : pH 6.8 and 30°C in a buffer containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1mM EDTA, 1mM DTT, 1mM AMP, 1% glycogen. [b] Value from Street, I.P. (1985), M.Sc Thesis University of British Columbia. [c] Not subjected to a full kinetic analysis.[d] Value from Sprang et al., 1982.

The possible substrate activity of 2FαGlcPF with glycogen phosphorylase *b* was also investigated by use of ^{19}F-NMR. After 48 hours incubation of 2FαGlcPF (10 mM) with 10.2 mg of glycogen phosphorylase *b* at 30°C (reaction mixture also contained 1 mM AMP and 0.5% glycogen), no fluorinated-glycogen products could be detected using ^{19}F-NMR. Under comparable conditions (4 mg of phosphorylase *a*, 48 hours incubation at 30°C) the production of 2-deoxy-2-fluoro-glucosylated-glycogen derivatives was evident from 2FαGlcP, hence based upon this observation and the detection limit of the NMR experiment, the turnover rate of 2FαGlcPF must be at least an order of magnitude slower than 2FαGlcP. Since neither experiment was conducted under saturating concentrations of substrate, this difference in turnover rate could be could be attributed to either a difference in binding

affinity, a difference in k_{cat} or both. The data in Table 4-II would suggest that these two compounds possess similar affinities for phosphorylase *b* and therefore the lack of detectable substrate activity for 2FαGlcPF must arise predominantly from a smaller value of k_{cat}, although under the limits of this experiment this difference could be as small as 3-fold.

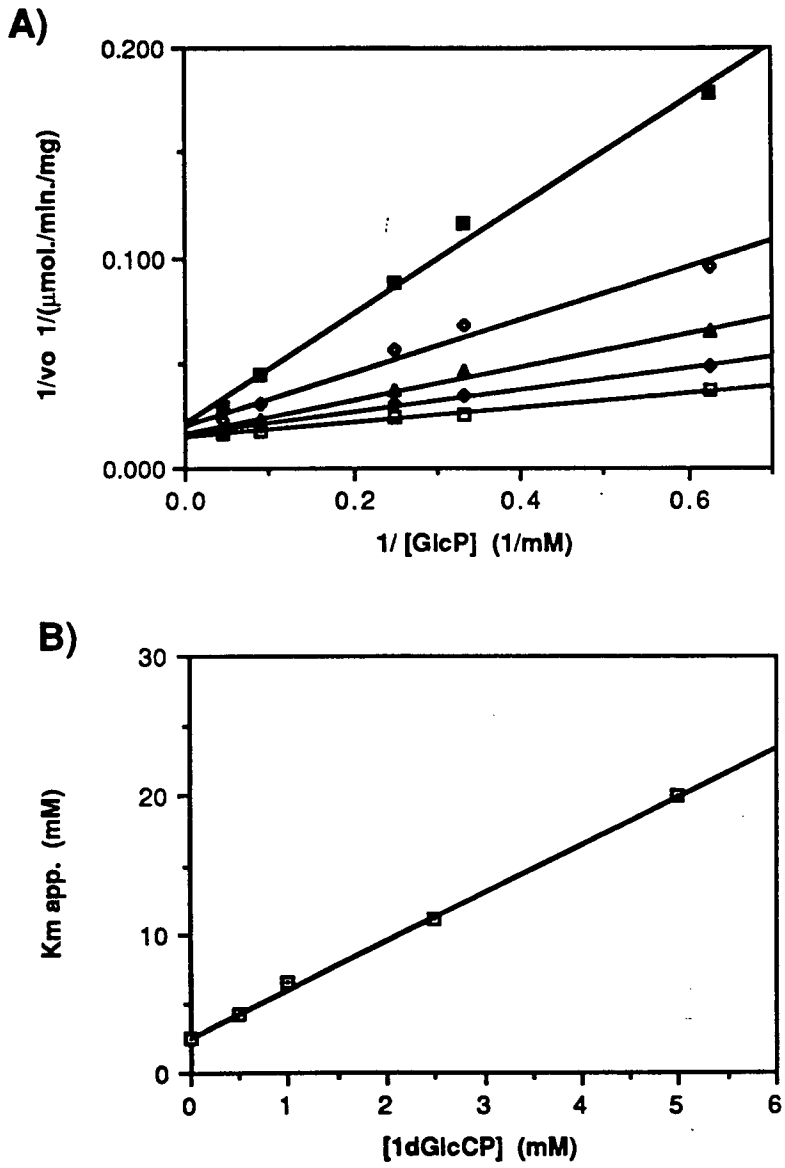


Figure 4-9. Determination of K_i for 1dαGlcCP.

Initial reaction rates were determined at pH 6.8 and 30°C in a buffer containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1mM EDTA, 1mM DTT, 1mM AMP, 1% glycogen and αGlcP at the following concentrations (mM): 1.6, 3.0, 4.0, 11.1, 22.2 The following inhibitors concentrations were used (mM): ❑ 0,◆ 0.5, ▲ 1.0,◊ 2.5, ■ 5.0.

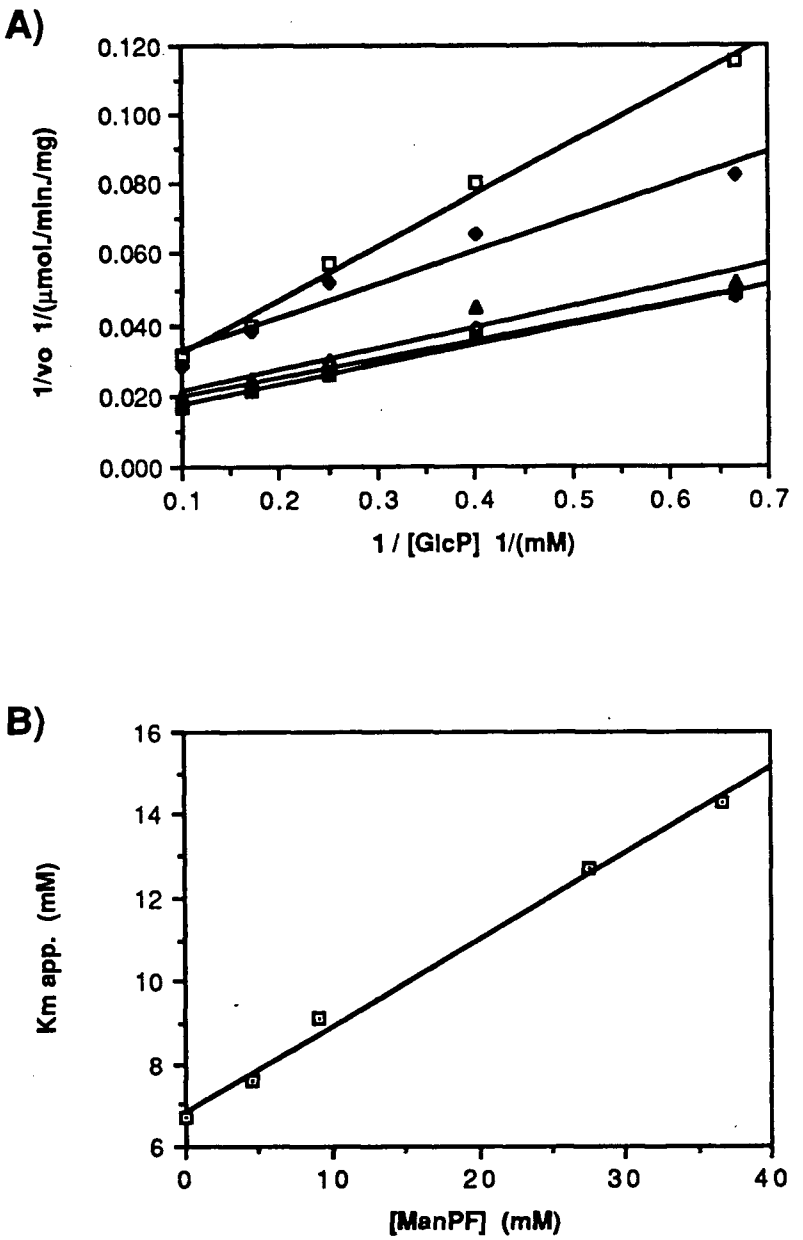


Figure 4-10. Determination of K_i for αManPF.

Initial reaction rates were determined at pH 6.8 and 30°C in a buffer containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1mM EDTA, 1mM DTT, 1mM AMP, 1% glycogen and αGlcP at the following concentrations (mM): 1.5, 2.5, 4.0, 6.0, 10.0. The following inhibitor concentrations were used (mM): ❑ 36.8,◆ 27, ▲ 9.2,◊ 4.6,■ 0.

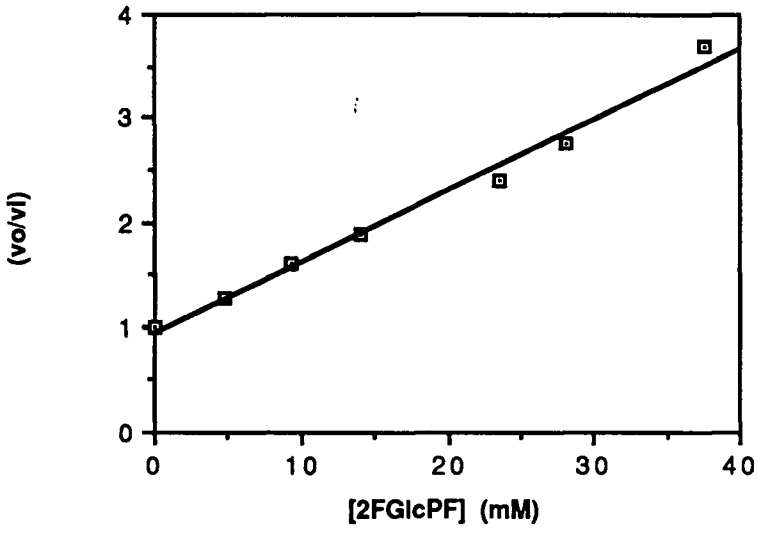


Figure 4-11. Determination of K_i for 2FαGlcPF.

Initial rates were determined as described in the text at a single fixed concentration of substrate (4.0 mM,αGlcP).The reaction contained 50 mM triethanolamine, 100 mM potassium chloride, 1 mM DTT, 1mM EDTA, 1mM AMP and 1 % glycogen. The following inhibitor concentrations were used (mM): 0, 4.7, 9.4, 14.1, 23.5, 28.2, 37.6.

The values for the inhibition constants for the series of substrate analogues presented in table 4-II allows a number of deductions to be made about the specificity of the phosphate subsite in glycogen phosphorylase. Since the phosphofluoridate analogue can only exist as a monoanion at the pH value of the experiment, and since this compound was a reasonable inhibitor of phosphorylase *b*, it can be inferred from this that the enzyme binds the monoanionic species of the natural substrate. However, the compound 2FαGlcP will exist predominantly in its dianionic state (82%) at pH 6.8 (The pKa_2 of 2FαGlcP has been determined to have a value of 5.9 by Withers et al., 1986), thus this observation is consistent with this compound binding to phosphorylase *b* predominantly as the dianionic species. A comparison of the K_i values obtained for 2FαGlcP and 2FαGlcPF would indicate only a 3 fold difference in affinity, consequently this would suggest that phosphorylase *b* can bind both monoanionic and dianionic species with approximately the same affinity. The slight decrease in affinity for the phosphofluoridate analogue is likely a result of the loss of some

weak interaction between substrate and enzyme. A similar comparison of the K_i values obtained for the compounds αManP and αManPF leads to the same conclusions. Again only a small difference in affinity for phosphorylase *b* is seen (approximately 2 fold) and like the previous compounds the phosphate ester exists predominantly as the dianionic species under the experimental conditions, while the phosphofluoridate analogue can only exist as a monoanion. Thus these results are also consistent with both the mono- and dianionic species being bound with approximately equal affinity.

The glucosyl derivative of phosphonic acid (1dαGlcCP) provides a more subtle probe than the phosphofluoridates into the specificity of the phosphate subsite. The relative proportion of the dianion / monoanion will be different, when compared to a similar phosphate ester the pKa_2 of the phosphonic acid is usually considerably higher. The pKa_2 for 1dαGlcCP was determined by ^{31}P-NMR (described later) to have a value of 7.3, thus this compound will be predominantly monoanionic (71%) at pH 6.8. The small increase in affinity shown towards phosphorylase *b* when compared to 2FαGlcP (and αGlcP, $K_m \approx 2$ mM) could thus be attributed to the specificity of phosphorylase for the monoanionic species and the relatively higher percentage of this species present at pH 6.8 in solutions of 1dαGlcCP. However the increase in affinity is relatively small and thus is still consistent with the hypothesis that phosphorylase *b* shows approximately equal affinity for the mono- and dianionic species of its substrate.

To further investigate the specificity of the substrate binding site with regards to its preference for monoanionic substrates the pH-dependence of the inhibition constants for the compounds 1dαGlcCP and 2FαGlcP were determined. For this analysis, a single buffer system was used (50 mM triethanolamine, 100 mM potassium chloride, 1 mM EDTA, 1mM DTT) as described by Withers et al., (1982b). This buffer system has a reasonable buffering capacity within the pH range 5.5 to 7.5, the pH range over which glycogen phosphorylase is most active. The results of this pH dependence study are presented in Figure 4-12.

The inhibition constant (K_i) is a true thermodynamic dissociation constant for the enzyme-inhibitor complex:

$$K_i = \frac{[E]\ [I]}{[E \bullet I]}$$

Where E, I are, respectively, the free enzyme and the free inhibitor and E•I is the non-covalent enzyme inhibitor complex. From this expression it can be seen that the value of K_i as a function of pH will be dependent on the product of the individual ionization constants of E and I, divided by those of E•I. Therefore it could be an almost impossible task to assign the contribution of a single ionizable group to the overall pH dependence of K_i (Knowles, 1976). However, both of the pH profiles shown in Figure 4-12 show an almost identical pH dependence of K_i for both 2FαGlcP and 1dαGlcCP. Interestingly, the pH dependence of

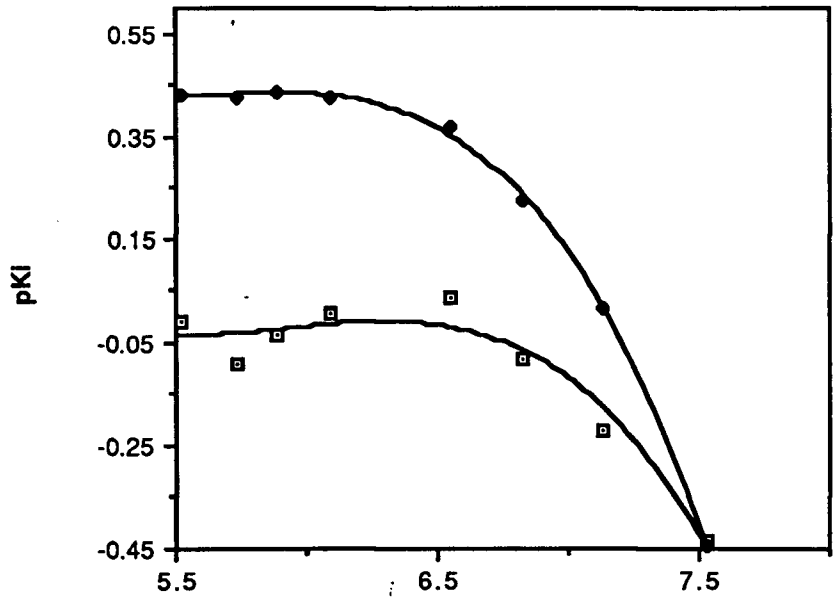


Figure 4-12. The pH dependence of K_i for 2FαGlcP and 1dαGlcCP.

The lines plotted on graph are the best fit of the data points to a 3rd degree polynomial, they do not represent any specific pH function. The values of K_i were determined as described in the text at a constant substrate concentration (αGlcP) of 2.76 mM in the presence of 1mM AMP and 1% glycogen. The following values of Ki were obtained (the pH is given in parentheses); o 2FαGlcP, 1.45 (5.40), 1.24, (5.73), 1.08 (5.89), 0.986 (6.09), 0.917 (6.55), 1.20, (6.83), 1.65 (7.13), 2.71 (7.53):u 1dαGlcCP, 0.369 (5.52), 0.373 (5.73), 0.365 (5.89), 0.377 (6.09), 0.426 (6.55), 0.593 (6.83), 0.968 (7.13), 2.80 (7.53).

binding for these two compounds shown here, is almost identical to the pH dependence of K_m for the enzymes natural substrate, αGlcP (Withers et al., 1982b). The pH dependence of binding can therefore be unambiguously assigned to the ionization of an enzymic functional group in the free enzyme or the enzyme-substrate (inhibitor) complex, since the free ligands have considerably different pKa values. Different pH dependencies would have been expected if the binding process had been dependent on the ionization state of the free ligand. The slight increase in K_i at low pH values seen for 2FαGlcP is probably not significant in this respect, since the ratio of the monoanionic to dianionic species present in solution changes by greater than 28 fold between pH 5.0 and pH 6.8, and yet the inhibition constants at the extremes of this range differ by only 10%.

The results from the pH dependence of K_i would strongly suggest that the binding of the sugar phosphate is independent of the ionization state of the substrate. That is, phosphorylase *b* binds both the monoanionic and dianionic substrates with approximately equal affinity.

NMR studies on the ionization state of the enzyme bound-substrate.

The kinetic experiments described above have demonstrated that phosphorylase *b* shows little discrimination with regards to the ionization state of its substrate, however these experiments do not provide information about the protonation events which occur in the enzyme-substrate complex. It might be suggested that for the dianionic substrate, protonation to produce the monoanion occurs after binding at the active site. To directly probe the ionization state of the enzyme-bound ligands two experiments designed to exploit the ^{31}P-NMR properties of 1dαGlcCP and the pH dependence of the ^{19}F-NMR resonance of 2FαGlcP were also performed.

^{31}P-NMR titration of 1dαGlcCP: the ^{31}P-NMR resonance of this compound is sensitive to pH and this was used to determine the ionization constants associated with the phosphonic acid group. A sample containing 1dαGlcCP (20 mM) in 0.1 M potassium

chloride in 50% deuterated water, was titrated over a wide range of pH values (0.48 to 10.70) by addition of small volumes of concentrated acid or base. The ^{31}P-NMR spectrum was recorded after every addition and the pH determined by meter reading. The titration curve obtained in this manner is presented in Figure 4-13. Values of 1.92 and 7.34 were determined respectively for pKa_1 and pKa_2 from this curve.

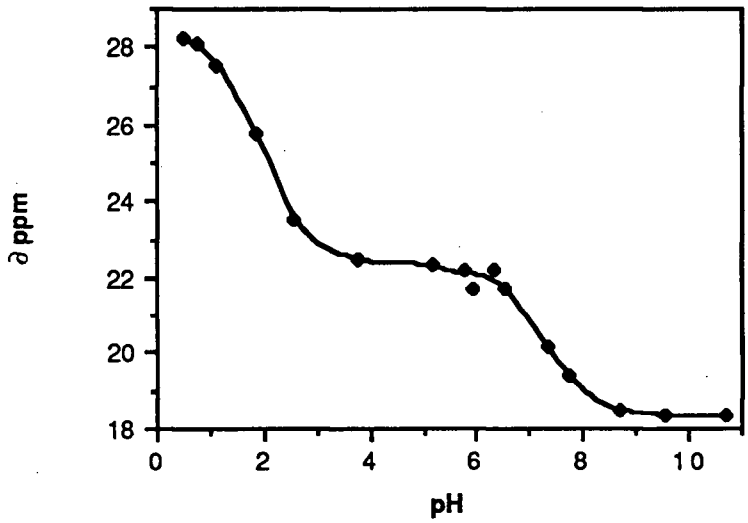


Figure 4-13. The ^{31}P-NMR titration curve for 1dαGlcCP.

The titration was carried out in a 50% deuterated solution in 100 mM potassium chloride, the concentration of 1dαGlcCP was 20 mM. The pH was adjusted by additions of small volumes of 2M potassium hydroxide or 3M hydrochloric acid.

The ionization state of enzyme-bound 1dαGlcCP: under conditions where a ligand is freely exchanging between two or more different environments effects on the linewidth and chemical shift of the NMR resonance can be expected. These effects are described for a few limiting conditions in Figure 4-14 of which the fast and slow exchange regimes are relevant to the discussion of the following experiments with phosphorylase *b*. Under slow exchange conditions, the two resonances will still be centred about ω_A and ω_B, but are broadened to $\Delta\omega(A)$ and $\Delta\omega(B)$ respectively, such that

$$\Delta\omega(A) - \Delta\omega_0(A) = \tau_A^{-1}$$

$$\Delta\omega(B) - \Delta\omega_0(B) = \tau_B^{-1}$$

where $\omega_0(A)$ and $\omega_0(B)$ are the linewidths in the absence of exchange. Under fast exchange conditions a single resonance is observed at an intermediate frequency (ω) with intermediate linewidth ($\Delta\omega$) given by

$$\omega = \rho_A \omega_A + \rho_B \omega_B$$

$$\Delta\omega = \rho_A \Delta\omega_0(A) + \rho_B \Delta\omega_0(B)$$

where ρ_A and ρ_B are the fractional populations of the exchanging species.

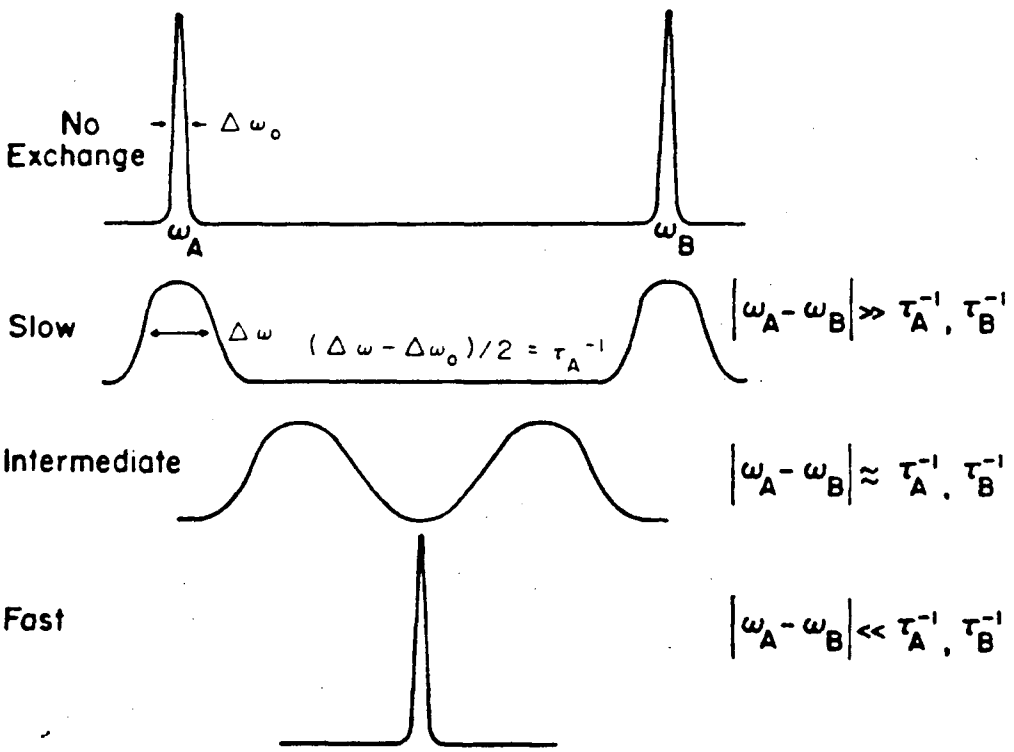


Figure 4-14. Effects of Chemical exchange on line shape and resonance position.

Figure from Cohn and Rao (1979) ω_A and ω_B are the resonance frequencies of species A and B and $\Delta\omega_0(A)$ and $\Delta\omega_0(B)$ are their respective linewidths, τ_A and τ_B are the lifetimes in the respective states when exchange takes place.

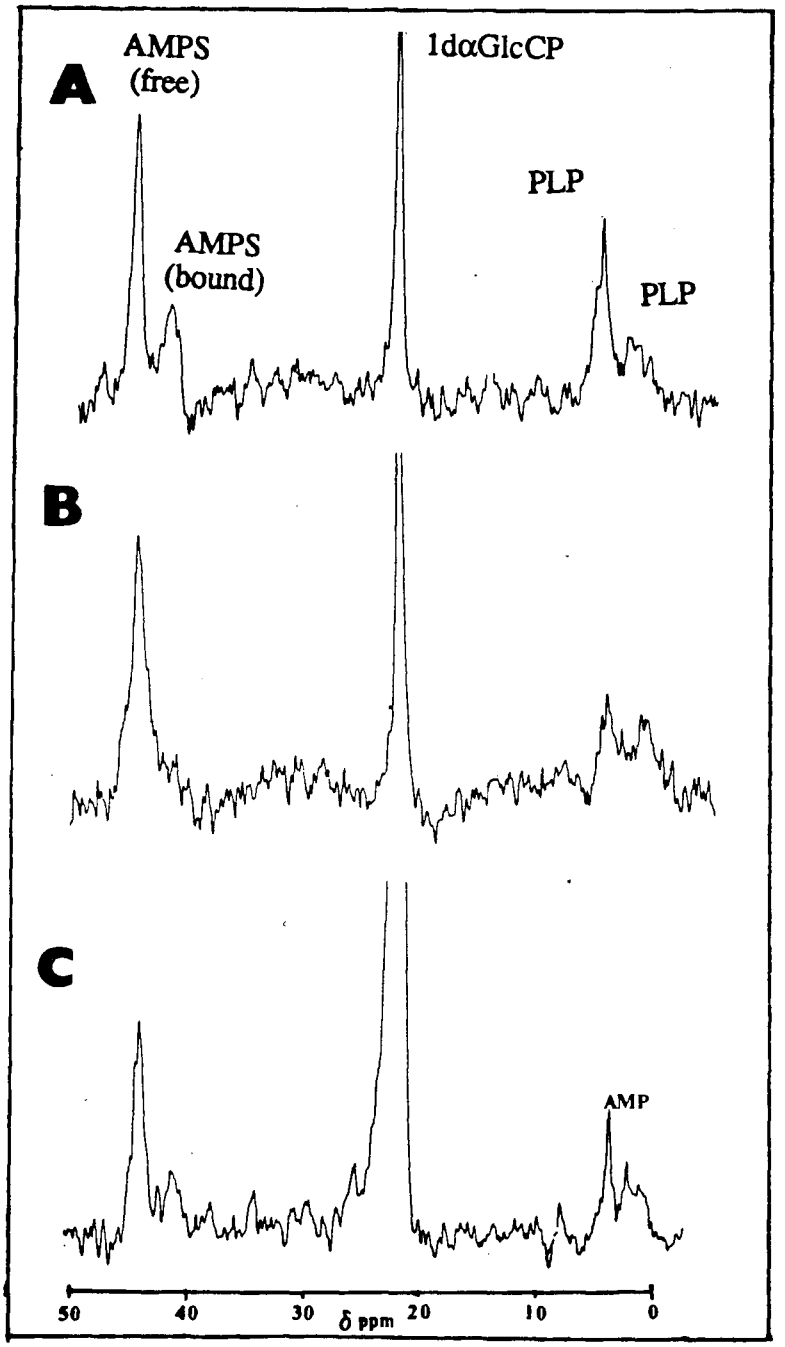


Figure 4-15. ^{31}P-NMR spectra of glycogen phosphorylase b in the presence of 1dαGclCP.

Reaction mixtures were in a 50% deuterated buffer containing 50 mM triethanolamine, 100 mM potassium chloride, 1 mM EDTA, and 1mM DTT at pH 6.8 and 30°C. A) Signal averaged over 10, 000 transients, mixture contained phosphorylase *b* (0.99mM), AMPS (1.84 mM), 1dαGlcCP (0.91mM). B) Signal averaged over 10, 000 transients, reaction mixture contained, phosphorylase *b* (0.96 mM), AMPS (1.78 mM), Glycogen (0.5%), 1dαGlcCP (0.88 mM). C) Signal averaged over 10, 000 transients, reaction contained phosphorylase b (0.89 mM), AMPS, (1.71 mM), 1dαGlcCP (4.88 mM).

The ^{31}P-NMR spectra of 1dαGlcCP in the presence of high concentrations of phosphorylase *b* (≈ 1 mM) and various effectors are shown in Figure 4-15. Spectrum A shows a sample containing phosphorylase *b* with approximately equimolar amounts of 1dαGlcCP (δ = 21.5 ppm) and nucleotide activator adenosine monophosphorothioate (AMPS). This compound is a good activator of phosphorylase *b* and is often used in ^{31}P-NMR studies, since it has the advantage that the resonance for the phosphorothioate group is well downfield of the resonance from the enzyme-bound PLP. The observation of distinct resonances for both the free and the bound AMPS demonstrates that this ligand is binding tightly to the enzyme and consequently is in the slow exchange limit on the NMR time scale.

The resonance due to free and enzyme-bound 1dαGlcCP is observed as a single exchange averaged signal (rapid exchange limit in Figure 4-14). Under these circumstances both the observed chemical shift and linewidth are a true weighted average of those from the free and bound species. Two resonances due to the enzymic cofactor, PLP are seen; one at δ = 3.9 ppm ($\Delta\upsilon_{1/2}$ = 120 Hz) and a signal of low intensity and large linewidth at δ ≈ 0.5 ppm ($\Delta\upsilon_{1/2}$ ≈ 290 Hz). The predominant resonance from PLP is due to the supposedly dianionic form of the cofactor, normally associated with AMPS-activated phosphorylase *b* . The broad resonance at δ = 0.5 ppm is probably the same form of the cofactor seen by Withers et al., (1981a) upon introduction of the competitive inhibitor glucose-1,2-cyclic phosphate to AMPS activated phosphorylase *b*.

Introduction of glycogen to this sample has two major effects: firstly, the binding of AMPS is apparently weakened, since the resonances due to both free and bound nucleotide are observed as a single broad exchange-averaged signal at δ = 41.0 ppm. A similar effect was observed by Withers et al. (1981a), upon addition of high concentrations of maltoheptaose to the phosphorylase *b*-AMPS-glucose-1,2-cyclic phosphate complex. The second effect of glycogen addition is seen in conversion of more of the PLP resonance from the AMPS activated form to the broad resonance at δ ≈ 0.5 ppm.

It has previously been observed that the ^{31}P-NMR resonance from PLP in the glucose-phosphorylase *b* complex also appears at $\delta \approx 0$ ppm (Withers et al., 1981a), but with a relatively narrower linewidth. Since the chemical shift of the high-field resonance due to PLP in the ternary enzyme-glycogen-substrate analogue complex, is similar to that of PLP in the enzyme-glucose complex, there remains the possibility that the shift of the PLP resonance is due to the introduction of contaminating glucose in the glycogen preparation. This would also be consistent with the apparent weakening of the AMPS binding, as phosphorylase *b* in the presence of this T-state effector shows a lower affinity for nucleotide activator (Withers et al., 1981a). To test this hypothesis samples of the glycogen preparation which were used in the above experiment were assayed for glucose content using the hexokinase / glucose 6-phosphate dehydrogenase coupled assay (described in Chapter 2). By use of this system it was ascertained that the glycogen preparation contained only negligible amounts of glucose, certainly insufficient amounts to cause the effects seen in Figure 4-15B. Similar conclusions were drawn by Withers et al. (1981a) after a chromatographic analysis of the glycogen preparation used in their NMR experiments.

Addition of further 1dαGlcCP to the above NMR sample (Figure 4-15C) resulted in an apparent increase in affinity of phosphorylase *b* for AMPS, as evidenced by the return of separate resonances for both free and bound nucleotide. A further increase in the proportion of the PLP at the high-field position is also seen. This effect on the PLP resonance is masked to some extent by the sharp resonance which appeared at $\delta = 3.3$ ppm, which is due to AMP liberated from AMPS as a result of its non-enzymic hydrolysis. However, effects on the PLP resonance seen here upon formation of the ternary enzyme-glycogen-1dαGlcCP complex are essentially identical to those seen by Withers et al.(1981a) in the ternary enzyme-maltoheptaose-glucose-1,2-cyclic phosphate complex.

An estimate of the chemical shift of the fully formed enzyme-ligand complex (and hence the ionization state in the enzyme-ligand complex) can be obtained from the following relationship (Dahlquist and Raftery, 1968)

$$[L_o] = \frac{[E_o]\,\Delta\delta_b}{\delta_F - \delta_{obs}} - K_d$$

Where, $[L_o]$ is the initial concentration of the added ligand, $[E_o]$ is the concentration of enzyme, $\Delta\delta_b$ is the change in chemical shift which accompanies formation of the fully bound enzyme-ligand complex, δ_F is the chemical shift of the ligand in free solution, δ_{obs} is the observed chemical shift of the exchanged averaged ligand and K_d is the dissociation constant for the enzyme ligand complex. The values of K_d and δ_b can be calculated from the NMR data by plotting the reciprocal of $(\delta_F - \delta_{obs})$ against the added concentration of ligand. Providing the enzyme concentration remains constant the slope of the plot yields $\Delta\delta_b$ and the X-intercept the value for K_d.

A separate ^{31}P-NMR experiment was conducted to determine the ionization state of enzyme-bound 1dαGlcCP. These samples were prepared under conditions of constant AMP (2mM) and glycogen (0.5%) concentrations, and additions of 1dαGlcCP were performed so that the enzyme concentration was not altered appreciably by these change in volume.The results of this experiment are presented in Table 4-III.

Table 4-III. Determination of the ionization state of bound 1dαGlcCP [a].

Enzyme Concentration (mM)	Ligand Concentration (mM)	[b] % Ligand Bound	[c] δ (ppm)
1.07	0.46	53.1	21.38
1.04	1.85	37.8	21.49
1.02	3.60	24.3	21.51
0	5.0	0	21.55

a Reaction were carried out in a 50% deuterated buffer containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1mM EDTA, 1 mM DTT at pH 6.8 and 30°C. [b] Calculated form a dissociation constant of 0.7mM. [c] Measured relative to 85% phosphoric acid.

From these data it can be seen that a small upfield shift from the resonance position of the ligand in free solution occurs as the percentage of the bound ligand increases. By reference to

the ^{31}P-NMR titration curve (Figure 4-13) this upfield shift corresponds to an increase in the dianionic form of the ligand. The data from Table 4-III was plotted as described above (Figure 4-16) and yielded values for $K_d = 0.6 \pm 0.4$ mM and $\Delta\delta_b = 0.16$ ppm, thus the value of K_d obtained here is consistent with the value obtained from steady state kinetics.

The shift of 0.16 ppm to higher field strengths accompanying the binding of the ligand to phosphorylase *b*, when interpreted solely in terms of a change in ionization state is consistent with a small increase in the concentration of the dianionic species upon binding to the enzyme.

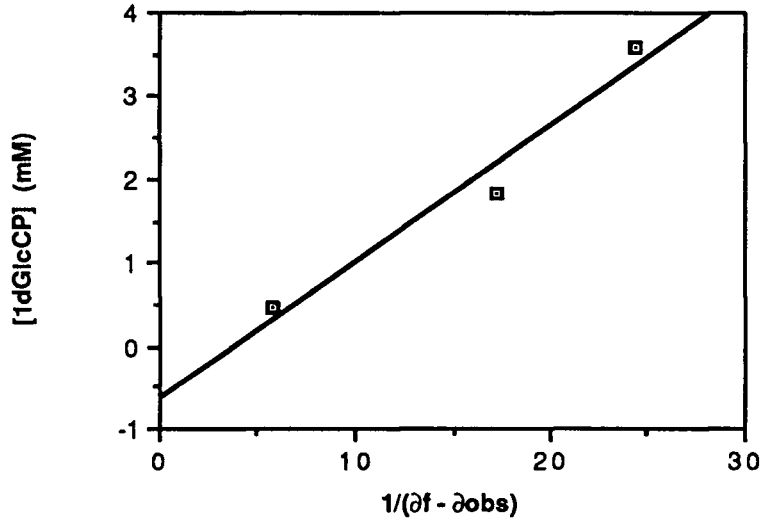


Figure 4-16. Determination of the ^{31}P-NMR chemical shift of the phosphorylase b-1dαGlcCP complex.

Considering that in the ^{31}P-NMR titration curve for the free ligand a difference in chemical shift of ≈ 4 ppm to higher field is observed between the monoanionic and dianionic forms of 1dαGlcCP, the shift of 0.16ppm seen upon binding to phosphorylase *b* does not constitute a very significant perturbation of dianion : monoanion ratio to that which is found in free solution at pH 6.8. It is also possible that this shift seen upon binding of the ligand to phosphorylase *b* is not due to changes in ionization state at all, but due to changes of the environment of the ligand on going from free solution to the active site. Since, in free solution at pH 6.8, 71% of 1dαGlcCP is present as the monoanionic species, the results

from the above NMR experiment are consistent with the majority of the ligand binding as the monoanionic species.

The ionization state of the phosphorylase b-2FαGlcP complex: the ^{19}F-NMR signal from 2FαGlcP shows a characteristic pH dependence (Withers et al., 1986, see also Figure 4-17) which has been used to determine the pKa values of the 1-phosphate. These values were found to be identical to those obtained by potentiometric methods. Thus this compound provides another useful NMR probe to determine the ionization state of this ligand while bound to phosphorylase *b*, which may hopefully avoid problems in intepretation which cloud the ^{31}P-NMR data.

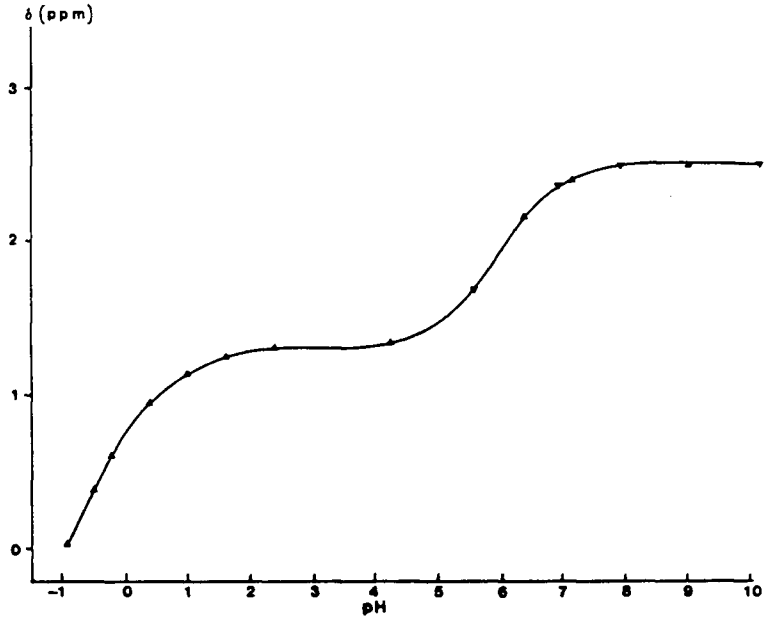


Figure 4-17. The ^{19}F-NMR titration of 2FαGlcP.

The ^{19}F-NMR spectra from mixtures containing 2αFGlcP (a small amount of the β-anomer is also present) and glycogen phosphorylase *b*, with 1 mM AMP, 0.5% glycogen at

pH 6.8 and 30°C are given in Figure 4-18 The relevant data pertaining to the samples are presented in Table 4-IV and the legend of Figure 4-18.

Table 4-IV. Determination of the ionization state of bound 2FαGlcP [a].

Enzyme Concentration (mM)	Ligand Concentration (mM)	[b] % Ligand Bound	Linewidth (Hz)	[c] δ (ppm)
0.68	0.8	21	103	199.20
0.68	1.6	17	65	199.19
0.63	2.2	14	55	199.20

[a] Samples were made up in a 50% deuterated buffered solution containing 50 mM triethanolamine, 100 mM potassium chloride, 1mM EDTA, 1 mM DTT at pH 6.8 and 30°C. [b] Calculated using a dissociation constant of 2.0 mM. [c] Measured relative to $CFCl_3$

The samples of 2FαGlcP used for this NMR experiment contained both α-(80%) and β-anomers (20%) of the deoxyfluoro sugar phosphate. The resonance due to the β-anomer appears slightly downfield from the resonance assigned to the α-anomer, at δ = 198.7 ppm. and since it does not bind to the enzyme (Graves and Wang, 1972) served as a useful internal reference. The resonances due to free and enzyme-bound 2FαGlcP are within the rapid exchange limit on the NMR time scale and are therefore observed as a single exchange-averaged signal. Thus the linewidth of the resonance due to the α-anomer increases as the percentage of the enzyme-bound form increases, while the linewidth of the resonance due to the β-anomer remains constant ($\Delta\upsilon_{1/2}$ = 35 Hz). The observed linewidth of the resonance due to 2FαGlcP is a weighted average of the linewidths from the ligand free-solution and enzyme-bound ligand.This value increases as the proportion of enzyme bound ligand increases, showing that binding is occurring. However, the chemical shift of the signal due to 2FαGlcP, which must also be a weighted average of the free and bound forms, remains constant, indicating that the enzyme-bound ligand has the same chemical shift as the ligand in

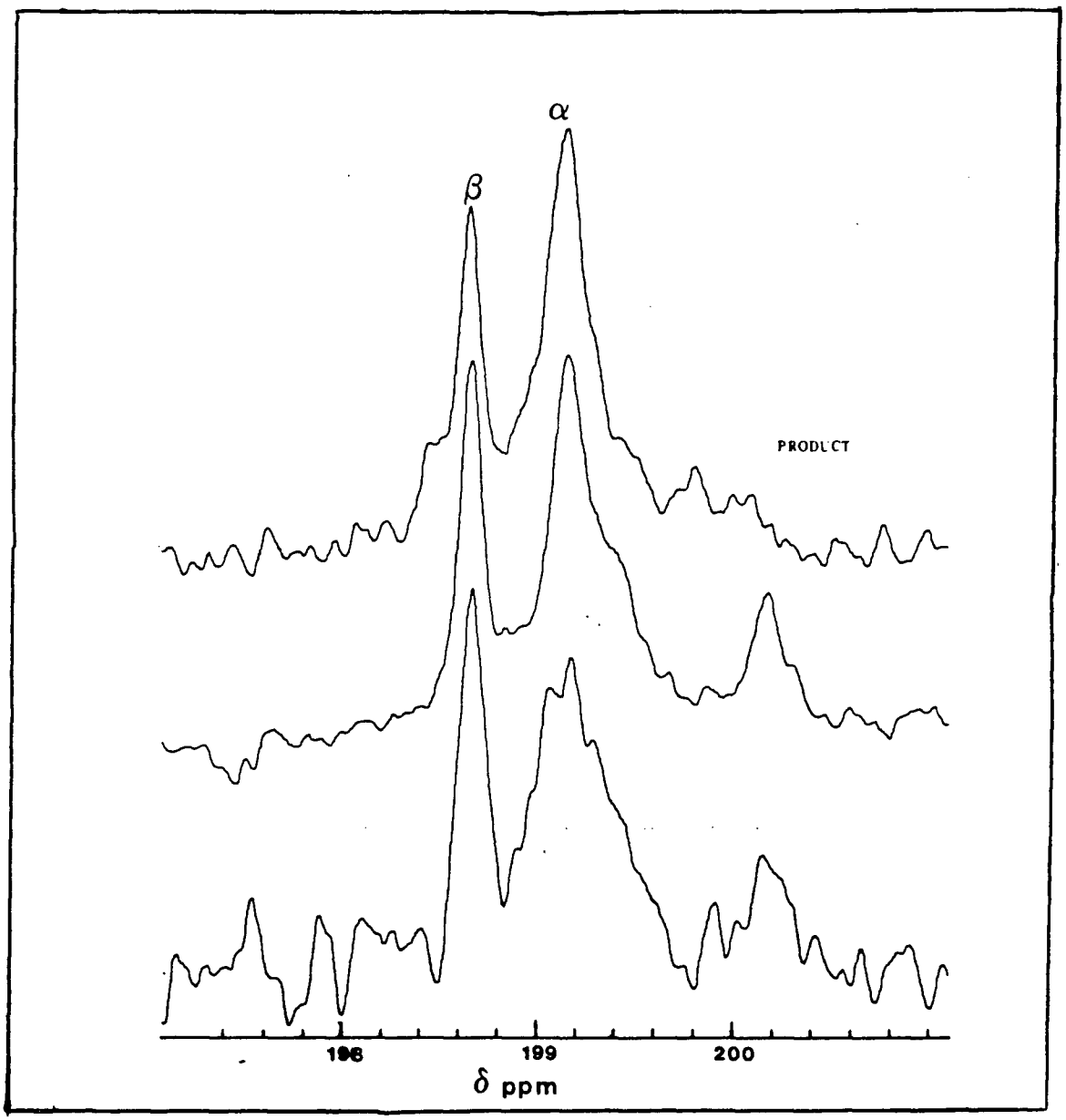


Figure 4-18. The ^{19}F-NMR proton-decoupled spectra of mixtures containing 2FGlcP and phosphorylase b.

Proton decoupling was performed either as continuous broad-band, or gated (decoupler on during acquisition only), the same results were obtained from both techniques. Samples were made up in a 50% deuterated buffered solution containing 50 mM triethanolamine, 100 mM potassium chloride, 1mM EDTA, 1 mM DTT at pH 6.8 and 30°C. A) Signal averaged over 45,000 transients, sample contained 0.68 mM phosphorylase b, 0.8 mM 2FαGlcP. B) Signal averaged over 45,000 transients, sample contained 0.68 mM phosphorylase b, 1.6 mM 2FαGlcP. C) Signal averaged over 15, 000 transients, sample contained 0.63 mM phosphorylase b, 2.2 mM 2FαGlcP.

free solution. Since any significant change in ionization state accompanying binding should be accompanied by a change in chemical shift and given that most of the ligand is present as the dianionic species in free solution at pH 6.8, this result suggest that this ligand binds to phosphorylase *b* largely as a dianionic species.

In summary, the inhibition constants obtained from the phosphofluoridate analogues demonstrated that these monoanionic substrates bound to phosphorylase *b* with approximately the same affinity as the corresponding phosphate ester. Since, under the experimental conditions employed the phosphate esters exist as a predominantly dianionic species these results suggest that both monanionic and dianionic forms of the substrate are bound to phosphorylase *b* with approximately equal affinity. The results of the NMR experiments also suggested that this was the case. The chemical shift of the ^{31}P-NMR resonance due to the phosphorylase-1dαGlcCP complex suggested that the ratio of monoanionic to dianionic enzyme-bound ligand was little different from the ratio of these ionic species in free solution. This result was consistent with the majority of the inhibitor binding as a monoanionic species. The ^{19}F-NMR experiment suggested that 2FαGlcP bound predominantly as the dianionic species and the lack of any chemical shift difference between the free and the enzyme bound forms of this ligand also suggests that the substrate is not protonated on binding at the active site. The mechanistic implications of this finding will be discussed in subsequent sections.

Discussion.

Transition State Structure in Reactions Catalysed by Glycogen Phosphorylase.

Essentially all kinetically based probes of reaction mechanism, are in effect interpreted by considering how some perturbing variable (temperature, concentration, isotopic composition, substituents) affects the free energy of activation for the process under consideration. From such experiments it is often possible to derive a reasonable model of the transition state. By use of the deoxy and deoxyfluoro analogues of αGlcP as kinetic probes for the transition

state in the glycogen phosphorylase-catalysed reaction and by comparison with how the same substitutions affect other reactions (which may be enzymic or non-enzymic) we should eventually be able to build up a reasonable picture for the transition state of the glycogen phosphorylase reaction.

From the kinetic data obtained with glycogen phosphorylase *b* and the deoxy and deoxyfluoro substrate analogues it can be seen that substitution of individual hydroxyl groups around the glucopyranose ring by fluorine or hydrogen causes a dramatic decrease in turnover rate. Indeed, 6dαGlcP and 6FαGlcP had previously been tested as substrates with glycogen phosphorylase and were thought to be inactive (Graves and Wang, 1972). Assuming that the glycogen phosphorylase reaction proceeds through transition states with substantial oxocarbonium ion-like character, then the observed rate reductions are probably a combination of two effects: firstly, electronic effects, caused by destabilization of an already electron deficient transition state by introduction of electronegative elements such as fluorine; as we have already seen in the case of β-glucosidase these effects can be quite dramatic. Secondly, destabilization of the enzymic transition state by disruption of the normal enzyme-ligand interactions.

Electronic Effects.

We have already seen from the preliminary discussion in Chapter 1 that the reaction mechanism for acid-catalysed hydrolysis of glucosides and glucopyranosyl phosphates likely involves a transition state with substantial oxocarbonium ion-like character. The effect of substitution of a hydroxyl group by the more electronegative fluorine was to decrease the rate of hydrolysis and presumably this was due to electronic destabilization by the fluorine of an already electron deficient transition state. The converse effect was also observed when the relatively electronegative hydroxyl group was replaced by hydrogen (deoxy sugars). If the enzymic reaction proceeded through a similar type of transition state and electronic considerations were the only factor associated with the substituents that affected the free

energy of the transition state then a similar relationship between rate and substituent should also be observed for the enzymic reaction. This type of relationship has often been used by the physical organic chemist to demonstrate similarities in transition state structure of related reactions and is known as a linear free energy relationship (LFER). These relationships are often expressed in the form:

$$k_A = C\, k_B^{\beta} \qquad (1)$$

Where k_A and k_B are the rate constants for reactions A and B and C and β are constants. It can be shown that this relationship applies directly to the transition state energy of the two reactions by the following:

From the Eyring equation

$$k_A = (kT / h)\ \exp\left(\frac{-\Delta G_A^{\ddagger}}{RT}\right) \qquad (2)$$

$$k_B = (kT / h)\ \exp\left(\frac{-\Delta G_B^{\ddagger}}{RT}\right) \qquad (3)$$

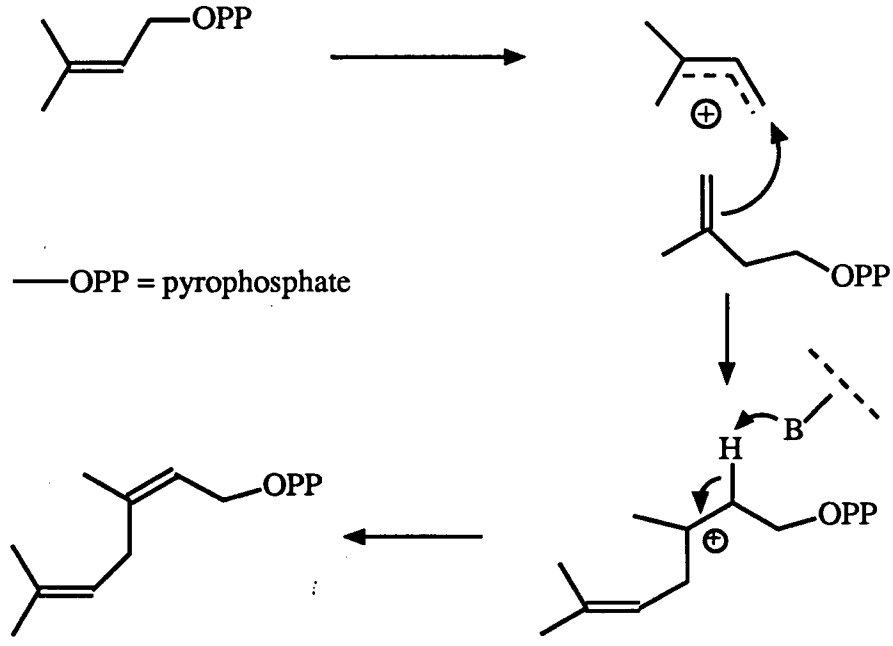
where $\Delta G^{\ddagger}$ is the Gibbs free energy of activation for the reaction A or B. By taking the logarithm of **1** and substituting the values for the activation energies from **2** and **3**, the following relationship can be derived.

$$\Delta G_A^{\ddagger} = \Delta G_B^{\ddagger}\, \beta + C$$

This is essentially the same relationship as shown by equation **1** but the term C now contains extra parameters from equations **2** and **3**, thus a plot of the logarithm of the rate constants for the two reactions under scrutiny, yields information about the similarity in the transition state structure of the two processes. The correlation coefficient provides a measure of the degree of certainty in the relationship, while the slope of the plot yields information on the similarity of the properties of the two transition states, that is charge development, concertedness etc.

Previously, in a study of farnesyl pyrophosphate synthetase, Poulter et al., (1981) were able to use electronic effects to demonstrate the intermediacy of an allylic cation in the enzyme-catalysed reaction (see Scheme 4-5). In this case it was possible to prove that

electronic effects were of paramount importance in the enzymic reaction by comparing the rates of the enzyme catalysed process for a series of fluoro-substituted substrates to those of their acid-catalysed hydrolysis, a process known to proceed via an allylic carbonium ion. A plot of the logarithm of the relative rate constant for acid-catalysed hydrolysis against logarithm of relative k_{cat} values for the enzymic process provided an excellent linear free energy relationship. Since the substituent effects on the acid-catalysed reaction are primarily electronic in origin, and since the linear free energy relationship demonstrated that there was a high correlation of substituent effects between the enzymic and non-enzymic reaction, a similar mechanism was implicated. In this case the authors were able to separate electronic from binding contributions to the overall stability of the enzymic transition state because the modifications performed on the substrate did not affect binding significantly.



Scheme 4-5. The reaction mechanism of farnesyl pyrophosphate synthetase.

In the context of the transition state structure in glycogen phosphorylase, again in the absence of any other factors which might destabilize the enzymic transition state, a comparison of the enzyme-catalysed process with the rates of acid-catalysed hydrolysis for the same series of deoxy and deoxyfluoro glucopyranosyl phosphates would yield information about the electronic structure of the enzymic transition state. However, these modifications to the structure of the substrate are also likely to cause significant perturbations of the enzyme-ligand interactions. From previous discussion in Chapter 1 (Binding Energy and Catalysis) it would be expected that these type of interactions would also be important to the stability of the enzymic transition state. The destabilization of this transition state due to deletion of specific enzyme-ligand interactions would thus be superimposed onto the perturbation in transition state energy due to electronic factors. Consequently this would make any LFER due to the electronic considerations only, harder to observe.

Plots of the logarithm for the enzymic rate constants (k_{cat} / K_m or k_{cat}) against the logarithm of the rate for the acid-catalysed hydrolysis for the deoxy and deoxyfluoro glucopyranosyl phosphates are presented in Figure 4-19. There appears to be little correlation between log (k_{cat} / K_m) versus log k (the first order rate constant for the acid-catalysed hydrolysis) as evidenced by the low value of the correlation coefficient (R). The correlation between log k_{cat} and log k (R = 0.9) is significant, indicating that the electronic factors are important in determining the stability of the transition state for the rate determining step in the enzyme-catalysed reaction. A rational for why this correleation should be present in one case, but not the other, as follows.

For all kinetic mechanisms where a single step has a transition state for which the free energy of activation greatly exceeds that of all others (a single rate determining step), relationships of kinetic parameters to reactant state and transition state energies can be described by a number of simple rules (Schowen, 1978).

1) The two parameters of enzyme kinetics k_{cat} and k_{cat} / K_m relate to the free energy difference between two states, a transition state and an initial reference state.

2) For k_{cat} / K_m the initial reference state is always the free enzyme and the free substrate(s); for k_{cat} it is the major form in which the enzyme accumulates in the reaction sequence.

3) For k_{cat} / K_m the effective transition state is always that of the first irreversible step in the reaction sequence. For k_{cat}, the effective transition state is that of the highest free energy following the state in which saturated enzyme (not necessarily the enzyme-substrate complex) accumulates (that is the transition state of the overall rate determining step)

For many kinetic mechanisms it is often the case that the first irreversible step and the rate determining step are the same, however this need not always be true. As an illustrative example consider the kinetic mechanism of a "retaining" glucosidase (Chapter 2). Reaction of the enzyme with a weakly activated substrate makes bond cleavage (glucosylation) the overall rate determining step in the catalytic process. In this case the first irreversible step is the same as the rate determining step. Reaction with a strongly activated substrate makes hydrolysis of the glucosyl-enzyme-intermediate the overall rate determining step, but in this case the first irreversible step is still bond cleavage. The bond cleavage step is effectively irreversible because the aglycone is free to diffuse out of the active site where it is diluted into solution making the rate of the reverse process effectively zero. Without a detailed knowledge of the kinetic mechanism for glycogen phosphorylase it is difficult to ascertain whether or not the plots shown in Figure 4-19 refer to the transition state of the same or different catalytic steps. The difference in the correlation coefficients for the two plots might therefore arise because the values k_{cat} and k_{cat} / K_m do not represent the same catalytic step. This would indicate that activation energy of the first irreversible step (as represented by k_{cat} / K_m) is not sensitive to the electronic effects of the substituents in the same manner as the acid-catalysed hydrolysis. Alternatively, it could be suggested that the perturbations in the activation energy caused by binding effects are much more important than the electronic effects to the stability of this transition state, and consequently the trend due to electronic effects is hidden.

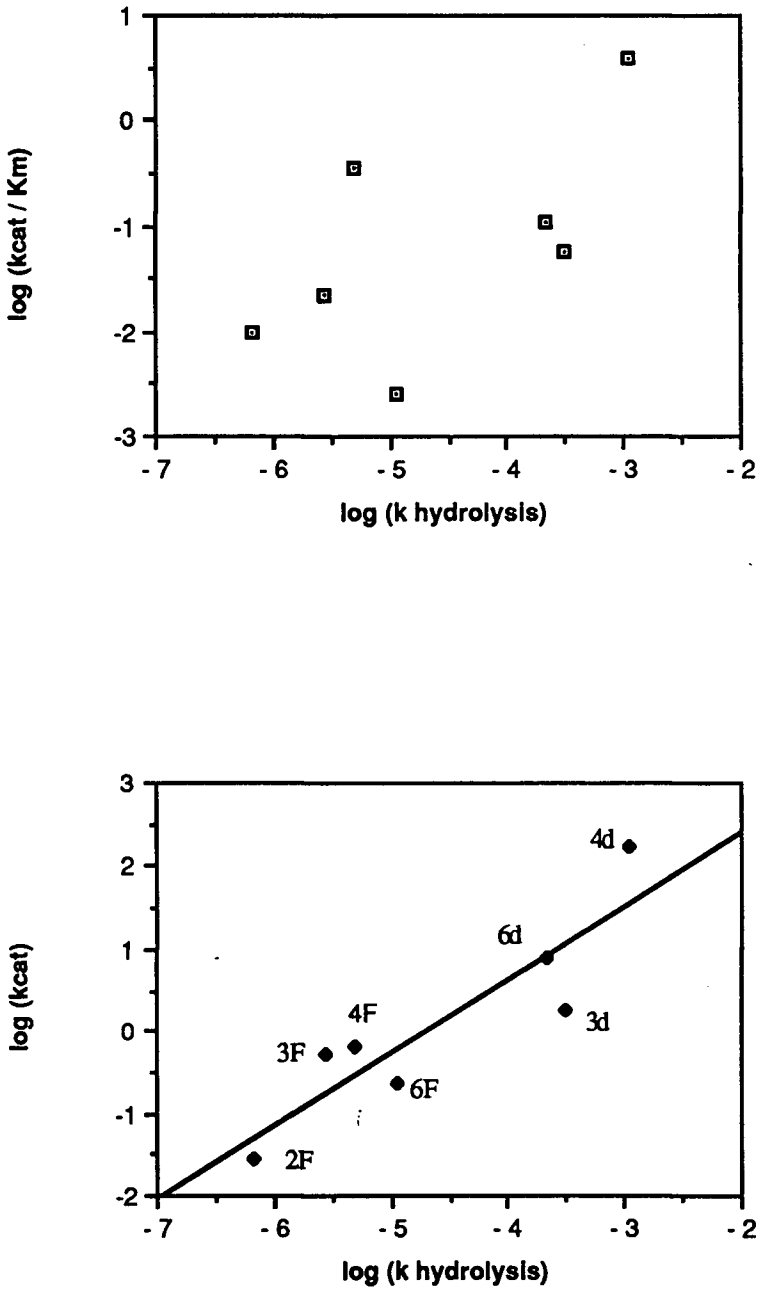


Figure 4-19. Linear free energy relationships between kinetic parameters for muscle glycogen phosphorylase and the acid catalysed hydrolysis rates for a series of deoxy and deoxyfluoro glucopyranosyl phosphates.

Both of these explanations are reasonable since the energy change represented by k_{cat}/K_m is from the free enzyme and free substrates to the transition state of the first irreversible step and therefore the perturbations in activation energy due to binding factors would be expected to be large. The activation energy represented by k_{cat} is from the *enzyme-bound* intermediate immediately preceding the rate determining step to the *enzyme-bound* transition state for the same step. Therefore the perturbations of the transition state free energy caused by binding factors are only the *difference* between the ground and transition state binding energies for the enzyme bound species. Thus much of the contribution due to modification of the enzyme-ligand interactions is subtracted out, making the trends due to electronic factors more easily observed.

It could be possible that the first irreversible step does not involve bond making or breaking processes. It has been proposed that a protein conformational change is at least partially rate determining in the bond cleavage step of O-aryl galactosides by E.coli β-galactosidase (Sinnott et al., 1978). For glycogen phosphorylase it has also been proposed that an protein conformational changes are an important part of the catalytic mechanism (Withers et al., 1982a; Chang and Graves, 1985; Chang et al., 1986).

The correlation of the trends in the perturbation of the activation energy for the overall rate determining step with those of the activation energy for the acid-catalysed hydrolysis due to the substituent effects is relatively good. Since the substituent effects on the rate of acid-catalysed hydrolysis for these compounds are thought to be mainly electronic in origin, then this suggests that this is also true for the enzymic reaction and would require that the enzymic transition state also had considerable oxocarbonium ion-like character. The trends in the relationship between k_{cat} and $k_{hydrolysis}$ extends over three orders of magnitude in rate for the enzyme catalysed process and the trend is observed despite interference from binding factors. The slope of the plot (β) is 0.8, indicating that the enzyme-catalysed reaction is slightly less sensitive to the electronic effects of substitution than the acid-catalysed hydrolysis. Expressed in a slightly different manner, the enzymic transition state has 80% of the oxocarbonium ion-

character of the transition state for the acid-catalysed reaction. This would suggest that the enzyme reaction is more pre-associative than the acid-catalysed hydrolysis, which could be rationalized in terms of the participation of an enzyme-bound nucleophile or nucleophilic participation by the incoming hydroxyl group of the saccharide acceptor. However, it is unlikely that an unstable species with considerable oxocarbonium ion-character would have a lifetime sufficient for transfer to the oligosaccharide acceptor to occur (unless preassociation with the acceptor-saccharide is ocurring, Sinnott, 1987) and would therefore have to be stabilized in some manner, probably as a covalent glucosyl-enzyme-intermediate.

The conclusions drawn from the data in this study have gained some support from other studies. The observation that the transition state analogue glucono-1,5-lactone binds tightly to the ternary enzyme-glycogen-phosphate complex (K_i = 0.025 mM) is also consistent with a transition state structure of this type. There are also a number of mechanistic implications from this result. The concerted process proposed by Klein et al. (1986) would seem less likely on this basis, since a truly concerted process cannot have a transition state with considerable oxocarbonium ion-character.

Enzyme-ligand interactions in R-state glycogen phosphorylase..

A study on the specificity of the glucose binding site in inactive T-state glycogen phosphorylase *b* (Street et al., 1986) was described in Chapter 3, but will be reviewed briefly here.

It was found that important hydrogen bonding interactions occurred between the enzyme and the 3- and 6-hydroxyl groups of the enzyme-bound glucose. The binding energy derived from the interactions between enzyme and ligand at these positions contributed ≈ 3 kcal mol^{-1} each to the overall binding energy of the enzyme-glucose complex. Potential enzymic hydrogen bonding partners for these hydroxyl groups were identified from X-ray crystallography and were found to be potentially charged residues (charge-neutral interactions). For the 6-position an interaction with a protonated histidine (His 376) was

postulated and for the 3-position an interaction with a glutamate residue (Glu 672). The interactions of the hydroxyl groups at the 1-, 2- and 4-positions were found to contribute 1.0 - 1.5 kcal mol $^{-1}$ each to the overall binding energy. From the X-ray crystallographic structure these were postulated to be interactions between the hydroxyl group of the enzyme-bound ligand and neutral amino acid residues (neutral-neutral interactions). These results are summarized in Figure 4-20.

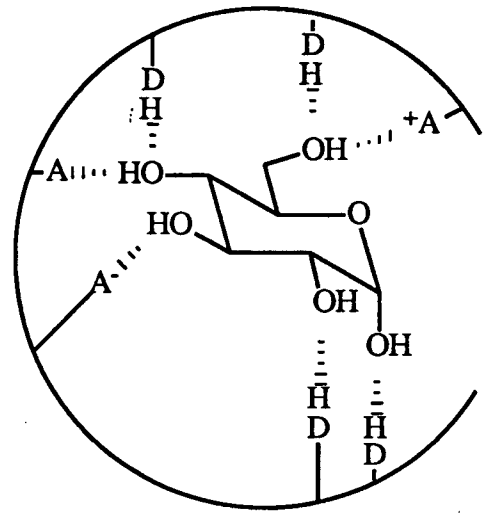
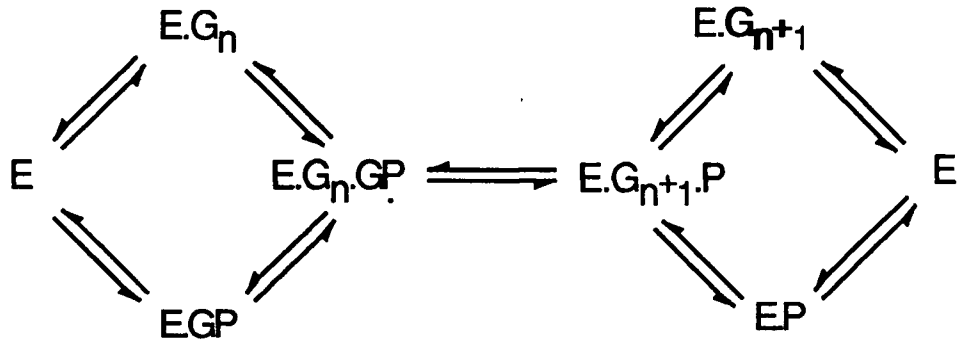


Figure 4-20. A schematic representation of the hydrogen bonding network in the glucose-phosphorylase b complex.

The kinetic data obtained from the deoxy and deoxyfluoro analogues of αGlcP pertains to the enzyme-ligand interactions in the active R-state enzyme and therefore enable us to make comparisons of the enzyme-ligand interactions of the phosphorylase *b*-glucose complex and the phosphorylase *b*-substrate complex. In the latter case these comparisons can be made for both the ground state of the enzyme-substrate complex as well as the transition state enzyme-ligand complex.

Enzyme-ligand interactions in the ground state enzyme-substrate complex: as mentioned briefly in the introduction the kinetic mechanism of glycogen phosphorylase has been determined as a rapid equilibrium random bi bi (see below):



Scheme 4-6. The kinetic mechanism of glycogen phosphorylase. E.GP is the enzyme glucose 1-phosphate complex, $E.G_n$ is the enzyme glycogen complex, $E.G_nGP$ and $E.G_{n+1}.P$ are the ternary enzyme substrate complexes and E.P is the enzyme phosphate complex.

The rate determining step is isomerization of the central ternary enzyme-substrate complex and under these conditions this model can be treated using the rapid equilibrium assumptions (that is isomerization of the central complex is slow compared with dissociation of the enzyme substrate complex). When one of the substrates is present at saturating concentrations (in our case this substrate is glycogen) then the model simplifies to that of a single substrate system. Under these conditions K_m will be equal to the dissociation constant of the enzyme-non-saturating substrate complex. However this is the simplest case, and often there are circumstances under which this generalization breaks down, as illustrated by the following examples.This usually occurs when there are several intermediates along the catalytic pathway and a slow step which allows accumulation of some of these intermediates (see below)

$$E + S \overset{K_d}{\rightleftharpoons} ES \overset{K}{\rightleftharpoons} E'S \overset{K'}{\rightleftharpoons} E''S \xrightarrow[\text{slow}]{k_4} EP$$

Where [E'S] = K [ES] AND [E''S] = K' [ES'] then

$$K_m = \frac{K_d}{(1+K+KK')}$$

In this case the value of K_m is a function of all of the different concentrations of the various intermediates. A similar situation can occur when other non-productive enzyme-substrate complexes accumulate (non-productive binding) Under most circumstances this is not the case and K_m can usually be taken as a measure of the affinity of a particular enzyme for a given substrate.

The affinity shown by glycogen phosphorylase for the various deoxy and deoxyfluoro substrates (as deduced from their K_m values) shows that there is an good qualitative agreement with the affinity of the glucose site (in the T-state enzyme) for the corresponding deoxy and deoxyfluoro analogue of glucose (Street et al., 1986). The observed order of binding affinities shown by the glucose analogues for the glucose subsite was $1 \approx 2 > 4 >> 6 \approx 3$. From the K_m values given in Table 4-I, again it can be seen important interactions occur with the 3 and 6 positions; both the 3 and 6 position analogues show low affinity for the enzyme (high K_m values), while interactions at the 2 and 4 positions, as evidenced by the comparatively high affinity for phosphorylase *b* (low K_m values) appear to be less critical.

Thus at least in a qualitative sense it can be seen that the enzyme-ligand interactions which were important in determining the specificity of the glucose binding site in T-state phosphorylase *b* remain so for the glucopyranose subsite in the R-state enzyme.

Enzyme-Ligand interactions at the transition state: the value of k_{cat} / K_m represents the free energy change on going from the free enzyme and free substrate to the transition state of the first irreversible step. Thus alterations in the ability of the ligand to interact with the enzyme at this transition state will be directly reflected in the stability of this transition state and can be measured by their effect upon k_{cat} / K_m. In the absence of any other factors which might influence the stability of this transition state, comparison of k_{cat} / K_m for the unaltered substrate with the same parameter for the substrate analogues would yield a value related to the energetic contribution to the stability of the transition state due to the enzyme-ligand

interactions at the position of substitution. These values are expressed as Gibbs free energies ($\Delta\Delta G^{\ddagger}$) in Table 4-I. However, we have already seen that perturbations in transition state energy due to the electronic factors of the substituents are also important. Therefore the values of $\Delta\Delta G^{\ddagger}$ represent not only the binding effects but are also due to the intrinsic electronic effects of the substituents. However, the decreased rates of turnover seen from the deoxy analogues must then arise *purely* from deletion of enzyme-ligand interactions, which are important to the stability of the enzymic transition state, as the intrinsic electronic effects will only serve to accelerate the reaction. In this case the values of $\Delta\Delta G^{\ddagger}$ provide a *minimum* estimate of the apparent binding energy associated with interactions at that position (Fersht, 1988). Even with this favourable electronic factor taken into account, the rate decreases observed on removal of a single hydroxyl group are quite considerable, suggesting that a lot of the catalytic efficiency of phosphorylase derives from non-covalent interactions. Comparison of k_{cat} / K_m values within the series of deoxy sugars shows again that deletion of the 3- and 6-positions causes the greatest loss of catalytic efficiency. This fact along with the preceding discussion on the ground state enzyme-substrate complex essentially shows that the glucopyranose binding site remains largely unchanged in the T to R state transition.

The work of A:R.Fersht and his colleagues (Fersht et al., 1988), which was discussed in Chapter 1 (Binding energy and catalysis) demonstrated that hydrogen bonding interactions were important for the stability of the transition state of the phosphoryl-transfer step in tyrosyl tRNA synthetase. The interactions of enzyme and ligand became more numerous and stronger at the transition state than in the ground state enzyme-substrate complex. This is also true of glycogen phosphorylase. Based on the energetic contributions of enzyme-ligand interactions in the ground state (from the energetic data obtained from the glucose-phosphorylase *b* complex and the qualitative data obtained from the K_m values) the charge-neutral hydrogen bonding interactions between the enzyme and the 3- and 6-hydroxyl groups of the ligand would be expected to contribute $\approx$ 3.0 kcal mol^{-1} each to the binding energy. From the values of $\Delta\Delta G^{\ddagger}$ in Table 4-I it can be seen that these same interactions

contribute 6.3 and 5.9 kcal mol $^{-1}$ each to the stability of the transition state-enzyme complex. It must also be remembered that these values represent a *minimum* estimate of the binding energy contributed by these interactions. Likewise, the neutral-neutral interaction at the 4-position contributes 3.7 kcal mol $^{-1}$ to the stability of the transition state, some 2 to 3 kcal mol $^{-1}$ of binding energy more than in the ground state complex.

Unfortunately, because the $\Delta\Delta G^{\ddagger}$ values for the deoxyfluoro analogues could not simply be interpreted in terms of hydrogen bonding interactions we were unable to assess the polarity and number of the interactions between ligand and enzyme at the transition state. Consequently the increased binding energy characteristic of the transition state-enzyme complex could be attributed to either increased strength of individual interactions, increased number of interactions or both. The enzyme could utilize the changing geometry of the ligand as it approached the transition state to maximize the strength of these hydrogen bonding interactions. Transition states which have considerable oxocarbonium ion-like character would have to adopt a boat or half-chair conformation. This change in conformation from the normal $^{4}C_{1}$ conformation of the ground state sugar would have to involve considerable movement of the hydroxyl groups to accommodate this change in geometry. Hence the new positions of the hydroxyl groups could provide more favourable alignments for interactions already accommodated in the ground state and possibly also gain new interactions as well.

A comparison of the kinetic results with the X-ray crystallographic structure of the heptulose 2-phosphate-phosphorylase b complex: although, the crystal structures of phosphorylase *a* or IMP-activated phosphorylase *b* are largely those of the T-state conformation, the crystals still show catalytic activity although at reduced rates (Kasvinsky and Madsen, 1976). Recently using IMP-activated phosphorylase *b* and a high intensity synchrotron X-ray source, L.N.Johnson and her group have been able to carry out time-resolved crystallographic studies and actually follow the conversion of an enzyme-substrate complex to an enzyme-product complex (Hajdu et al., 1987). Although the protein

conformational changes are undoubtedly attenuated to some extent by crystal packing forces, the results obtained in this study probably represent a qualitative description of at least a small part of the conformational changes which occur in the T to R state transition. It should also be remembered that heptulose 2-phosphate is considered to be a transition state analogue, by virtue of the unusual conformation that the phosphate group of this compound adopts, both in free solution and when bound to phosphorylase *b*.

A refinement of the crystallographic data is still in progress but a tentative structure for the heptulose 2-phosphate–phosphorylase *b* complex is presented in Figure 4-21 (L.N. Johnson personal communication). A comparison between the potential hydrogen bonding interactions in the T-state structure of the phosphorylase *a*–glucose complex with the phosphorylase *b*–heptulose 2-phosphate complex is also given in Table 4-V:

Even at the present level of refinement the glucopyranose binding site in the phosphorylase *b*–heptulose 2-phosphate complex bears a remarkable similarity to that of the phosphorylase *a*–glucose complex. There is also an excellent agreement with the results of our kinetic experiments. Potentially strong interactions between a protonated histidine (His 377) and the hydroxyl group at the 6-position are seen, while substitution of the 6-hydroxyl group with either fluorine or hydrogen resulted in both a large increase in K_m and a dramatic decrease in k_{cat}. The second important interaction as deduced from the kinetic experiments occurred with the hydroxyl group at C-3. In the phosphorylase *a*–glucose complex we were able to correlate this with a second charge–neutral hydrogen bonding interaction with Glu 672. In the phosphorylase *b*–heptulose 2-phosphate complex, and at the present level of refinement this interaction is not so clear. The residue Glu 672 appears to be almost 4 Å away from the oxygen of the 3-OH group and at present is thought to be involved in hydrogen bonding interactions with a second protein residue, Tyr 573. However, from the kinetic experiments the 3-position still appears to be important for catalytic activity and we can be fairly sure that as refinement progresses the interaction between this position and Glu 672 will become more apparent. It is also possible that the crystal structure binding mode of

heptulose 2-phosphate is not perfect and that the structure observed is a compromise forced on the ligand by the rigidity of the crystalline lattice.

As in the T-state enzyme the 2- and 4-positions are involved in much weaker interactions, possibly involving hydrogen bonding between neutral–neutral partners. This situation remains unaltered in the R-state enzyme, deletion of the hydroxyl group at the 4-position causing the least dramatic loss of activity. From the crystal structure, possible neutral-neutral interactions between the hydroxyl group of the ligand and both the backbone nitrogen of residue 675 and the carbonyl group of Asn 484 are apparent.

Table 4-V. A comparison of potential hydrogen bonding interactions in the active site of glycogen phosphorylase a and b.

	Potential Hydrogen bonding interactions	
Sugar Hydroxyl Group	Phosphorylase *a* glucose	phosphorylase *b* Heptulose 2-phosphate
1-O-	Leu 136 N (D)	Leu 136
2-OH	Tyr 573 OH (D/A) Asn 284 Nδ2 Glu 672 Oε2 (A)	Tyr 573 Glu 672
3-OH	Glu 672 Oε2 (A) Ser 675 (D)	Glu 672 Ser 675
4-OH	Asn 484 Oδ1 (A) Gly 675 N (D)	Asn 484 Gly 675
6-OH	His 377 Nδ1 (D) Asn 484 Oδ1 (A)	His 377 Asn 484

Data for phosphorylase a–glucose complex from Street et al., 1986. Data for phosphorylase *b*–heptulose 2-phosphate complex from L.N. Johnson personal communication. D and A denote the implied role of the ligand as a donor or acceptor in hydrogen bond formation.

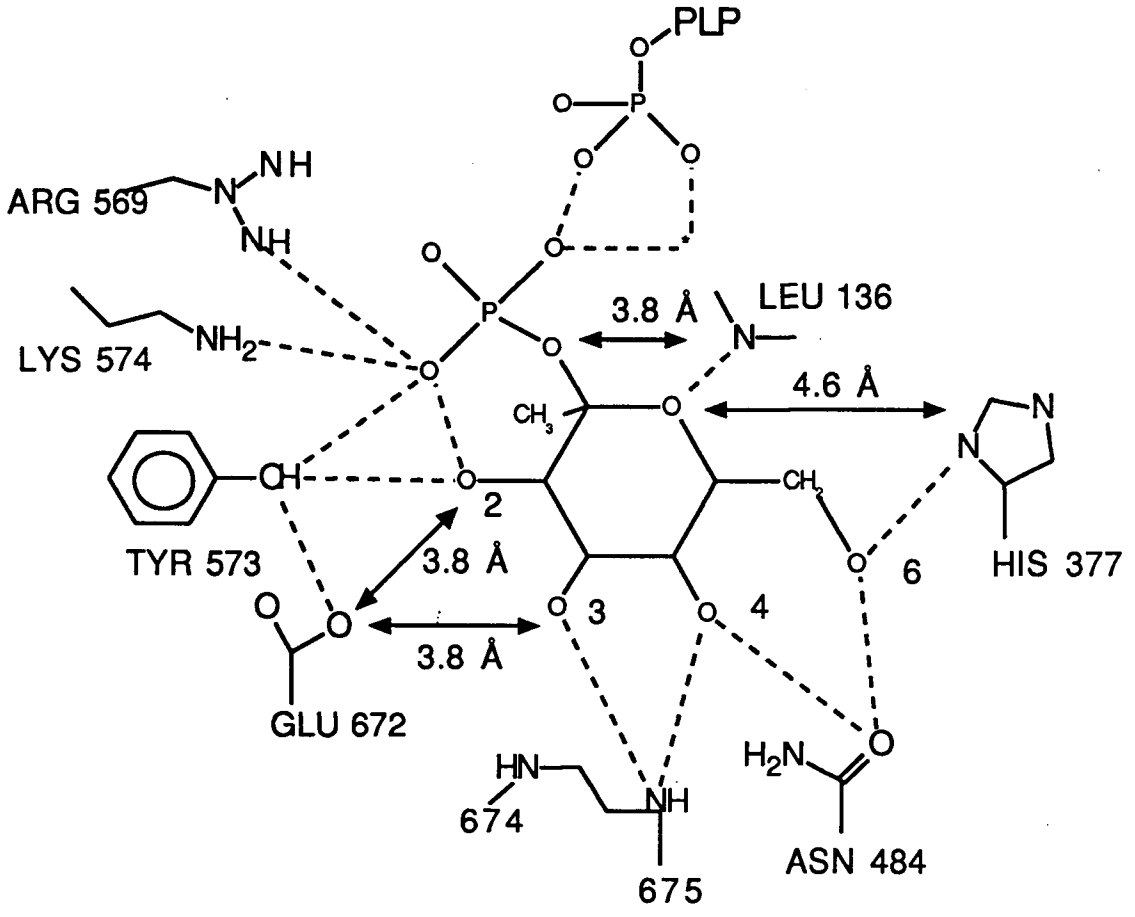


Figure 4-21. The X-ray crystallographic structure of phosphorylase b active site–heptulose 2-phosphate complex.

Data supplied by L.N.Johnson, personal communication. Potential hydrogen bonding interactions are shown as dotted lines, water molecules involved in hydrogen bonding networks are denoted by *.

Unfortunately, due to our inability to obtain pure preparations of 2dαGlcP we cannot readily assess the importance of the hydrogen bonding interactions at the 2-position. However, from potential interactions deduced from the crystal structure of the phosphorylase *b*–heptulose 2-phosphate complex, again only neutral-neutral interactions seem possible. This

conclusion is born out to some extent from the kinetic data since in terms of K_m, 2FαGlcP bound at least as tightly as the enzymes natural substrate.

Lastly, it may be noted that for the glucose-phosphorylase *a* complex there is an asparagine residue that apparently hydrogen bonds to the 2-hydroxyl group of the ligand, but this residue is appears to be missing in the heptulose 2-phosphate-phosphorylase *b* complex. This residue (Asn 284), is part of a polypeptide loop which extends between residues 282-286 which moves out of the active site during the T- to R-state transition. This movement was first observed upon comparison of the T-state glucose-phosphorylase a complex and the complex of the same enzyme containing the R-state inhibitor glucose-1,2-cyclic phosphate bound at the active site (Withers et al., 1982a). The movement of the peptide loop is thought to be an essential part of the protein conformational transition which accompanies formation of the binding site for the phosphate group of the substrate.

The kinetic data presented in this section show that essentially the structure of the glucopyranose binding site is conserved during the T- to R-state transition and that the enzyme-ligand interactions which occur with this part of the substrate are important for stabilization of the enzymic transition state. In time, as more and better X-ray crystallographic data become available for the R-state enzyme, it will be interesting to see if the predictions made here based upon results from the kinetic experiments remain in good agreement with the structural data.

The Specificity of the Substrate's Phosphate Binding Site.

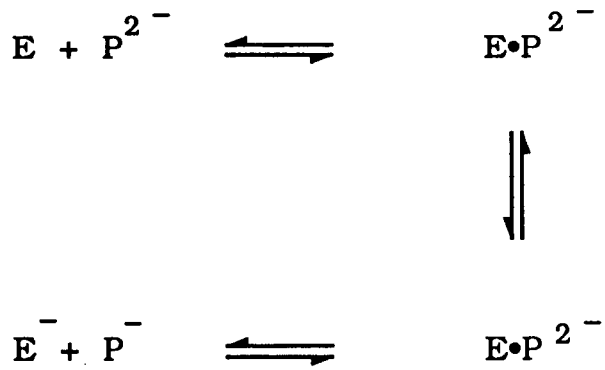
The observation that glycogen phosphorylase *b* can bind both the monoanionic and dianionic from of its substrate with approximately equal affinity was somewhat unexpected. From the introductory discussion on the contributions of hydrogen bonding to binding energy (Chapter 3), it was seen that the hydrogen bonds between the solvent and the solvated ligand are exchanged for hydrogen bonds within active site of the enzyme upon binding. Likewise, the interactions between the solvated binding site of the protein and the solvent, are

exchanged for those of the enzyme-ligand complex, thus the numbers and types of hydrogen bonding interactions are conserved in the binding process and the binding energy is derived from an enthalpic contribution associated with differences in geometry of the solvent and enzyme complexes, and from the favourable entropic factor derived from the release of ordered water from the solvated species into bulk solution. On this basis, it might have been expected that a binding site which had evolved to interact with a charged species, such as a phosphate group, would be very specific for the ionization state of this ligand. This is simply because the enzyme-ligand interactions involved in binding a monoanionic as opposed to a dianionic species to the same binding site would produce at least 1.5 - 3.0 kcal mol $^{-1}$ difference in binding energy. This difference in binding energy would lead to 2 to 3 orders of magnitude greater affinity for the dianionic species.

There are a number of instances where this type of behaviour has been observed, one example of which pertains to the AMP binding site in glycogen phosphorylase *b* (Withers and Madsen, 1980). The affinity of the AMP binding site of phosphorylase *b* for AMP is high (K_d = 0.04 mM), but in this case the phosphofluoridate analogue of AMP (which can only exist as a monoanion) exhibited greatly reduced affinity for the enzyme (K_d = 2.7 mM) and did not show any of the properties of an allosteric activator which would normally be associated with AMP. Similarly, the enzyme phosphoglucomutase has been observed to bind the substrate analogues glucose-6-phosphofluoridate, αManPF and 2FαGlcPF, on average three orders of magnitude less tightly than the corresponding phosphate ester (Percival, M.D., 1988, Ph.D. thesis University of British Columbia). For, this system it was calculated that an average of 2.7 kcal mol $^{-1}$ of binding energy was lost upon binding the monanionic phosphofluoridate as opposed to the dianionic phosphate ester. It appears then that the respective binding sites in these two enzymes maximize the binding energy available from their substrates by binding the dianionic species.

Thus to bind both the monoanionic and dianionic forms of the substrate with approximately the same affinity, phosphorylase *b* must maintain the number of charge-neutral

(or ion pair) and neutral-neutral interactions for both species. The following scheme shows how this can be accomplished.



Scheme 4-7. *A kinetic model for binding of monoanionic and dianionic substrates to phosphorylase b with equal affinity.* P^{2-} and P^{-} are the dianionic and monoanionic forms of the substrates, E, and E^{-} are the neutral and anionic forms of the enzyme.

To conserve the number and types of interaction , the monoanionic substrate could only bind to the charged form of the enzyme, while the dianionic substrate could only bind to neutral form of the enzyme. Therefore in each case the energy, derived from binding the different forms of the substrate is equal to the difference in the hydrogen bonding energies resulting from two charge-neutral interactions (all other interactions between enzyme and ligand remaining constant). This would result in the enzyme showing approximately the same affinity for both the monanionic and dianionic forms of the substrate.

The application of ^{31}P-NMR spectroscopy to the study of ionization states of phosphate esters within an enzyme-ligand complex has been quite extensive (Cohn and Rao, 1979). The chemical shift of the biologically important phosphate esters are apparently relatively insensitive changes in local magnetic environment (Gorenstein et al., 1976), but are quite dependent in changes in the phosphate-oxygen bond angles and phosphate ester torsional angles (Gorenstein, 1975; Gorenstein, and Kar, 1975). A change in ionization state of a phosphate ester often leads to a change in the phosphorus-oxygen bond angle, thus the secondary ionization of a phosphate monoester produce a 4 ppm downfield shift (Crutchfield et al., 1967) which is most likely attributable to this change in bond angles. However it need

not be the case that the converse argument is also true, a change in phosphorus-oxygen bond angle does not necessarily mean a change in ionization state.

The literature of glycogen phosphorylase has many examples where this fact is not taken into account when interpreting the results obtained from ^{31}P-NMR studies (see Chapter 3 for discussion on the work of Klein et al, (1984) and Heorl et al., (1979) and at best this must lead to an over-simplistic view of the processes which are occurring in the system under study. However, with careful interpretation and by corroboration from results obtained with techniques other than ^{31}P-NMR it is possible to gain insight into the ionization processes which occur in an enzymic reaction (see Gorenstein et al.,1976; Hull et al., 1976; Campbell et al., 1978).

By using a combination of steady-state kinetics, ^{19}F-NMR and ^{31}P-NMR techniques it has been demonstrated that glycogen phosphorylase *b* binds both the monoanionic and dianionic species of its substrate, and that the monoanion : dianion ratio of these species in free solution is perturbed little on binding at the active site of the enzyme. These results are thus incompatible with the hypothesis that the PLP and the phosphate of the substrate are involved in a proton transfer relay,.at least in the ground state enzyme-substrate complex as no appreciable proton donation was observed by ^{19}F-NMR in the 2FαGlcP-enzyme complex. However, it does not exclude the possibility that reaction occurs via a small percentage of the protonated substrate or that extensive proton donation only occurs in the transition state. Since the hypothesis of the proton-shuttle was largely based on the results obtained from ^{31}P-NMR studies performed on ground-state enzyme-ligand complexes in the non-regulatory α-glucan phosphorylases, and since we have already seen that extensive proton-donation does not occur in the ground-state ligand-phosphorylase *b* complex, the results obtained in the present study must cast doubt upon the interpretation of the results on which this hypothesis was based. Further, evidence against the proton shuttle hypothesis can be gathered from the ^{31}P-NMR experiments performed on the 1dαGlcCP-phosphorylase-glycogen complex. These experiments demonstrated that the formation of this ternary-enzyme

complex was accompanied by a change in the PLP resonance, from a signal at δ 3.8 ppm ($\Delta\upsilon_{1/2}$ = 130 Hz) to a much broader resonance at δ ≈ 0.5 ppm ($\Delta\upsilon_{1/2}$ = 290 Hz). These changes in the spectral properties of the PLP resonance are essentially the same as those observed by Withers et al., (1981a) upon formation of the ternary complexes, phosphorylase *b*-maltoheptaose-glucose-1,2-cyclic phosphate and phosphorylase *b*-and-PLPPαGlc (Withers 1985). These changes have been interpreted by Withers et al. (1981a) as either representing the formation of a protonated coenzyme phosphate in the intermediate exchange regime, or a constrained and distorted dianionic phosphate moiety. From the data available at present we cannot distinguish between these possibilities. It has been argued by Klein et al.(1984), that the analogue glucose-1,2-phosphate has a very low pKa value and therefore could not be protonated at physiological pH values, consequently the changes in the ^{31}P-NMR resonance elicited by the binding of this compound would not be typical of those for the normal substrate. This would also be true of the PLPPαGlc-phosphorylase *b* complex since again, the glucosidic phosphate in this complex would also have a low pKa value. However, the compound 1dαGlcP also produced a similar response in the ^{31}P-NMR signal of cofactor PLP and this analogue has a pKa_2 value of 7.34, and would be easily protonated over the pH range where phosphorylase is most active. This strongly suggests that the results obtained with the compounds PLPPαGlc and glucose-1,2-cyclic phosphate were not atypical of those that would be expected from the normal substrate. The observation of these similar results for both types of compound, might then suggest that the cofactor phosphate is not involved in a direct proton donation to the phosphate group of the substrate. Since extensive proton donation could not occur to the compounds of low pKa value and yet these compounds produce the same changes in the ^{31}P-NMR resonance of the cofactor PLP as compounds to which proton donation can occur. If then the ^{31}P-NMR resonance which is characteristic of the ternary enzyme-ligand complex is due to an exchangeable protonated phosphate, it is unlikely that it is exchanging directly with the phosphate group of the substrate, but more likely with an amino acid residue of the protein.

CONCLUSION.

By analogy with the reaction mechanism of "retaining" glucosidases, it has been postulated that the mechanism of glycogen phosphorylase also involves a transition state with considerable oxocarbonium ion-like character. Previously, evidence gathered from kinetic isotope effects and potentially tight binding "transition state analogues" which might have supported this hypothesis was inconclusive. Largely due to this lack of convincing evidence a mechanism which involves a concerted transition state has recently been proposed (Klein et al.,1986).

In this study we prepared and examined the substrate activity of a series of deoxy and deoxyfluoro analogues of αGlcP. All of these compounds were found to act as substrates for glycogen phosphorylase *b* although at greatly reduced rates (1 to 5 orders of magnitude) when compared with the normal substrate. These dramatic rate reductions were interpreted as being the result of a deleterious combination of electronic and binding effects of the substituents on the stability of the enzymic transition state. It was demonstrated that a linear free energy relationship existed between the logarithm of rate constant for the overall rate determining step of the enzyme-catalysed process and the logarithm of the first order rate constant for the acid-catalysed hydrolysis of the same series of deoxy and deoxyfluoro-α-glucopyranosyl phosphates, suggesting that the substitutions on the glucopyranosyl ring affected the activation energy for both processes in a similar manner. Since the acid-catalysed hydrolysis involves a transition state with considerable oxocarbonium ion-like character, and the effects of the substituents are largely electronic in nature, this would suggest that the same electronic effects are important to the stability of the enzymic transition state. This result provides new evidence to support the hypothesis that transition states with considerable oxocarbonium ion-like character are involved in the reaction catalysed by glycogen phosphorylase and serves to make the analogy with the reaction mechanism of glucosidase even stronger. It would also make the possibility of a reaction mechanism involving a truly concerted transition state less likely.

The results obtained principally from the deoxy substrate analogues indicated that the structure of the glucopyranose binding site remained intact during the T to R-state protein conformational change. It was observed in a previous study that interactions between the 3- and 6-hydroxyl groups of the sugar ring and the enzyme were important to the stability of the T-state phosphorylase *b* -glucose complex (Street et al., 1986). The results obtained from the deoxy substrate analogues suggest that interactions between enzyme and substrate at these positions are also important in stabilizing of the ground-state enzyme-substrate complex and the enzymic transition state.

Through a series of compounds designed to probe the specificity of the phosphate subsite of the active site in glycogen phosphorylase *b* it was found that this enzyme binds both the monoanionic and dianionic forms of its substrate with approximately equal affinity. The results of ^{19}F- and ^{31}P-NMR studies on the active-site complex formed by 2FαGlcP and 1dαGlcCP with phosphorylase *b* indicated that little protonation of the substrate occurred on binding to the enzyme. Thus these results would cast into doubt the proposal of the role of PLP as the essential acid catalyst. Further, the effects on the PLP resonance in the ternary enzyme-glycogen-1dαGlcCP complex were found to be virtually the same as those observed in a similar ternary complexes formed with glucose-1,2-cyclic phosphate (Withers et al., 1981a) and PLPPαGlc (Withers 1985), thus the ^{31}P-NMR resonance from PLP in these complexes would appear typical of the normal ternary enzyme-substrate complex. Since the compounds glucose-1,2-cyclic phosphate and PLPPαGlc have pKa values low enough to preclude their participation in a "proton shuttle" mechanism this result suggests that direct phosphate-phosphate interaction does not involve proton transfer.

CHAPTER 5.

β–Glucosides as Anti-Fungal Agents

Introduction. The Cellulase Complex.

It is now well established that the cellulase enzyme complex is formed from at least three different types of enzyme, all of which are essential for decomposition of crystalline cellulose: exo-β-1,4-glucanase (or β-1,4-glucan cellobiohydrolase), endo-β-1,4-glucanase and a β-glucosidase or cellobiase (Eriksson and Pettersson, 1975a; Pettersson, 1975; Wood, 1972; 1975; Halliwell and Griffin, 1973). Each of these enzymes fulfills a specific role in cellulose saccharification (see Figure 5-1): exo-β-1,4-glucanase shortens cellulose chains by removing disaccharide units (cellobiose) from the non-reducing end (Eriksson and Pettersson, 1975b, Pettersson, 1975; Wood, 1972, 1975), while endo-β-1,4-glucanase cleaves the chain at random internal points thus increasing the number of end points on which the exo-glucanase can act (Eriksson and Pettersson, 1975b; Wood, 1975). The combined effect of these two enzymes is of both solubilising cellulose and reducing the overall chain length of the polysaccharide. Finally β-glucosidase catalyses the hydrolysis of cellobiose and to a certain extent some of the higher cellodextrins (usually up to cellopentaose) to produce glucose, which is then utilized by the cellulolytic organism as a carbon and energy source. As the ultimate enzyme in the saccharification process β-glucosidase plays an essential role in regulation of the entire cellulase system. The removal of cellobiose is crucial to the function of cellulase, as both exo- and endo-glucanases are inhibited by high concentrations of this disaccharide (Berghem et al., 1975; Halliwell and Griffin, 1973; Pettersson, 1975; Wood, 1975).

Our strategy for development of new and potentially environmentally sound control agents of cellulolytic fungi is based on our knowledge of β-glucosidase. The strategy was originally two fold: The first approach was to inhibit β-glucosidase using 2-deoxy-2-fluoro β-glucosyl fluorides which were being developed in conjunction with this work. It was postulated that this would have the effect of depriving the organism of its primary source of energy and carbon by stopping the hydrolysis of cellobiose to glucose. A secondary effect, namely the inhibition of the remaining components of the cellulase system due to the

subsequent build up of cellobiose and other higher cellodextrins, could also be realized. However due to the small quantities of these compounds that were available this was never tested.

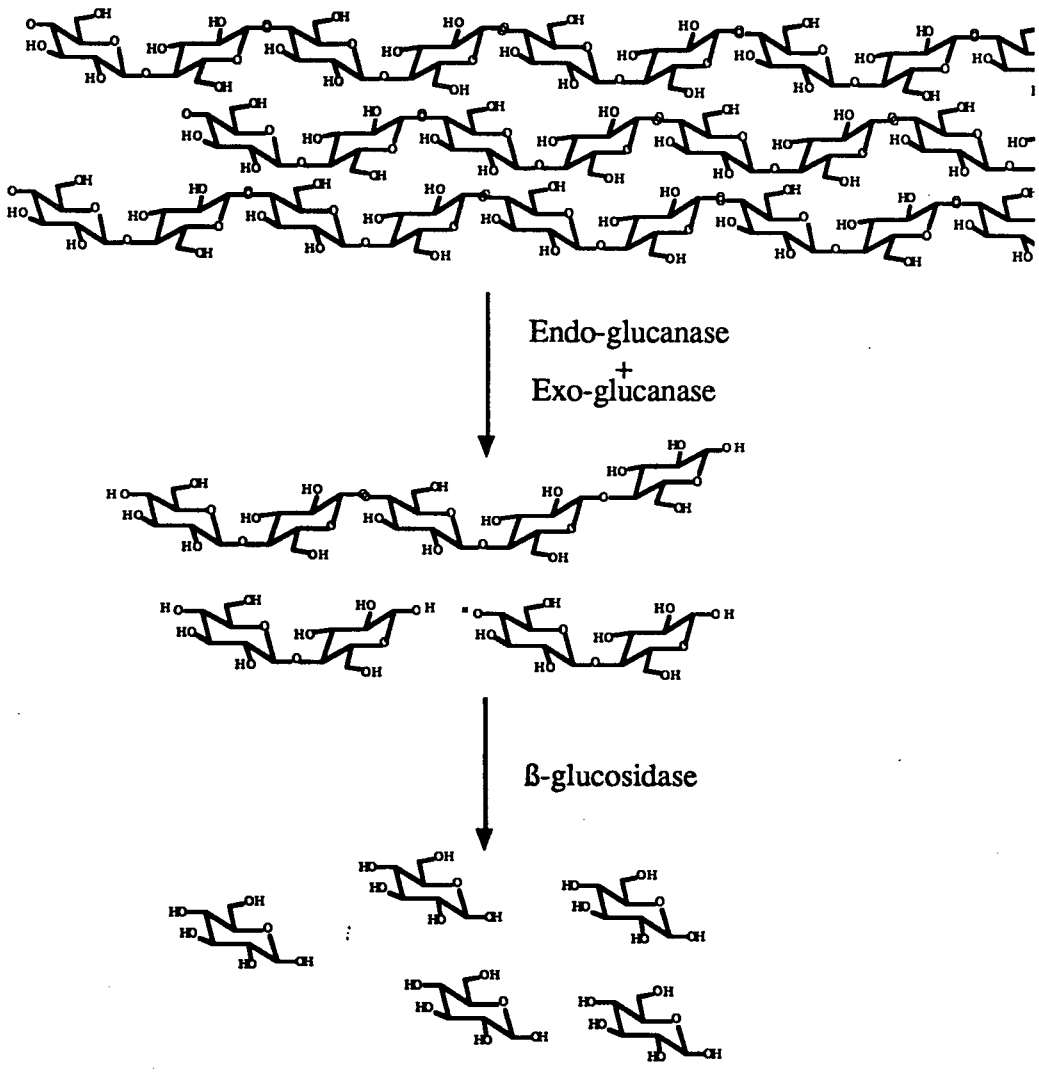


Figure 5-1. Degradation of crystalline cellulose by the cellulase complex.

The second approach relied on the activity of the β-glucosidase rather than its inhibition. It was considered that the hydrolytic process of this enzyme could be used to activate a normally inert and non-toxic form of a fungicide in which a known fungicide is conjugated to glucose by a β-glucosidic linkage. In this way the environmental effects of the fungicide could be minimized, as the toxicant would be present only when it was required and only in small quantities. As most β-glucosidases have relatively lax specificity for the aglycone portion of the glycoside, it was envisaged that this part of the structure could eventually be varied through a wide variety of structural types until an ideal balance between toxicity and reactivity was obtained. However for the purposes of this early developmental work simple phenolic glucosides were used, as these are readily prepared and in most cases phenols are also quite toxic. By using a β-glucoside which would release a coloured phenolate when hydrolysed, a simple method for detection of β-glucosidase activity in cultures of cellulolytic fungi could also be developed.

Results and Discussion.

The aim of the early part of this work was to develop a method which would require only small quantities of p-nitrophenyl β-D-glucopyranoside (βGlc PNP) to assess its fungicidal effects. Initial experiments with this aim were attempted by introducing a 1.3 cm filter paper disk which contained between 0 to 516 μg of βGlcPNP directly onto the surface of an agar plate (1.5% malt extract) which was then inoculated with *T. pseudokoningii* . However, after 7 days incubation no fungicidal activity or production of the yellow phenolate was apparent. Control experiments performed by introducing a solution of sweet almond β-glucosidase onto disks which had been incubated for the same period but in the absence of a fungal culture also failed to produce the yellow phenolate. It was deduced from the latter experiment that the water soluble glucoside, quite rapidly diffused from the filter disk where it was diluted below its detection limits. This was confirmed by taking 1.3 cm filter disks which had been soaked in a mineral salts solution containing up to 225 μg of βGlcPNP. These disks

were suspended on a wire gauze over a damp filter paper in a sterile petri dish. Approximately one week after inoculation of the filter disk with a culture of *T. pseudokoningii* a yellow colour from the released phenol became apparent. Although this experiment demonstrated in a qualitative sense that it was possible to detect β-glucosidase activity it was essentially impossible to quantify fungal growth in this system. To circumvent problems caused by water solubility the specially modified petri dishes depicted in Figure 5-2 were prepared. Each dish contained five wells designed to hold approximately 1 mL of test compound which had been solidified in an agar medium. The remainder of the dish is then filled to a depth of 1 or 2 mm below the rim of the wells with the same agar medium. This provided an effective barrier to diffusion of the test compound but not to the fungal mycelia, which are quite capable of surmounting small physical barriers. This system was tested using a nutrient medium (1.5 % malt extract) and a commercial fungicide TCMTB (2-(thiocyanomethylthio) benzothiazole). After inoculation with cultures of *T. pseudokoningii* and *A. pullulans* incubation was continued until growth was essentially complete on the control well (no further change over a period of 5 days). The toxic limit was thus defined as the lowest concentration at which fungal growth could not be observed in the well at the end of the incubation period. Toxic limits for TCMTB were determined at 0.0008 % for *T. pseudokoningii* and 0.001 % for *A. pullulans* after 14 days incubation.

Tests to determine toxic limits for βGlcPNP were conducted on a mineral salts medium which contained cellulose as the sole carbon source. This ensures maximum induction of all of the components of the cellulase system (Deshpande at al., 1978; Hirayama, 1979; Mandels, 1975). Representative samples of white rot, brown rot and sap stain fungi were selected on the basis of their ability to grow rapidly on this medium. The toxic limits determined for βGlc PNP with five different fungi are presented in Table 5-I, photographs of the cultures of *P. chrysosporium* and *C. globosum* taken after 14 days incubation are shown in Figure 5-3.

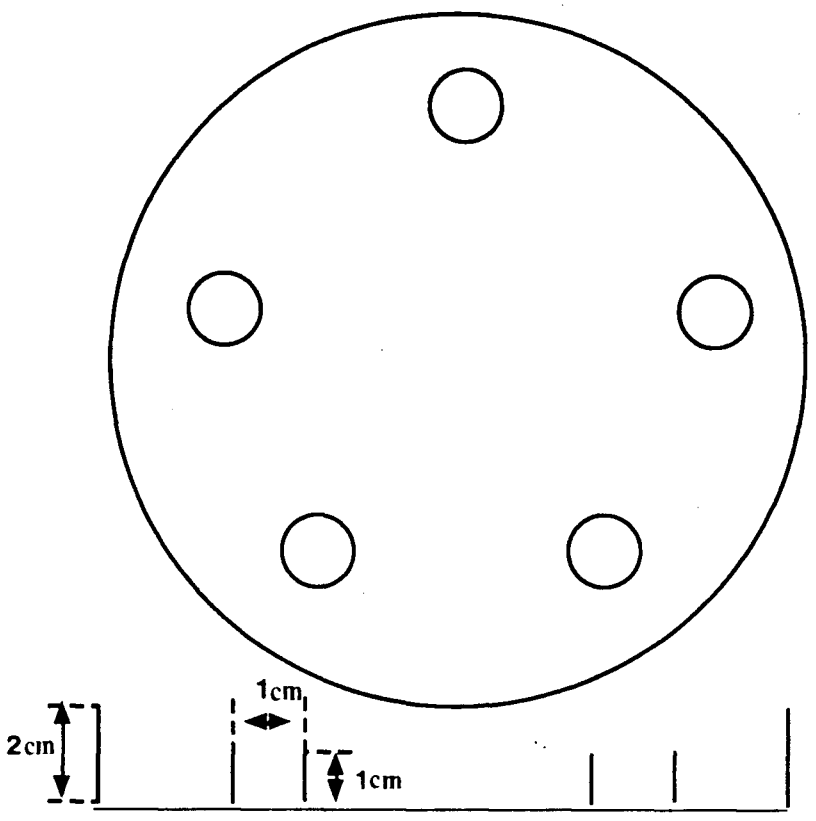


Figure 5-2. A specially modified petri dish used for determination of toxic limits.

Table 5-I. Toxic limits of βGlcPNP for wood decay fungi.

Organism	[a] Toxic Limit ($\mu g\ mL^{-1}$)	[b] p-Nitrophenol ($\mu g\ mL^{-1}$)	Incubation (Days)
C. versicolor	1000	1000	25
P. chrysosporium	250	250	25
C. globosum	500	500	25
C. puteana	1000	*1000	25
T. reesei	250	**-	13

* Only just detectable by eye.** Not detectable due to the secretion of a yellow pigment by the fungus.[a] As defined in the text.[b] Minimum concentration at which the yellow phenolate could be seen.

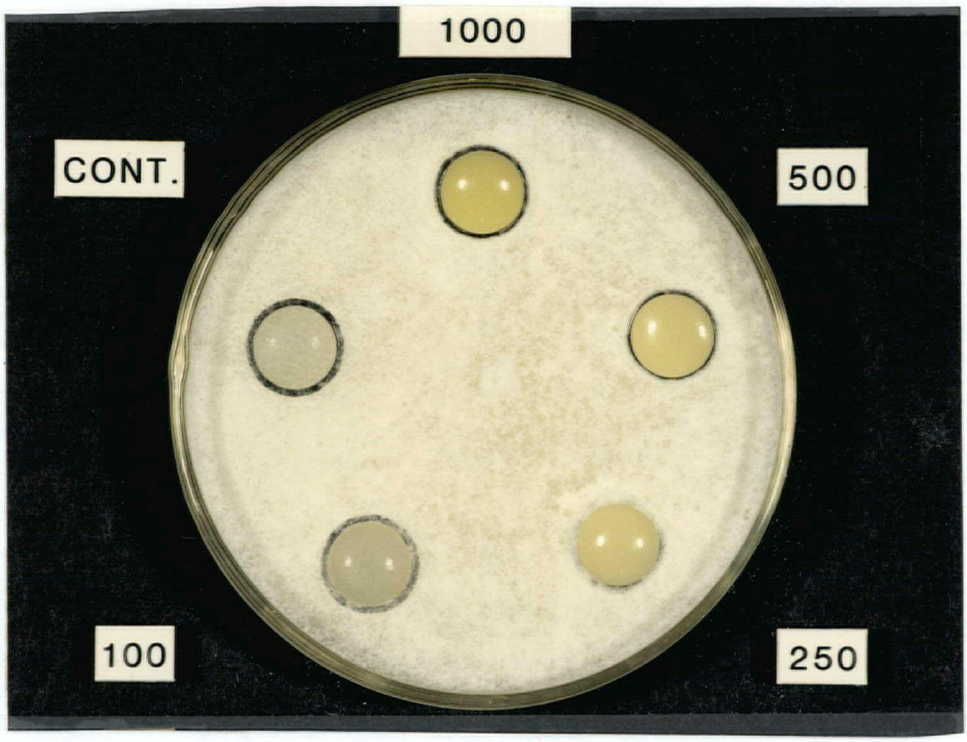


Figure 5-3. P. chrysosporium toxicity limit determination for βGlcPNP at 14 days incubation. Concentrations of βGlcPNP are given in μg mL^{-1}.

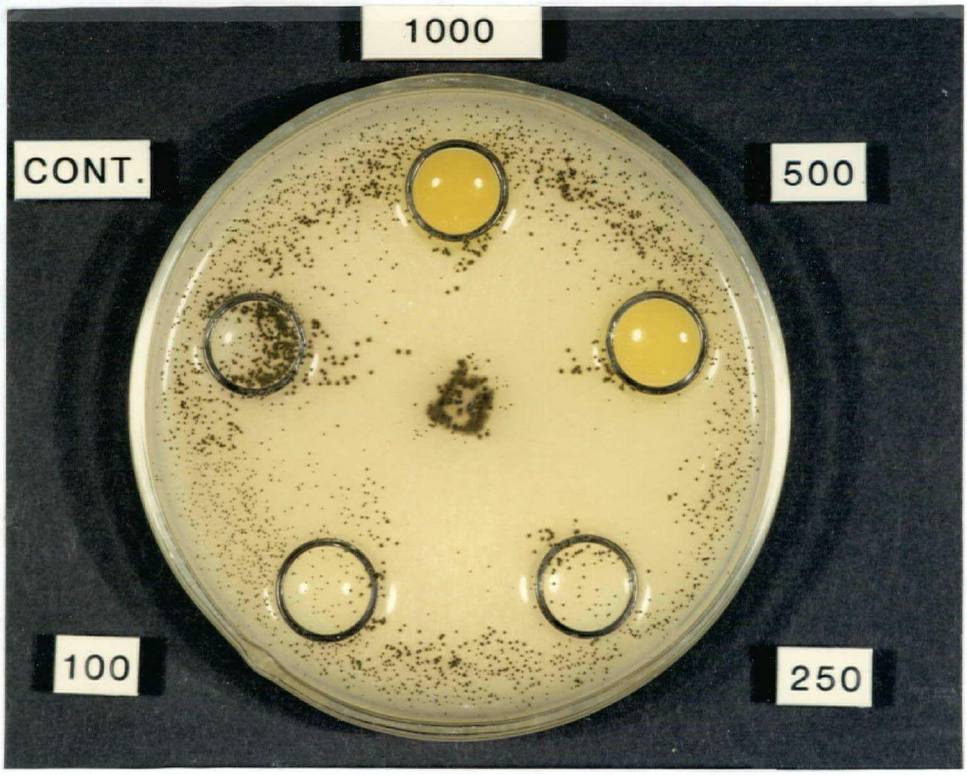


Figure 5-4. C. globosum toxicity limit determination for βGlcPNP at 14 days incubation. Concentrations of βGlcPNP are given in μg mL^{-1}.

The data in Table 5-I shows a good correlation between the presence of the p-nitrophenolate and the toxic limit, which suggests that the phenolate is the toxicant rather than the intact glucoside. However it is also possible to argue that all that is being observed is a species variation in tolerance to the phenolate and/or glucoside. The apparent variation of the phenolate detection limit is worthy of some note as it could reflect either: differing quantities of β-glucosidase produced by different organisms under normal growth conditions or the substrate specificity of different fungal β-glucosidases. It is likely that both factors contribute to some extent, as it has been observed in cultures of *T.reesei* that the major product of cellulose saccharification is in fact cellobiose rather than glucose (Sternberg et al., 1977). This is probably a reflection of the low amounts of β-glucosidase which are produced by this organism. While most β-glucosidases (at least those that have been purified to homogeneity) readily hydrolyse both aryl β-glucosides and cellobiose (Berghem and Pettersson, 1974; Deshpande et al., 1978; Herr et al., 1978; Shewale and Sadara, 1981), there are a few exceptions: Youatt (1958) and Jermyn (1955) have purified an aryl β-glucosidase devoid of cellobiase activity. Bucht and Eriksson (1969) have also isolated an aryl β-glucosidase with insignificant cellobiase activity and purified a separate protein with the converse specificity. As the β-glucosidases from most of the organisms used in this test are largely uncharacterised, it remains a possibility that the high toxicity limits seen with cultures of *C. versicolor* and *C. puteana* are due to the inability of the fungal β-glucosidase to hydrolyse aryl glucosides efficiently.

Preliminary experiments to assess the ability of βGlcPNP to prevent fungal degradation in samples of wood were also conducted. Decreases in dry weight of the samples were used as the criteria for measurement of cellulolytic activity. Samples of both a softwood (ponderosa pine) and a hardwood (aspen) doweling were used. Sterile samples of doweling were treated with βGlc PNP and placed into the wells of the specially modified petri dishes. Cellulose/agar medium was used in the remainder of the dish. After inoculation, incubation was continued at 20°C and residual dry weights were recorded at six and ten weeks. Weight

losses obtained from cultures of *P. chrysosporium* , *C. versicolor, C. globosum* and *C. puteana* are presented in Figures 5-5 to 5-10. Experiments were also carried out with cultures of *T. reesei* and *T. harzianum* , however essentially no weight loss was produced by these organisms. Within the limits of this experiment, a good correlation is seen between these results and those obtained on agar. Very little protection was seen against weight loss from cultures of *C. puteana* or *C. versicolor,* (on either ponderosa pine or aspen) and both of these organisms were the least susceptible to βGlcPNP on the cellulose / agar medium. With cultures of *P. chrysosporium* (Figures 5-7 and 5-8) on both softwood and hardwood samples at six weeks, protection against weight loss was quite pronounced. However, at ten weeks weight losses appeared quite uniform across the sample. Probably the most convincing results are obtained from *C. globosum* (Figure 5-9) although the results at six weeks incubation indicate a relatively small and uniform weight loss, by ten weeks there is a clear correlation between increasing glucoside concentration and decreasing weight loss. Again in this case good results were obtained on the cellulose / agar medium. Although these results are rather disappointing in terms of overall protection of the wood samples against cellulolytic degradation, it is almost certain that better results would have been obtained with higher β-glucoside concentrations. However, a great deal of variability between duplicates was seen and this was probably due to differential uptake of βGlcPNP by the doweling samples. As in this experiment it was possible only to calculate an average uptake of βGlc PNP per sample (based on 24 samples) no accounting for this variability could be made. Therefore, results in future experiments could probably be improved by devising a more accurate method to measure βGlcPNP uptake in individual samples.

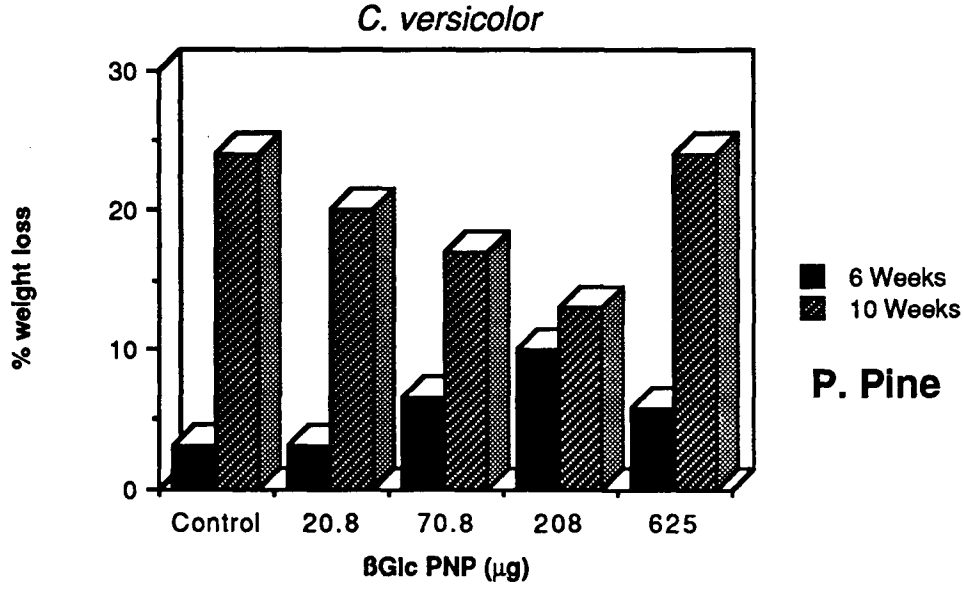


Figure 5-5. Weight loss caused by C.versicolor ponderosa pine doweling.

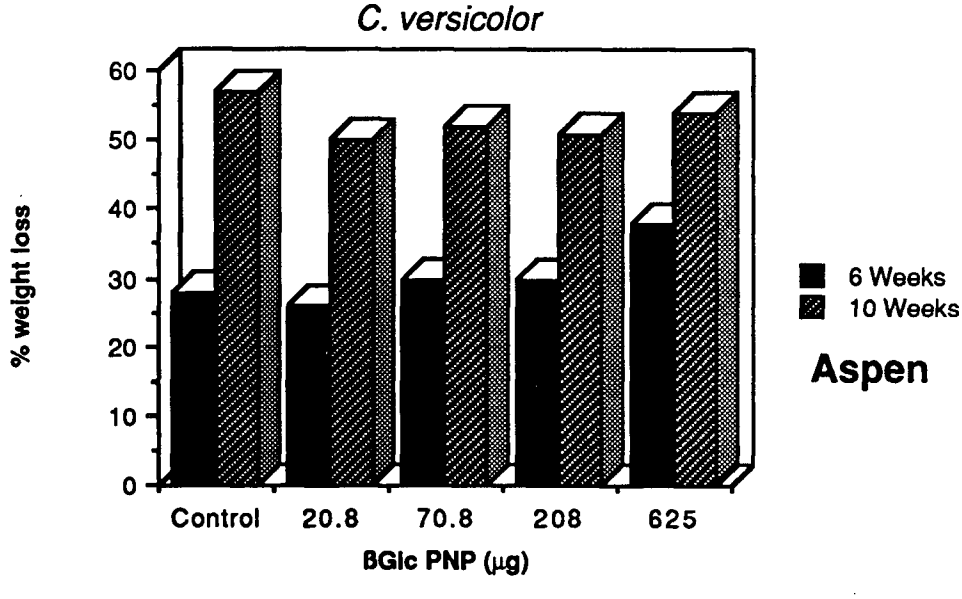


Figure 5-6. Weight loss caused by C. versicolor on samples of aspen doweling.

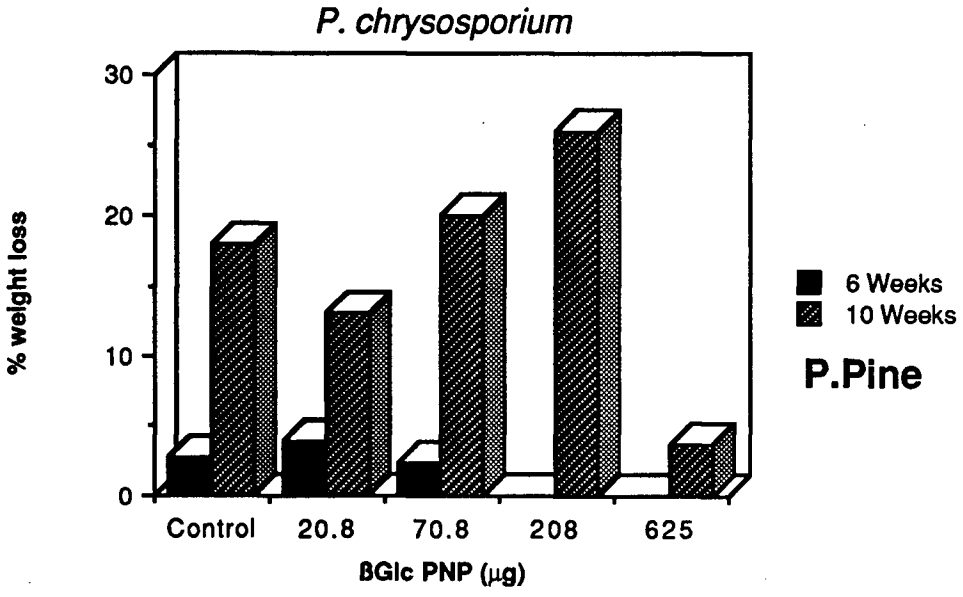


Figure 5-7. Weight loss caused by P. chrysosporium on samples of ponderosa pine doweling.

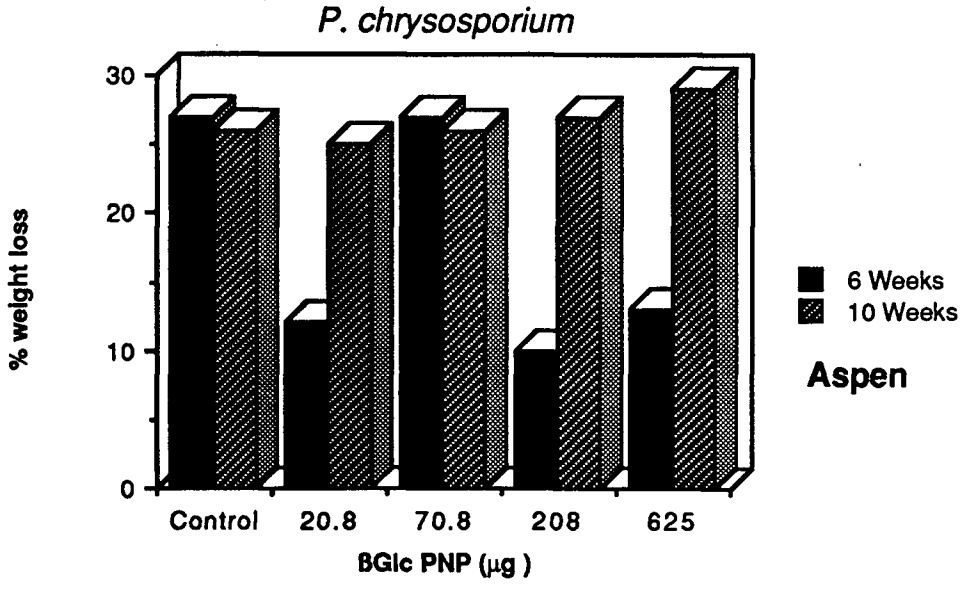


Figure 5-8. Weight loss caused by P. chrysosporium on samples of Aspen doweling.

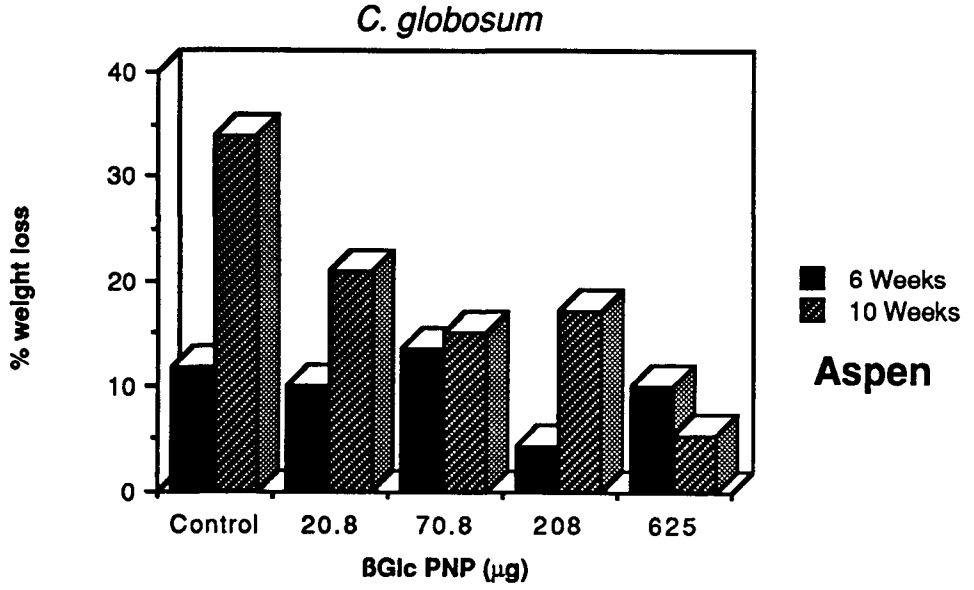


Figure 5-9. Weight loss caused by C. globosum on samples of aspen doweling.

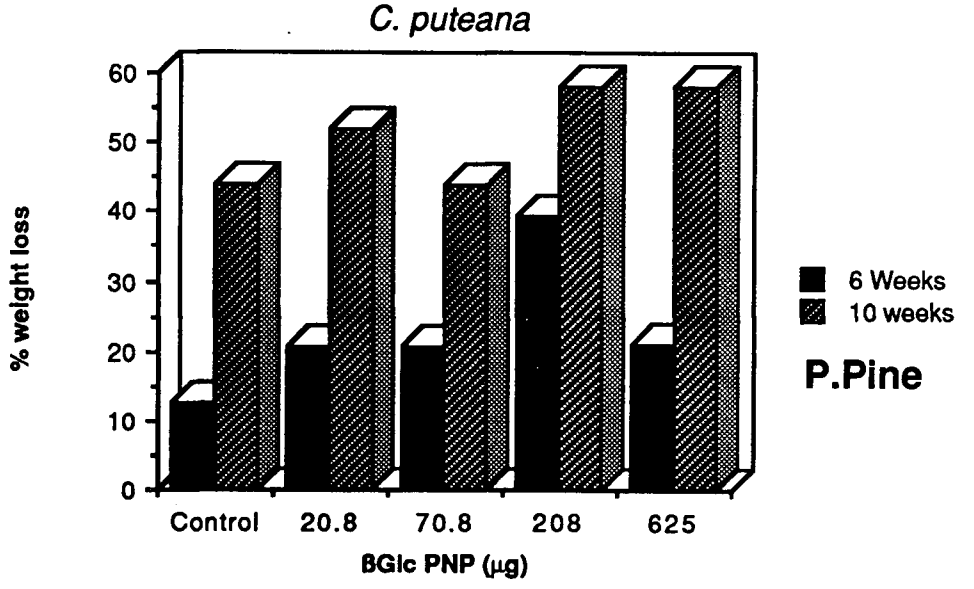


Figure 5-10. Weight loss caused by C. puteana on samples of ponderosa pine doweling.

Toxicity Sudies on the Intact βGlcPNP

To try and assess the toxicity of the intact glucoside as opposed to its hydrolysis products, toxicity limits were determined on a nutrient medium containing a relatively rich carbon source, maltose. It was proposed that by providing maltose (an α-1,4-linked disaccharide) as the sole carbon and energy source that the production of β-glucosidase would largely be repressed and consequently we would be able to determine toxicity of the unhydrolysed glucoside. However, as can be seen from the data presented in Table 5-II this was not the case.

Table 5-II. Toxic limits of βGlcPNP for wood degrading fungi on maltose medium.

Organism	[a] Toxic Limit ($\mu g\ mL^{-1}$)	[b] p-Nitrophenol ($\mu g\ mL^{-1}$)	Incubation (Days)
C. versicolor	>1000	1000	15
P. chrysosporium	100	100	15
C. globosum	500	500	15
C. puteana	1000	1000	15
T. reesei	100	100	15

[a] As defined in the text.[b] Minimum concentration at which the yellow phenolate could be seen.

In all cases production of yellow phenolate was evident at similar or lower limits to those seen on cellulose. This was surprising as in most cellulolytic fungi that have been studied β-glucosidase is an inducible enzyme (Berghem and Pettersson, 1974; Deshpande et al., 1978; Gussem et al., 1978; Luis and Becker, 1973; Meyer and Canerascuni; 1981; Shewale and Sadarna 1978). However, again there are a number of exceptions: *Stachybotris atra* produces both inducible and constitutive β-glucosidases (Gussem et al., 1978) and *Chaetomium thermophile* synthesizes three different constitutive β-glucosidases in relatively large amounts (Luis and Becker, 1973). There is also the possibility that βGlcPNP is in itself inducing β-glucosidase. This rather intriguing possibility that the pro-fungicide is in fact

inducing the specific metabolic machinery necessary for its own activation is based upon the observations of several workers. Deshpande et al. (1978), Hirayama et al. (1979), Shewale and Sadara (1978) and Smith and Gold (1979) have all observed that a number of different β-glucosides (methyl β-glucoside, gentiobiose and cellobiose) are efficient inducers of β-glucosidase in cellulolytic organisms.

In conjunction with this work, an assessment of toxicity for the intact βGlcPNP on *Daphnia* has been carried out by J. Servici and D. Martins at D.F.O. Cultus Lake B.C. They were able to demonstrate that at least initially, βGlcPNP was much less toxic than the free phenolate. In cultures of *Daphnia* concentration of 8μg mL^{-1} of p-nitrophenol were sufficient to cause complete mortality, however concentrations of 80μg mL^{-1} of βGlcPNP were non-toxic for up to 3 days. After 3 days sufficient quantities of the glucoside had hydrolysed to cause mortality. Subsequent sterile ageing of βGlcPNP solutions, which were then contaminated at various times suggested strongly that the hydrolysis of the glycoside arose from bacterial contamination.

Some Suggestions for Future Research..

Clearly the development of β-glycosides as control agents for cellulolytic fungi has a long way to go before they become a commercially viable proposition. However, the initial results described here (which were obtained with just a simple phenolic β-glucoside) hold promise for this approach.

Some of the already commercially available fungicides may be readily converted to glycosides, which may in turn, produce a beneficial change in their toxicological properties The compound TCMTB is a case in point. Although an excellent fungicide it is also quite toxic to fish, thus its conversion to a β-glucoside may remove the unwanted toxicity. As many β-glucosidases will readily hydrolyse carbon-nitrogen, carbon-sulphur, carbon-halogen as well as carbon-oxygen bonds, the aglycone is in no way restricted to simple phenolic, or alkoxyl groups. Hence one direction for future research would be to test a wide

variety of different structural types to find a beneficial balance between reactivity and toxicity in a β-glucoside.

Much of the usefulness of this approach depends upon the ability of the β-glucoside to be hydrolysed selectively by cellulolytic organisms. The problems encountered in *Daphnia* cultures which were probably caused by non-cellulolytic bacteria will present a significant developmental challenge to overcome. With this aim in mind, there are a number of approaches which can be followed: Firstly, in many bacterial systems β-glucosidase is an intracellular enzyme, whereas in fungal cultures which are growing on a largely insoluble substrate like cellulose, β-glucosidase is often excreted from the cell. A β-glucoside bearing a charged group of some kind (for example a pryidinium glucoside) would be less amenable to transport into a cell and therefore be more specific for organisms which produce extracellular β-glucosidase. A second approach which relies on other components of the cellulase system as well as β-glucosidase may also be useful. Instead of coupling the toxicant to a monosaccharide, an oligosaccharide or even cellulose itself could be used. As most β-glucosidases have insignificant activity with oligosaccharides greater than 5 or 6 residues long, the combined action of two or more components of the cellulase system would then be required to activate the pro-fungicide. In this way the pro-fungicide could be made very specific for cellulolytic organisms.

CHAPTER 6.

Materials and Methods

SYNTHETIC METHODS .

Unless otherwise stated the following are implied:

Melting points (m.p.) were determined on a Fisher-Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectroscopy was performed on the following instruments; Proton: 270 MHz (FT) spectra on a Bruker model HXS-270, equipped with a Nicolet 1180 computer and a Diablo disk drive, 300 MHz (FT) spectra on a Varian model XL-300 and 400 MHz spectra an a Bruker model WH-400. ^{19}F : 254 MHz (FT) spectra on a Bruker model HXS-270, equipped with a Nicolet 1180 computer and a 5mm ^{19}F probe. ^{31}P: 109 MHz (FT) spectra were recorded on a Bruker HXS-270 equipped with a Nicolet 1180 computer and a 10 mm ^{31}P probe. For ^{1}H spectra signal positions are given in the delta scale (δ) with reference to internal tetramethylsilane (δ = 0.00 ppm) (all solvents except D_2O). Samples dissolved in D_2O are referenced to external 2,2-dimethyl-2-silapentane-5-sulphonate (δ = 0.015 ppm). For ^{19}F spectra signal positions are given on the delta scale (δ) with reference to $CFCl_3$ (δ = 0.00 ppm). Signals occurring up-field of this resonance are given a positive value. External trifluoroacetic acid in D_2O (δ = 76.53 ppm) or hexafluorobenzene in $CDCl_3$ (δ = 162. 9 ppm) were used as references. For ^{31}P spectra, signal positions are given on the delta (δ) scale with reference to 85% phosphoric acid (δ = 0.00 ppm). Signals occurring downfield of this resonance are given a positive value. External 85% phosphoric acid was used as the standard. Signal multiplicity, coupling constants (where necessary), integrated peak area and resonance assignments are indicated in parentheses. Mass spectral data were collected on an AEI-MS-9 (low resolution) spectrometer employing electron impact ionization. Micro-analyses were performed by Mr P. Borda, Micro-analytical laboratory, University of British Columbia, Vancouver.

Solvents and reagents used were either reagent grade, certified or spectral grade and were distilled prior to use. Dry solvents or reagents where indicated, were prepared as follows; methylene chloride was washed several times with concentrated sulphuric acid , followed by several washings with water and a saturated solution of sodium bicarbonate. The

solvent was then pre-dried with sodium sulphate and distilled from calcium hydride. Pyridine was pre-dried for several days by standing over pellets of potassium hydroxide and then distilled from barium oxide. Toluene, N,N'-dimethylacetamide (DMAc) and acetonitrile were distilled from calcium hydride. Methanol was distilled from magnesium methoxide prepared *in situ* by reaction of methanol with magnesium turnings in the presence of catalytic amounts of iodine. Tetrahydrofuran (THF) was distilled from sodium and benzophenone. Carbon tetrachloride was distilled from phosphorus pentoxide. Crystalline phosphoric acid was obtained from BDH Chemicals and was dried *in vacuo* over magnesium perchlorate for several days prior to use.

Thin layer chromatography was conducted on aluminium backed plates of (0.2 mm thickness) Kieselgel 60 F_{425}. After development in the appropriate solvent, compounds were visualized by fluorescence quenching under U.V. light or by charring with 10% sulphuric acid in methanol. Column chromatography was carried out according to the method of Clark-Still et al.(1977) ("Flash Chromatography"), using a silica gel column of Kieselgel 60 (180-230 mesh).

The compound 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside was prepared by Dr. P.Bird (Withers et al. 1987). The following compounds were obtained from Dr. D.H.Dolphin as their per-O-acetates: 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride, 2-deoxy-2-fluoro–β-D-mannopyranosyl fluoride and 2-deoxy-2-fluoro-α-D-galactopyranosyl fluoride. The following compounds had been prepared previously (Withers et al, 1986): 2-, 3-, 4- and 6-deoxyfluoro-α–D-glucopyranosyl [bis(cyclohexylammonium) phosphate].The following compounds were prepared by D.Percival: α-mannosyl [mono(cyclohexyl-ammonium) phosphofluoridate] and 2-deoxy-2-fluoro-α–D-glucopyranosyl [bis(cyclohexyl-ammonium) phosphate].

6-Deoxy-α–D-glucopyranosyl [bis(cyclohexylammonium)phosphate] (1).

1,2,3,4-Tetra-O-acetyl-6-deoxy-β-D-glucopyranose (Arita et al., 1982) (2g, 6 mmol) was stirred with 4.0g of anhydrous molten phosphoric acid under vacuum for 2 hours at 50°C. Aqueous 2M lithium hydroxide (80mL) was then added and the mixture kept overnight at room temperature. After filtration through celite to remove excess lithium phosphate the pH of the filtrate was adjusted to approximately 8 with Dowex 50W - X8 (H^+) cation exchange resin. Lyophilization of this solution gave a powder which was redissolved in a minimal volume of water. The bislithium salt of the sugar phosphate was precipitated by addition of methanol-acetone 1:4 and isolated by filtration. After drying, final purification was accomplished by conversion to the bis cyclohexylammonium salt by passage down a column of Dowex 50W-X8 (cyclohexylammonium) followed by repeated recrystallization from a minimal volume of water by addition of acetone (0.824g, 30%): ^{1}H-NMR data (D_2O, 400 MHz): δ 5.38 (dd, 1H, $J_{1,2}$ 3.5, $J_{1,P}$ 7.0 Hz, H-1), 3.95 (dt, 1H, $J_{3,2}$ 7.0, $J_{3,4}$ 9.0Hz, H-3), 3.70 (t, 1H, $J_{3,4}$ 9, $J_{4,5}$ 9 Hz, H-4), 3.45 (ddd, 1H, $J_{2,1}$ 3.5, $J_{2,P}$ 2.0, $J_{2,3}$ 7.0 Hz, H-2), 3.11 (m, 3H, H-5, CH-NH_3^+), 1.95-1.24 (20H, 2, cyclohexyl), 1.23 (d, 3H, $J_{6,5}$ 5.5Hz, C-6 CH_3); Elemental analysis: Required for $C_{18}H_{39}N_2O_8P$: C,48.89; H, 8.88; N, 6.33. Found, C,48.63; H, 8.50; N, 6.46.

Methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-α-D-glucopyranoside (2).

Methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (Reist et al., 1965) (10g, 20 mmol) was dissolved in 50 mL of anhydrous pyridine and cooled to 0°C. Sulphuryl chloride (3.5g, 26 mmol) was added in a dropwise fashion and the mixture left at room temperature for 18 hours. The reaction was quenched by addition to a large volume of iced water and the product extracted into methylene chloride. The organic phase was washed with 1 M hydrochloric acid, water and dried with sodium sulphate. The product crystallized on removal of the solvent and was recrystallized from hot ethanol to give **2** (8.72g, 85%); m.p. 136-138°C; ^{1}H– NMR data ($CDCl_3$, 300 MHz) δ 8.12 - 7.31 (15H, 3 OBz), 6.08 (t, 1H, $J_{3,2}$ 9.8,

$J_{3,4}$ 9.8 Hz, H-3), 5.22 (2H, H-1, H-2), 4.75 (AB multiplet, 2H, $J_{6,6'}$, $J_{6,5}$, $J_{6',5}$, Hz, H-6, H-6'), 4.35 (m, 1H, H-5), 4.22 (t, $J_{4,5}$ 10.2, $J_{4,3}$ 10.2 Hz, H-4).

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-α-D-xylohexopyranoside (3).

Compound **2** (7.0g, 13.3 mmol) and α,α'-azobisisobutyronitrile (20 mg) were dissolved in dry toluene (100 mL) and placed under dry nitrogen. Tributyltin hydride (4.7g, 16 mmol), was then added and the reaction temperature maintained at 80°C for 24 hours. After the solution had cooled and the solvent had been removed *in vacuo*, the resulting gum crystallized on trituration with n-pentane. Recrystallization from diethyl ether by addition of n-pentane gave **3** (5.48g, 84%); m.p. 119 - 120°C; ^{1}H-NMR data ($CDCl_3$, 300 MHz): δ 8.12 - 7.31 (15H, 3 OBz), 5.80 (dt, 1H, $J_{3,4ax}$ 11.2, $J_{3,2}$ 11.2, $J_{3,4eq}$ 5.3 Hz, H-3), 5.19 (d, $J_{1,2}$ 3.8 Hz, H-1), 4.52 - 4.37 (3H, H-6, H-6', H-5), 3.47 (s, 3H, -OCH_3, 2.50 (dt, 1H, $J_{4eq,5}$ 6.4, $J_{4eq,3}$ 6.4, $J_{4eq,4ax}$ 12.0 Hz, H-4eq), 1.93 (q, 1H, $J_{4ax,4eq}$ 12.0, $J_{4ax,5}$ 12.0, $J_{4ax,3}$ 12.0 Hz, H-4ax).

2,3,6-Tri-O-benzoyl-4-deoxy-α-D-xylohexopyranosyl chloride (4).

Compound **3** (1.0g, 2.0 mmol) was dissolved in 3 mL of 1,1-dichloromethyl methylether and stirred with a catalytic amount of freshly fused zinc chloride under nitrogen at 65°C. After 3 hours the reaction had gone to completion so the remaining ether was removed *in vacuo*. The resulting gum was dissolved in methylene chloride and washed three times with a saturated solution of sodium bicarbonate. After drying the organic phase with sodium sulphate and removal of the solvent, the remaining gum was crystallized from diethyl ether by addition of pentane to give **4** (0.798g, 80%). m.p. 154-155°C; ^{1}H-NMR data ($CDCl_3$, 300 MHz): δ 8.10 - 7.33 (15H, 3 OBz), 6.52 (d, 1H, $J_{1,2}$ 4.0 Hz, H-1), 5.87 (m, 1H, H-3), 5.48 (dd, 1H, $J_{2,1}$ 4.0, $J_{2,3}$10 Hz, H-2), 4.72 (m, 1H, H-5), 4.50 (m, 2H, H-6, H-6'), 2.58(dt, 1H, $J_{4eq,5}$ 6.4, $J_{4eq,3}$ 6.4, $J_{4eq,4ax}$ 12.0 Hz, H-4eq), 2.02 (q, 1H, $J_{4ax,4eq}$ 12.0, $J_{4ax,5}$ 12.0, $J_{4ax,3}$ 12.0 Hz, H-4ax).

1-O-Acetyl-4-deoxy-2,3,6-tri-O-benzoyl-β-D-xylohexopyranose (5).

Compound **4** (7.5g, 15 mmol) was dissolved in 10 mL of glacial acetic acid and 9.5g (30 mmol) of mercuric acetate added. The mixture was left stirring at room temperature for 1 hour. After an extractive workup (methylene chloride-water) followed by drying (sodium sulphate) and removal of the solvent *in vacuo*, the remaining gum was crystallized from diethyl ether by addition of n-pentane. Recrystallization from methanol gave **5** (7.31g, 100%). m.p. 115-116°C; ^{1}H-NMR data ($CDCl_3$, 300 MHz): δ 8.05 - 7.45 (15H, 3 OBz), 5.98 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 5.54 (dd, 1H, $J_{1,2}$ 8.0 $J_{2,3}$ 8.9 Hz H-2), 5.49 (m, 1H, H-3), 4.50 (m ,2H, H-6, H-6'), 4.22 (m, 1H, H-5), 2.53 (m, 1H, H-4eq), 2.08 (s, 3H, OAc), 1.92 (m, 1H, H-4ax).

4-Deoxy-α–xylohexopyranosyl [bis(cyclohexylammonium)phosphate] (6).

Compound 5 (2.0g, 4.1 mmol) and 4.05g of anhydrous phosphoric acid were heated *in vacuo* for 1.5 hours at 55°C as detailed in the preparation of 1. The mixture was then dissolved in 30 mL of dry THF prior to addition of 80 mL of 2M lithium hydroxide. The remainder of the purification was performed exactly as for 1, yielding 6 (0.4g, 22%). 1H-NMR data. (D2O, 400 MHz): δ 5.48 (dd, 1H, $J_{1,2}$ 3.3, $J_{1,P}$ 7.2 Hz, H-1), 4.18 (m, 1H, H-5), 3.85 (dt, 1H, $J_{3,4eq}$ 5.0, $J_{3,4ax}$ 9.2, $J_{3,2}$ 9.2, Hz, H-3), 3.59 (AB multiplet, 2H, $J_{6,5}$ 3.0, $J_{6',5}$ 6.5, $J_{6,6'}$ 12.1 Hz, H-6, H-6'), 3.38 (ddd, 1H, $J_{2,1}$ 3.1, $J_{2,3}$ 9.1, $J_{2,P}$ 1.5 Hz, H-2), 3.13 (m, 2H, 2 CH-NH3+), 1.96 - 1.17 (22H, 2 cyclohexyl, H-4eq, H-4ax); Elemental analysis Required $C_{18}H_{39}N_2O_8P$. $3H_2O$: C, 43.54; H, 9.13; N, 5.64: Found: C, 44.05; H,9.00; N, 5.59.

1,2:5,6-Di-O-isopropylidine-3-chloro-3-deoxy-α–D-glucofuranose (7).

1,2:5,6-Di-O-isopropylidine-α-D-allofuranose (Stevens, 1972) (10g, 38 mmol) was dissolved in 60 mL of dry pyridine cooled in an ice bath and sulphuryl chloride added (6.7g,

50 mmol) over a period of 15 min. The reaction was then allowed to warm to room temperature and left to stir for 18 hours. The reaction was quenched by careful addition of 2 mL of water. The pyridine was removed *in vacuo* and the remaining oil dissolved in methylene chloride, washed several times with water, and dried with sodium sulphate. After removal of the solvent the product mixture was purified by flash chromatography (methylene chloride - ethyl acetate 9:1). Fractions containing the major component were pooled, and the solvent removed to give **7** as a pale yellow oil (5.8g, 55%). ^{1}H-N.M.R. ($CDCl_3$, 270 MHz): δ 5.93 (d, 1H, J 3.5 Hz, H-1), 4.71 (d, 1H, J 3.5 Hz H-2), 4.40 (d, 1H, J 2.8 Hz, H-3), 4.33 (m, 1H, $J_{5,4}$ 8.5, $J_{5,6}$ 4.5, $J_{5,6'}$ 8.5 Hz, H-5), 4.15 (dd, $J_{4,5}$ 8.5, $J_{4,3}$ 3.0 Hz, H-4), 4.09 (AB multiplet, 2H, $J_{6,6'}$ 8.9, $J_{6,5}$ 8.5 $J_{6',5}$ 4.5 Hz, H-6, H-6'), 1.50 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.31 (s, 3H, CH_3).

3-Chloro-3-deoxy-D-glucose (8).

Compound **7** (6.6g, 23.6 mmol) was dissolved in 50 mL of 60% v/v aqueous trifluoroacetic acid and stirred at room temperature for 2.5 hours. The reaction mixture was then evaporated to yield a gum from which the **8** crystallized from methanol on addition of ether (3.42g 72 %); m.p.142-143°C.

1,2,4,6-Tetra-O-acetyl-3-chloro-3-deoxy-β-D-glucopyranose (9).

A solution of **8** (3.4g, 17 mmol) in 32 mL of dry pyridine was acetylated by addition of 18 mL of acetic anhydride (0°C to room temperature). After 20 hours methanol was added to remove excess acetic anhydride and all solvents were removed *in vacuo*. The resulting gum was converted directly to the per-O-acetylated α-bromide by dissolution in 25mL of 45% hydrogen bromide in acetic acid. After 1 hour at room temperature methylene chloride was added and the mixture washed three times with ice water, followed by drying of the organic phase with sodium sulphate. After removal of the solvent the α-bromide was converted to the β-per-O-acetate by reaction with 6g of mercuric acetate in 70 mL of glacial acetic acid as

detailed for **3**. Recrystallization of the product from ether-pentane gave **9** (4.6g, 74%). m.p. 121 - 122°C; ^{1}H - NMR data ($CDCl_3$, 270 MHz): δ 5.66 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 5.23 (2H, H-2, H-4), 4.20 (AB multiplet, 2H, $J_{6,6'}$ 12.0, $J_{6,5}$ 2.5, $J_{6',5}$ 4.5, H-6, H-6'), 4.01 (t, 1H, $J_{2,3}$ 10, $J_{3,4}$ 10 Hz, H-3), 3.78 (m, 1H, H-5), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.01 (s, 3H, OAc).

1,2,4,6-Tetra-O-acetyl-3-deoxy-β-D-ribohexopyranose ***(10).***

A solution of **9** (4.5g, 12.2 mmol) in 30 mL of anhydrous toluene containing 20 mg αα'-azobisisobutyronitrile was reduced with 5.5g of tributyltin hydride at 80°C under dry nitrogen for 20 hours. The solvent was removed *in vacuo* and the product crystallized on trituration with n-pentane. Recrystallization from methanol gave pure **10** (3.42g, 84%). m.p. 134-135°C; ^{1}H-NMR data ($CDCl_3$, 270 MHz): δ 5.70 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1), 4.93 (2H, H-3 + H-4), 4.18 (AB multiplet, $J_{6,6'}$12.1, $J_{6,5}$ 7.2, $J_{6',5}$ 3.8 Hz, H-6 + H-6'), 3.83 (m, 1H, H-5), 2.65 (dt, 1H, $J_{3ax,3eq}$ 12.3, $J_{3eq,4}$ 5.0, $J_{3eq,2}$ 5.0 Hz, H-3eq), 2.11 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.57 (m,1H, $J_{3ax,4}$ 10.9, $J_{3ax,2}$ 10.9, $J_{3ax,3eq}$ 12.0 Hz, H-3ax).

3-Deoxy-α-D-ribohexopyranosyl [bis(cyclohexylammonium)phosphate] ***(11).***

Reaction of **10** (2.0g, 5.96 mmol) with 4.0g of anhydrous phosphoric acid was performed exactly as for **1**, yielding **11** (0.84g, 32%). ^{1}H-NMR data (D_2O, 270 MHz): δ 5.32 (dd, 1H, $J_{1,2}$ 4.0, $J_{1,P}$ 7.0 Hz, H-1), 3.82 - 3.48 (5H, H-2, H-4, H-5, H-6, H-6'), 3.09 (m, 2H, CH-NH_3^+), 2.11 - 1.14 (22H, cyclohexyl, H-3ax, H-3eq); Elemental analysis required for $C_{18}H_{39}N_2O_8P$. $2H_2O$: C, 45.18; H, 9.06; N, 5.85. Found: C, 44.79; H, 8.76; N, 5.48.

3,4,6-Tri-O-benzyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (12) and 3,4,6-tri-O-benzyl-α-D-mannopyranosyl fluoride (13).

The compound 3,4,6-tri-O-benzyl-D-mannopyranose (Franks and Montgomery, 1968) (0.5g, 1.1mmol) was dissolved in dry methylene chloride (10 mL) and cooled to -20°C. Anhydrous conditions were maintained while DAST (0.4 mL, 3.3 mmol) was added and the reaction mixture allowed to warm slowly to room temperature. After a period of 24 hours the reaction mixture was cooled to 0°C and, after cautious addition of 5 mL of methanol the reaction was stirred at room temperature for a further 2 hours. The solvents were removed *in vacuo* and the resulting oil purified by flash chromatography (methylene chloride-ethyl acetate, 19:1). The chromatographically more mobile fraction was purified further on a second flash column (n-pentane- ethyl acetate, 9:1) to yield **12** as a colourless gum (0.183g, 36%). ^{1}H-NMR data ($CDCl_3$, 400 MHz): δ 7.34 - 7.14 (15H, aromatic OBn), 5.33(ddd, 1H, $J_{1,F-1}$ 52.7, $J_{1,F-2}$ 3.9, $J_{1,2}$ 6.5 Hz, H-1), 4.89 - 4.51 (6H, benzylic, OBn), 4.49 (m, 1H, $J_{2,F-2}$ 51.0, $J_{2,F-1}$ 13.4, $J_{2,1}$ 6.5., $J_{2,3}$ 8.0 Hz, H-2), 3.79, (dt, 1H, $J_{3,2}$ 8.2, $J_{3,4}$ 10.3 Hz, H-3), 3.78 - 3.70 (3H, H-4, H-6, H-6'), 3.61 (m, 1H, H-5); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ 141.05 (dt, 1F, $J_{F-1,H-1}$ 52.7, $J_{F-1,H-2}$ 13.8 , $J_{F-1,F-2}$ 13.8 Hz F-1), 197.78 (m, 1F, $J_{F-2,H-2}$ 50.6, $J_{F-2,H-1}$ 3.47, $J_{F-2,H-3}$ 15.1, $J_{F-1,F-2}$ 13.8 Hz, F-2); Mass spectral data, *m/z* : 454.

The second fraction (from the first column) gave **13** as a colourless gum (0.092g, 18%).^{1}H-NMR data ($CDCl_3$, 400 MHz): δ 7.35 - 7.17 (15H, aromatic OBn), 5.66 (dd, 1H, $J_{1,2}$ 1.7, $J_{1,F}$ 49.6 Hz, H-1), 4.82 - 4.52 (6H, benzylic OBn), 4.08 (m, 1H, H-2), 3.92 (t, 1H, $J_{4,5}$ 8.5, $J_{4,3}$ 8.5 Hz, H-4), 3.90 (m, 1H, H-5), 3.86 (dd, 1H, $J_{3,2}$ 1.9, $J_{3,4}$ 8.1 Hz, H-3), 3.74 (AB multiplet, 2H, $J_{6,6'}$ 11.0, $J_{6,5}$ 1.8, $J_{6',5}$ 3.9 Hz, H-6, H-6'), 2.82 (broad s, removed on addition of D_2O, C-2-OH); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ 140.98 (d, $J_{F,H-1}$ 49.5 Hz, F-1); Mass spectral data *m/z* : 452.

3,4,6-Tri-O-benzyl-2-O-methyl-α- and β-D-glucopyranosyl fluorides (14 and 15).

Methyl 3,4,6-tri-O-benzyl-D-mannopyranoside (Franks and Montgomery, 1968) (0.222g, 0.48 mmol), was dissolved in dry methylene chloride (10 mL) and cooled to -20°C under anhydrous conditions. DAST (1.24 mmol) was added and the reaction temperature maintained at -20°C for 30 min. After allowing the reaction to warm to room temperature it was heated to 40°C for 90 min. The mixture was then cooled to 0°C and after cautious addition of 5 mL of methanol, stirred for 1 hour at room temperature. After removal of the solvents the product mixture was separated by flash chromatography (n-pentane-ethyl acetate, 9:1). The first compound to be eluted was found to be **14** (0.048g, 23%). ^{1}H-NMR Data ($CDCl_3$, 400 MHz): 7.36 - 7.13 (15H, aromatic OBn), 5.73 (dd, 1H, $J_{1,2}$ 6.7, $J_{1,F}$ 52.0 Hz, H-1), 4.88 - 4.54 (6H, benzylic OBn), 3.72 - 3.57 (5H, H-3, H-4, H-5, H-6, H-6'), 3.58 (d, 3H, J 1.5 Hz, OCH_3), 3.25 (m, $J_{2,1}$ 6.5 $J_{2,3}$ 8.1, $J_{2,F-1}$ 12.1 Hz, H-2); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ 138.61 (dd, $J_{F,H-1}$ 53.3 Hz, $J_{F,H-2}$ 12.1 Hz); Mass spectral data. *m/z* : 466.

Further elution with the same solvent gave the chromatographically less mobile compound **15** (0.054g, 24%).^{1}H-NMR data 7.36 - 7.17 (15H, aromatic OBn), 5.73 (dd, 1H, $J_{1,2}$ 2.7, $J_{1,F}$ 54.0 Hz, H-1), 4.91 - 4.50 (5H, H-3, H-4, H-5, H-6, H-6'), 3.56 (s, 3H, OCH_3), 3.55 (m, 1H, $J_{2,1}$ 2.7, $J_{2,3}$ 9.46, $J_{2,F}$ 25.6 Hz, H-2); ^{19}F-NMR data ($CDCl_3$, 254 MHz) δ 149.87 9 (dd, $J_{F,H-1}$ 53.6, $J_{F,H-2}$ 25.7 Hz, F-1); Mass spectral data. *m/z*: : 466.

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-β-D-galactopyranosyl bromide (16).

2-Deoxy-2-fluoro-α-D-galactopyranosyl fluoride (1.0g, mmol) was dissolved in 10 mL of 45% hydrogen bromide in glacial acetic acid, 5 drops of acetic anhydride added and the reaction left to stir at room temperature for 3 hours. After an extractive work up (methylene chloride - water) the product was dried with sodium sulphate and the solvent removed *in vacuo* . The product crystallized from ether on addition of n-pentane to give **16** as a

colourless solid (0.88g, %). m.p.79-80°C ; ^{1}H-NMR data ($CDCl_3$, 400 MHz): δ 5.62 (d, 1H, $J_{1,2}$ 4.3 Hz, H-1), 5.55 (dt, 1H, $J_{4,5}$ 1.4, $J_{4,3}$ 3.2 Hz, H-4), 5.49 (dt, 1H, $J_{3,4}$ 3.2, $J_{2,3}$ 10.0, $J_{3,2}$ 10.0 Hz, H-3), 4.77 (ddd, 1H, $J_{2,1}$ 4.1, $J_{2,3}$ 10.0, $J_{2,F}$ 50.5 Hz, H-2), 4.53 (t, 1H, $J_{5,6}$ 6.7, $J_{5,6'}$ 6.5 Hz, H-5), 4.16 (AB multiplet, 2H, $J_{6,6'}$ 11.5, $J_{6',5}$ 6.5, $J_{6,5}$ 6.8 Hz, H-6, H-6'), 2.16 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.06 (s, 3H, OAc); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ 195.71 (dd, $J_{F,H-3}$ 9.7, $J_{F,H-2}$ 48.5 Hz).

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride (17)

Compound **16** (0.7g,1.89mmol) was treated with 1.4g of silver fluoride in 10 mL of acetonitrile for 48 hours at room temperature. The silver salts were removed by filtration through silica gel using ethyl acetate as the eluant to give **17** as a colourless gum. (0.569g, 97%). ^{1}H-NMR data ($CDCl_3$, 400MHz): δ 5.46 (m, 1H, H-4), 5.38 (ddd, 1H, $J_{1,2}$ 6.9, $J_{1,F-2}$ 5.1, $J_{1,F-1}$ 52.4 Hz, H-1), 5.15 (m, 1H, $J_{3,4}$ 3.5, $J_{3,2}$ 9.6, $J_{3,F-2}$ 13.0 Hz, H-3), 4.67 (m, 1H, $J_{F-2,2}$ 51.7, $J_{2,3}$ 9.7, $J_{2,1}$ 6.8, $J_{2,F-1}$ 13.0 Hz, H-2), 4.20 (d, 2H, J 7.2 Hz, H-6, H-6'), 4.07 (t, 1H, J 7.1Hz, H-5), 2.16 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.07 (s, 3H, OAc); ^{19}F-NMR data ($CDCl_3$, 254 MHz) δ 142.38 (dt, 1F,$J_{F-1,H-2}$ 14.2, $J_{F-1,F-2}$ 14.2, $J_{F-1,H-1}$ 52.4 Hz, F-1), 209.55 (dt, 1F, $J_{F-2,H-3}$ 13.9, $J_{F-2,F-1}$ 13.9, $J_{F-2,H-2}$ 51.4 Hz, F-2),

2-Deoxy-2-fluoro-β-D-galactopyranosyl fluoride (18).

Compound **17** was deacylated using a strong base ion-exchange resin according to the method of Reed *et al.*, (1981) Compound **17** (0.56g, 1.8 mmol) was dissolved in 20 mL of anhydrous methanol and a catalytic amount of AG1-X8 ($^-$OH) anion exchange resin added. The reaction was left to stir at room temperature for 3 hours then filtered and the solvent removed *in vacuo*. The product was purified by flash chromatography (ethyl acetate) to give **18** as a colourless gum (0.285g, 86%). ^{1}H-NMR data (D_2O, 400 MHz): δ 5.45 (ddd, 1H, $J_{1,F-2}$ 4.3, $J_{1,2}$ 6.8, $J_{1,F-1}$ 53.1 Hz, H-1), 4.52 (m ,1H, $J_{2,F-1}$ 2.5, $J_{2,F-1}$ 52.5, $J_{2,1}$ 7.0, $J_{2,3}$ 9.3 Hz, H-2), 4.01- 3.77 (5H, H-3, H-4, H-5, H-6, H-6'); ^{19}F-NMR data (D_2O, 254

MHz): 144.50 (dt, 1F, $J_{F-1,F-2}$ 15.9, $J_{H-1,F-1}$ 53.7, $J_{F-1,H-2}$ 15.9 Hz, F-1), 211.57 (m, 1F, $J_{F-2,H-2}$ 51.0, $J_{F-1,H-3}$ 15.6, $J_{F-1,F-2}$ 15.6 Hz, F-2).

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (19).

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl bromide (Street, I.P.,1985 M.Sc. Thesis University of British Columbia) (0.7g, 1.89 mmol) was reacted with 1.7g of silver fluoride exactly as detailed for **16** to give a colourless gum which crystallized from ether on addition of n-pentane yielding **19** as a colourless solid (0.489g, 84%). m.p. 104-105°C; ^{1}H-NMR data ($CDCl_3$, 400 MHz): δ 5.45 (ddd, 1H, $J_{1,F-2}$ 4.0, $J_{1,2}$ 6.2, $J_{1,F-1}$ 52.0 Hz, H-1), 5.34 (dt, 1H, $J_{3,F-2}$ 15.3, $J_{3,4}$ 9.2, $J_{3,2}$ 9.2 Hz, H-3), 5.13 (t, 1H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, H-4), 4.51 (m, 1H, $J_{2,1}$ 6.2, $J_{2,3}$ 8.0, $J_{2,F-2}$ 50.1, $J_{2,F-1}$ 14.1 Hz, H-2), 4.31- 4.17 (2H, H-6, H-6'), 3.91 (m, 1H, H-5), 2.10 (6H, OAc), 2.05 (s, 3H, OAc); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ. 140.5 (m, $J_{F,F}$ 15, $J_{F-1,H-2}$ 14, $J_{F-1,H-1}$ 48 Hz, F-1), 200.5 (m, $J_{F,F}$ 14, $J_{F,H-3}$ 14, $J_{F,H-2}$ 52 Hz, F-2).

2-Deoxy-2-fluoro-β-D-glucopyranosyl fluoride (20)

The per-O-acetate **19** (0.472g, 1.52 mmol) was deacetylated exactly as for **17** in 20 mL of dry methanol. The product crystallized from methanol on addition of diethyl ether to give **20** as a colourless solid (0.2g, 71%). m.p.104-105°C; ^{1}H-NMR data (D_2O, 400MHz): δ 5.49 (ddd, 1H, $J_{1,2-F}$ 3.2, $J_{1,2}$ 7.0 $J_{1,F-1}$ 53.2 Hz, H-1), 4.34 (m, 1H, $J_{2,1}$ 7.0, $J_{2,3}$ 9.3, $J_{2,F-1}$ 13.5, $J_{2,F-2}$ 51.4 Hz, H-2), 3.82 (AB multiplet, 2H, $J_{6,6'}$ 12.4, $J_{6,5}$ 2.0, $J_{6',5}$ 5.5 H-z, H-6, H-6'), 3.80 (dt, 1H, $J_{3,4}$ 9.0, $J_{3,2}$ 9.0, $J_{3,F-2}$ 15.6 Hz, H-3), 3.59 (ddd, 1H, $J_{5,4}$ 9.4, $J_{5,6}$ 2.0, $J_{5,6'}$ 5.6 Hz, H-5), 3.53 (t, 1H, $J_{4,3}$ 9.0, $J_{4,5}$ 9.0 Hz, H-4); ^{19}F-NMR data (D_2O, 254 MHz): δ.144.8 (m, $J_{F,F}$ 16, $J_{F-1,H-2}$ 16, $J_{F-1,H-1}$ 48 Hz, F-1), 203.4 (m, $J_{F,F}$ 15, $J_{F,H-3}$ 16, $J_{F,H-2}$ 50 Hz, F-2).

2-Deoxy-2-fluoro-α-D-glucopyranosyl fluoride (21).

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride (0.25g, 0.81mmol) was deacetylated using strong base ion exchange resin exactly as detailed for **17**. After purification by flash chromatography (ethyl acetate) **21** was obtained as a colourless gum (0.115g, 77%). ^{1}H-NMR data (D_2O, 400 MHz): δ 5.87 (dd, 1H, $J_{1,2}$ 2.9, $J_{1,F-1}$ 53.8 Hz, H-1), 4.46 (m, 1H, $J_{2,1}$ 3.0, $J_{2,3}$ 10.0, $J_{2,F-1}$ 24.5, $J_{2,F-2}$ 53.8 Hz, H-2), 3.99 (dt, 1H, $J_{3,F-2}$ 13.5, $J_{3,4}$ 9.6 Hz, H-3) 3.88-3.71 (2H, H-6, H-5), 3.76(dd, 1H, $J_{6',5}$ 4.8, $J_{6,6'}$ 11.9 Hz, H-6'), 3.55 (t, 1H, $J_{4,3}$ 9.8, $J_{4,5}$ 9.8 Hz, H-4); ^{19}F-NMR data (D_2O, 254 MHz): δ.136.3 (m, $J_{F,F}$ 16, $J_{F-1,H-2}$ 24, $J_{F-1,H-1}$ 54 Hz, F-1), 200.1 (m, $J_{F,F}$ 16, $J_{F-2,H-3}$ 16, $J_{F-2,H-2}$ 48 Hz, F-2).

1–Deoxy–β-D-glucosyl benzene (22).

2,3,4,6-Tetra-O-acetyl-β-D-glucosyl benzene was prepared by the action of phenyl-magnesium bromide on 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (5.0g, mmol) according to the method of Bonner (1963). The product was recrystallized twice from propan-1-ol to give the per-O-acetate of **22** as a colourless crystalline solid (2.63g, %). m.p. 158 -159°C (lit m.p. 155 - 156°C); ^{1}H-NMR data ($CDCl_3$, 270 MHz): δ 7.35 (5H, phenyl), 5.34 (t, J 9.5 Hz), 5.25 (t, J 10.0 Hz), 5.12 (t, J 9.5 Hz) (H-2, H-3, H-4), 4.24 (AB multiplet, 2H, $J_{6,5}$ 4.5, $J_{6',5}$ 2.0, $J_{6,6'}$ 12.5 Hz, H-6, H-6'), 4.18 (m, 1H, $J_{5,6}$ 4.6, $J_{5,6'}$ 2.2, $J_{5,4}$ 9.5 Hz, H-5), 2.09 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.80 (s, 3H, OAc).

Deacetylation of the per-O-acetate was carried out by dissolving 2.0g of the compound in 50 mL of 0.1M sodium methoxide in methanol. After about 20 minutes the reaction had gone to completion and the entire mixture was allowed to percolate down a column of Dowex 50W -X8 acidic ion exchange resin. The column was then washed with methanol and the eluant collected. After removal of the solvent the residue was purified by

flash chromatography (ethyl acetate, methanol 9:1) and the product dried *in vacuo* over potassium hydroxide pellets, yield (1.06g, %).

3,4,6-Tri-O-acetyl-2-fluoro-D-glucal (23).

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl bromide (0.224g,0.6 mmol) was dissolved in 10 mL of acetonitrile, 0.9 mL of triethylamine added and the reaction heated to reflux for 22 hours. After evaporation of the solvent, triethylammonium bromide was removed from the residue by flash chromatography (hexane-ethyl acetate, 4:1) to give **23** as a colourless gum (0.145g, 87%). ^{1}H-NMR data ($CDCl_3$, 400 MHz): δ 6.78 (d, 1H, $J_{1,F}$ 4.2 Hz, H-1), 5.63 (t, 1H, $J_{3,4}$ 4.0, $J_{3,F}$ 4.0 Hz, H-3), 5.21 (dt, 1H, $J_{4,3}$ 4.0, $J_{4,5}$ 5.5 Hz, H-4), 4.30 (m, 1H, H-5), 4.29 (AB multiplet, 2H, $J_{6,6'}$ 12.0, $J_{5,6}$ 7.0, $J_{5,6'}$ 3.8 Hz, H-6, H-6'), 2.12 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.10 (s, 3H, OAc); ^{19}F-NMR data ($CDCl_3$, 254 MHz): δ 167.08 (t, $J_{F,1}$ 3.9, $J_{F,3}$ 3.9 Hz, F-2).

2-Fluoro-D-glucal (24).

The triacetate **23** (0.145g, 0.5 mmol) was deacylated in 3 mL of 0.1M sodium methoxide in anhydrous methanol at room temperature. After 20 minutes the reaction had gone to completion and the remaining base was neutralized by addition of Dowex 50W-X8 (H^+) acidic ion exchange resin. After filtration and removal of the solvent the residue was purified by flash chromatography (ethyl acetate) to give **24** as a colourless gum.(0.064g, 80%) The gum could be induced to crystallize on standing for several months at -20°C, however repeated attempts to recrystallize the product from a number of different solvents failed. Deprotection of this compound was also carried out using the strong base ion exchange resin as detailed for **17**. No significant difference in yield was obtained. ^{1}H-NMR data (D_2O, 400 MHz): δ 6.74 (d, 1H, $J_{1,F}$ 4.8 Hz, H-1), 4.36 (dd, 1H, $J_{3,F}$ 1.8, $J_{3,4}$ 5.5 Hz, H-3), 3.93, 3.85 - 3.74 (4H, H-4, H-5, H-6, H-6'); ^{19}F-NMR (D_2O, 254 MHz): δ 169.57 (dd, $J_{F,H-1}$

5.0, $J_{F,H-3}$ 2.0 Hz); Elemental analysis: Required for $C_6H_9O_4F$; C, 43.91; H, 5.53. Found; C, 44.12; H, 5.70.

(2,3,4,6-Tetra-O-benzyl-α-D-glucopyranosyl) methylmercuric chloride (25).

A three neck flask equipped with a condenser and stirring bar was flame dried and allowed to cool under an atmosphere of dry nitrogen. The apparatus was then charged with triphenylmethylphosphonium iodide (27g, 66.8 mmol) and 300 mL of dry THF. n-Butyl lithium in hexane (104 mmol) was then added slowly while the reaction temperature was maintained at 0°C . After the addition was complete the reaction was warmed to room temperature and stirred for 1 hour to allow complete formation of the purple coloured phosphorane. 2,3,4,6-Tetra-O-benzyl-D-glucopyranose (Tate and Bishop, 1963) (17.0g, 31.4 mmol) in dry THF followed by 8.5g of sublimed solvent-free potassium t-butoxide was added to the reaction and stirring continued for 20 hours. The reaction was quenched by cooling to -20°C and the precipitated triphenylphosphine oxide removed by filtration. The filtrate was then washed with a saturated solution of sodium chloride, dried with sodium sulphate and the solvent removed *in vacuo*. Partial purification of the product was achieved by column chromatography (methylene chloride) to give a mixture of the 3 major products. These were then redissolved in 50 mL of dry THF and stirred overnight with 4.2g of mercuric acetate. Potassium chloride (1.6g) in 0.5 mL of water was added and stirring continued for a further 1 hour. After an extractive workup the organic phase was dried (sodium sulphate) and the solvent removed *in vacuo* . The residue crystallized on addition of n-pentane to a solution of the crude preparation in diethyl ether, followed by seeding with an original sample of **25.** Further product was obtained by flash chromatography of the remaining gum (hexane - ethyl acetate, 9:1) followed by crystallization as before. The crops were combined and recrystallized to give pure **25** as a white crystalline solid (2.156g, 8.9%). m.p. 103°C, (Lit m.p.(Pougny et al., 1981) 104.5°C); ^{1}H -NMR data ($CDCl_3$, 270 MHz): δ 7.33 - 7.10 (20H, aromatic OBn), 4.90 - 4.59 (8H, benzylic OBn), 4.10 (ddd, 1H, $J_{2,1}$

6.7, $J_{2,1'}$ 11.0, $J_{2,3}$ 4.5 Hz, H-2), 3.72 - 3.59 (6H, H-3, H-4, H-5, H-6, H-7, H-7'), 1.95 (AB multiplet, 2H, $J_{1,1'}$ 11.3, $J_{1,2}$ 6.7, $J_{2,1'}$ 11.5 Hz, H-1, H-1').

(2,3,4,6-Tetra-O-benzyl-1-deoxy-α-D-glucopyranosyl) methyl iodide (26).

To a solution of iodine (0.753g) in 60 mL of dry methylene chloride, 2.1g (2.7mmol) of **26** was added and left stirring for 18 hours at room temperature. Iodine was removed by washing with 10% sodium sulphite and 5% sodium iodide followed by drying with sodium sulphate. After removal of the solvent, crystallization of the residue from methanol gave **27** as a white solid (1.55g, 91%). m.p. 87-88°C; ^{1}H-NMR data ($CDCl_3$, 270 MHz): δ 7.34 - 7.10 (20H, aromatic OBn), 4.89 - 4.47 (8H, benzylic OBn), 4.15 (dt, 1H, $J_{2,3}$ 4.3, $J_{2,1}$ 11.5, $J_{2,1'}$ 4.3 Hz, H-2), 3.80 - 3.49 (6H, H-3, H-4, H-5, H-6, H-7, H-7'), 3.48 (AB multiplet, 2H, $J_{1,1'}$ 11.5, $J_{1,2}$ 4.1, $J_{2,1'}$ 11.5 Hz, H-1, H-1').

Diethyl (2,3,4,6-Tetra-O-benzyl-1-deoxy-α-D-glucopyranosyl) methylphosphonate (27).

A solution of **27** (1.5g, 2.3 mmol) in 17 mL of triethylphosphite was heated at reflux for 2.5 hours. The volatile products and excess reagent were removed by distillation under reduced pressure and the residue purified by flash chromatography (methylene chloride-ethyl acetate, 4:1) to give **27** as a colourless oil (1.245g, 80%). ^{1}H-NMR data ($CDCl_3$, 270 MHz): δ 7.31 - 7.12 (20H, aromatic OBn), 4.90 - 4.45 (8H, benzylic OBn), 4.09 (q, 2H, J 8.0 Hz, O-$\underline{CH_2}$-CH_3), 4.06 (q, 2H, J 8.0 Hz, O-$\underline{CH_2}$-CH_3) 4.01 (ddd, 1H, $J_{2,3}$ 4.5, $J_{2,1}$ 7.6, $J_{2,1'}$ 3.2 Hz, H-2), 3.79 - 3.62 (6H, H-3, H-4, H-5, H-6, H-7, H-7'), 2.20 (m, 2H, $J_{2,1'}$ 7.6, $J_{2,1}$ 3.2, J_P 18 Hz, H-1, H-1'), 1.29 (t, 3H, J 8.2 Hz, O-CH_2-$\underline{CH_3}$). 1.27 (t, 3H, J 8.2 Hz, O-CH_2-$\underline{CH_3}$).

Cyclohexylammonium (1-deoxy-α-D-glucopyranosyl) methylphosphonate (28).

To a solution of **27** (0.982g, 1.5 mmol) in dry carbon tetrachloride (0°C) under dry nitrogen was added iodotrimethylsilane (1.9 mL, 13.4 mmol) and stirring continued for 45 min. The

reaction was quenched by addition of 2 mL of water followed by removal of all solvents *in vacuo*. The residue was then repeatedly extracted with diethyl ether until this procedure failed to remove any further colour. The gum was redissolved in methanol and an excess of cyclohexylamine added. Addition of ether followed by cooling in a dry ice acetone bath precipitated the product which was isolated by filtration and quickly dried *in vacuo*. This procedure was repeated four times to give **29** as a white amorphous solid (0.33g, 62%). ^{1}H-NMR data (D_2O, 270 MHz): δ 4.30 (m, 1H, $J_{2,3}$ 4.8 $J_{2,1}$ 4.0, $J_{2,1'}$ 10.1, $J_{2,P}$ 10.1Hz, H-2), 3.76 - 3.26 (6H, H-3, H-4, H-5, H-6, H-7, H-7'), 3.06 (m, 1H, CH-NH_3^+), 1.89 - 1.07 (12H, cyclohexyl, H-1, H-1'); ^{31}P-NMR data (D_2O (20mM), 109.29 MHz): δ 22.20 (m, $J_{P,H-2}$ 9.47, $J_{P,H-1}$ 17.55, $J_{P-H-1'}$ 27.19). Elemental analysis. Required for $C_{13}H_{28}O_8PN$. C, 43.70; H, 7.90, N, 3.92. Found, C, 43.28; H, 8.20; N, 3.70.

ENZYMOLOGY (II)

Absorbance measurements were carried out on a Pye Unicam PU-8800 UV/visible spectrophotometer, equipped with a Julabo circulating water bath. Measurements of pH were carried out on a Radiometer PHM 62 pH meter equipped with a Sigma Trizma glass / calomel combination electrode and standardized with BDH standard buffers. Rabbit muscle was obtained from Pel-Freez biologicals. Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex1-Cl column and assayed by the method of Dishe (Ashwell, 1957). Ammonium sulphate (ultra pure grade) was obtained from Schwarz / Mann Biotech. AMP and AMPS were obtained from Sigma Chemical Co, stock solutions were made up in water and their concentration determined by optical density at 260 nm (E= 1600 M^{-1} cm^{-1}). NADP, ATP, βGlcPNP, βGalPNP, were also obtained from Sigma.

The following isolates of wood degrading fungi were obtained from the culture collection of Forintek Canada Corp; *Chaetomium globosum* Kze. : Fr., *Coniophora puteana* (Schum. : Fr.) Karst, *Coriolus versicolor* (L.: Fr.) Quel, *Phanaerochaete chrysosporium* Burds, *Trichoderma reesei* Simmons, *Trichoderma harzianum* Rifai. Stock cultures of *E.*

coli strain JM109 containing the plasmid pABG5 were obtained from A.J.Warren , Microbiology department University of British Columbia. Quantitative amino acid analysis were performed by M.Mauk, Department of Biochemistry, University of British Columbia. All media were sterilized in an autoclave at 121°C for 20 minutes. Stock solution of ampicillin, and isopropyltio β-D-galactopyranoside were sterilized by passage through a sterile 0.22 μm Millipore filter and added to the sterile media just prior to inoculation of the culture.

Gel electrophoresis was conducted on a Mini-Protean^{TM} Dual slab cell (Bio-Rad Laboratories). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the specifications Laemmli (1970) using a 12% separating gel containing 0.1 % SDS and a 4.0% stacking gel. Samples were prepared in the specified sample buffer and heated to 100°C prior to application to the gel.

PROTEIN PURIFICATION

Glycogen Phosphorylase.

Glycogen phosphorylase *b* (E.C. 2.4.1.1.) was prepared from rabbit muscle by the method of Fischer and Krebs (1962) using DTT instead of cysteine and recrystallized at least three times before use. Protein concentrations were determined from absorbance measurements at 280 nm using an absorbance index $E_{280}^{0.1\%} = 1.32$ mL mg^{-1} cm^{-1} (Buc and Buc, 1968) Phosphorylase *a* was prepared from phosphorylase *b* by the action of phosphorylase kinase (E.C. 2.7.1.38) (Krebs et al., 1964).

β-Glucosidase (pABG5).

The enzyme described here as pABG5 β-glucosidase was originally isolated from an *Agrobacterium* species (ATCC 21400) (Han and Srinivasan, 1968; Day and Withers, 1986), however the protein used for work in this thesis was obtained from the cloned *Agrobacterium* gene expressed in *E. coli* (strain JM109) (Wakarchuck et al., 1986). The following procedure

is based on the purification of the wild type enzyme and was carried out with technical assistance from Mr C.Sherwood.

β-Glucosidase activity was quantified by adding aliquots of the enzyme to a 1cm pathlength cuvette containing 1.0 mL of 1.5 mM βGlcPNP in 50 mM sodium phosphate buffer pH 6.8. The cuvette was equilibrated to 37°C in the spectrophotometer prior to addition of the sample. Optical density readings at a wavelength of 400nm were integrated over 15 second intervals (for a total time of 2 minutes) and the results averaged to give the final rate in absorbance units / minute. Total units of β-glucosidase activity in the original sample were calculated from the following equation:

$$U = \frac{(A_{400} / \text{min.})\ x.y}{7.28.z}$$

Where: U is the number of units of β-glucosidase activity; 1 unit being defined as the total amount of enzyme which is required to hydrolyse 1μmol of βGlcPNP in 1 minute at pH 6.8 and 37°C; A_{400} / min. is the average absorbance change at 400 nm in 1 minute; x is the total sample volume (mL); y is the volume in the cuvette (mL) ; z is the sample volume added to the cuvette (mL) and 7,280 M^{-1} cm^{-1} is the molar extinction coefficient of p-nitrophenol at pH 6.8 and 37°C.

Large scale preparation of the enzyme was carried out by growing 5 to 20 L fermenter cultures of *E. coli* strain JM109 in M9 minimal medium using 1% glycerol as the carbon source. The media also contained isopropylthio β-D-galactopyranoside (0.2 mM) and ampicillin (100mg L^{-1}). The cultures were grown at 37°C using an aeration rate of 6L / minute. Stock cultures of JM109 were stored at -20°C as a 50 % cell suspension in glycerol. These preparations were then used to inoculate 150 mL shake flask cultures in Luria broth (containing ampicillin at 100 mg L^{-1}) which were grown overnight prior to introduction into the fermenter. Cell growth was monitored by following optical density at 650 nm. β-Glucosidase activity was monitored by removing 1mL aliquots of the culture and disrupting

the cells by sonication using a Bronwill Biosonik 4 equipped with a micro probe and used on the high power setting. Three 30 second bursts interspersed with 1 minute cooling in ice-water were sufficient to cause complete lysis of the sample. Aliquots of the lysate were then assayed in 1ml of 1.5 mM βGlcPNP (50 mM sodium phosphate buffer pH 6.8) as described above. A time course for cell density and total β-glucosidase activity in a 5L fermenter culture is shown in Figure 6-1. To avoid the sharp decline in total activity which is seen in late log phase, cultures were harvested on attaining an optical density of 1.2. Cells were harvested in a Sorvall RC5B centrifuge (15 min, 5000 g) and the cell paste stored at -20°C until needed. All subsequent manipulations were carried out at 4°C except for the Pharmacia FPLC chromatography, which was done at room temperature. Cell extracts were prepared by grinding with 2.5 times their weight in alumina powder (Schleif and Wensink, 1981). The extraction buffer was 25 mM sodium phosphate pH 7.0, 10 mM 2-mercaptoethanol and the alumina pellet precipitated by centrifugation at 5,000g for 10 min. The pellet was resuspended twice in this buffer before discarding. Nucleic acids were removed from the crude extract by precipitation with 1.5% streptomycin sulphate (3 hours at 0°C) followed by centrifugation (20 minutes, 20,000 g). The clarified extract was then pumped onto a DE-52 column (22 cm x 5 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.0. This column was eluted with a 2 x 1L linear gradient of 0 to 1M sodium chloride in the starting buffer (see Figure 6-2). Fractions containing β-glucosidase were pooled and concentrated by ultrafiltration using a Millipore CX-30 membrane. The concentrated eluate was loaded onto a Sephadex S-200 column (55 cm x 3 cm) and eluted with 50 mM sodium phosphate buffer pH 7.0 (Figure 6-3). Fractions containing activity were pooled and then loaded onto a Pharmacia Mono Q anion exchange column that had been equilibrated in buffer A (See Figure 6-4). The column was eluted (see Figure 6-4) with a 15 mL gradient of 0.1 to 0.4 M sodium chloride, fractions containing the highest specific activity were pooled and stored at 4°C in the presence of 1 mM sodium azide. The final purification step gave a homogeneous preparation by SDS-PAGE, giving a monomer molecular weight for the pABG5 β-glucosidase of 50,000

and a specific activity (SA) of 186 U mg^{-1}. Yields, specific activities and purification factors for this protocol are given in Table 6-I.

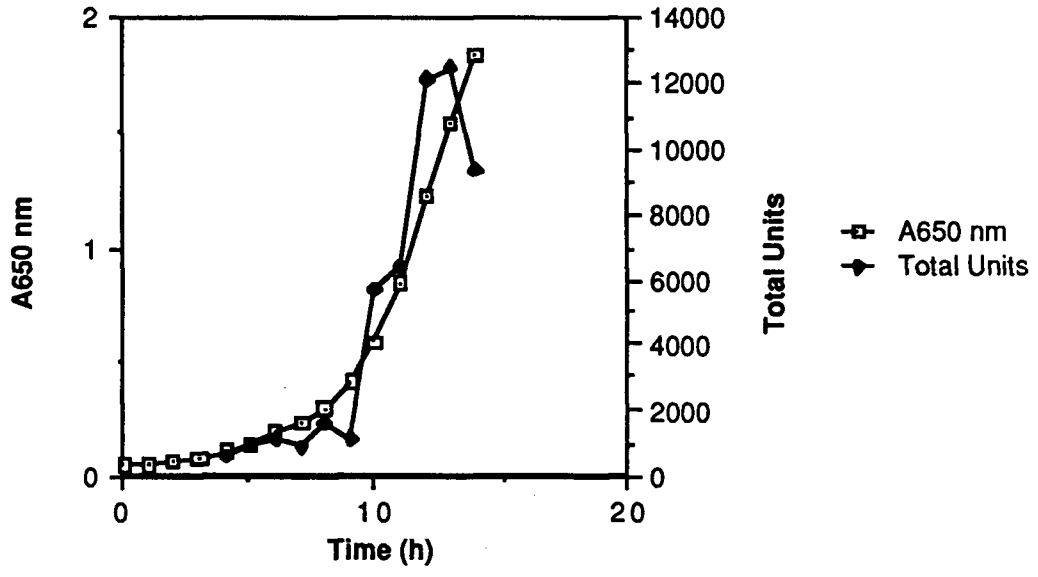


Figure 6-1. Variation of cell density and total β-glucosidase activity with time.

Table 6-I. Purification of pABG5 β-glucosidase from E.coli (JM109)[a].

Fraction	Total Units	Yield (%)	Specific Activity (U mg^{-1})
Crude Lysate	5,200	-	-
DE-52	4,100	78	45
S200	3,900	95	90
Mono Q	-	72	186

[a] Yields are an average of 3 separate purifications from 10L cultures of JM 109 grown on the M9 minimal media and harvested as described in text.

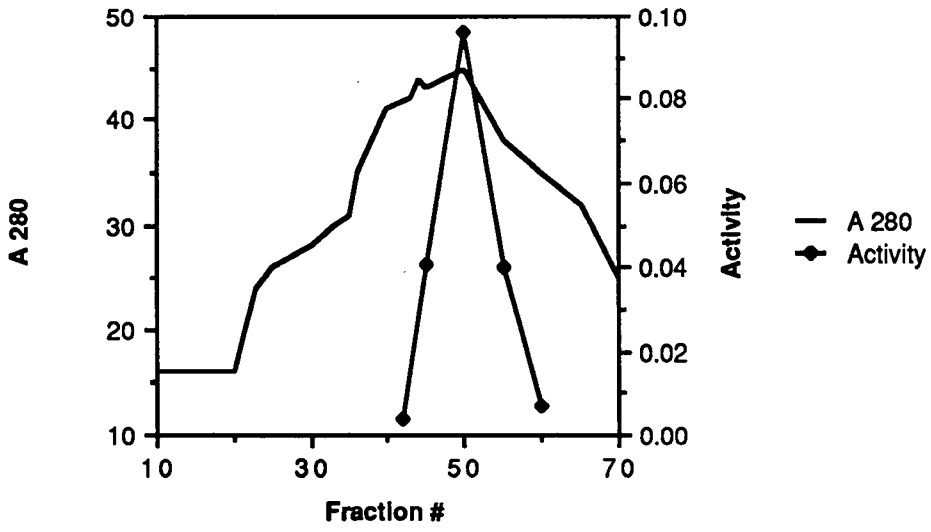


Figure 6-2. Elution profile for β-glucosidase pABG5 from DE52 anion exchange column.

Activity is given as the optical density change at 400 nm / minute in 1 mL of 1.5 mM βGlcPNP at 37°C and pH 6.8 for 10μL of a 50 fold dilution of the column eluate. Flow rate 60mL / hour, fraction volume 15 mL.

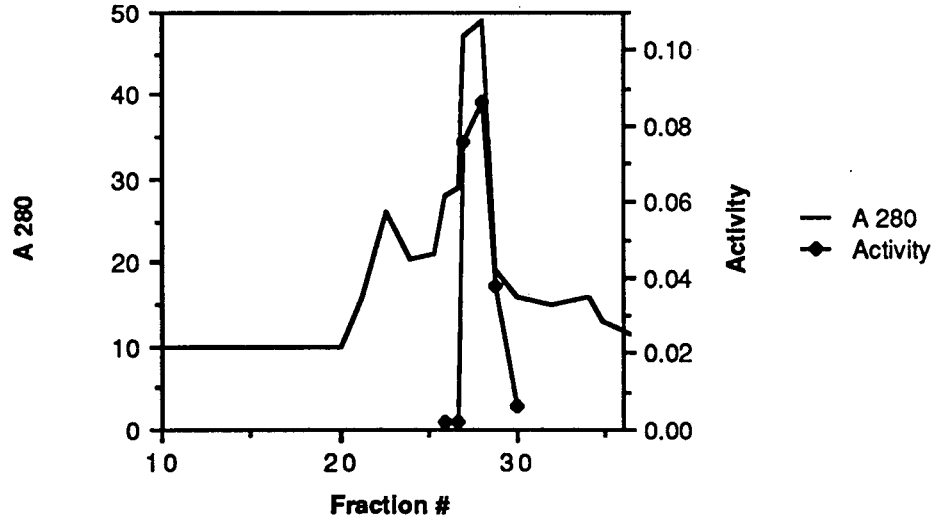


Figure 6-3. Elution of pABG5 β-glucosidase from Sephadex S-200.

Flow rate 20 mL / hour, fraction volume 5 mL. Activity is given in optical density change at 400 nm / minute in 1mL of 1.5 mM βGlcPNP at 37 °C and pH 6.8 for 5 μL of a 100 fold dilution of the column eluate.

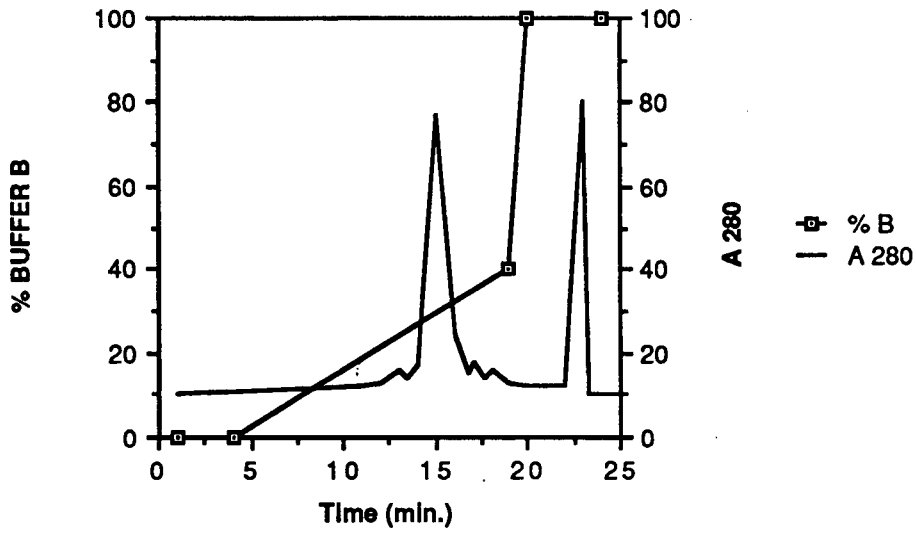


Figure 6-4 . Elution profile of β–glucosidase from Mono Q anion exchange column.

Buffer A: 50 mM sodium phosphate pH 6.8, Buffer B: 50 mM sodium phosphate pH 6.8 , 1M sodium chloride. Flow rate 1 mL/minute.

Determination of β-glucosidase concentration.

METHOD A) Quantitative amino acid analysis: aliquots of a homogeneous preparation of pABG5 β-glucosidase (0.5 mL, S.A. 186 U mg^{-1}) were dialysed against 4L of 10 mM ammonium bicarbonate buffer (pH 7.8) for 18 hours at 4°C. The protein was filtered through a 0.22 μm filter and an optical density of 0.571 at 280 nm recorded. A 300 μL aliquot was accurately measured and added directly to a hydrolysis tube. Norleucine (10 nmol, Sigma norleucine standard) along with 500 μL of ultra pure water was added to the sample and the mixture lyophilized. The lyophilisate was redissolved in 500 μL of ultra pure water and lyophilized again. This process was repeated twice more to remove ammonium bicarbonate from the sample. Hydrochloric acid (6N, analytical grade reagent) was added and the mixture degassed by freeze / thaw under vacuum. Hydrolysis was completed by heating *in vacuo* at 110°C for 24 hours. The sample was dried in a Speed Vac. and the pellet taken up in 75μL of citrate buffer (pH 2.2). This solution was filtered through a 0.45 μm filter and 20μL aliquots

used for the amino acid analysis. The results obtained from this analysis are given in Table 6-II. An absorbtivity index $E_{280}^{0.1\%}$= 2.20 $cm^{-1}$for optical density readings at 280 nm was calculated using the protein concentration determined in this analysis. Enzyme concentrations for all kinetic experiments were determined using this value and are based on a monomer molecular weight of 50,983 (Wakarchuck, W.W. (1987) Ph. D. thesis, University of British Columbia).

Table 6-II. Results from a quantitative amino acid analysis of pABG5 β-glucosidase.

Amino Acid	[a] nmol Detected	[b] nmol In Sample	[c] nmol Corrected	# RESIDUES DNA	Analysis	[d] nmol Protein.
ASP + ASN	20.707	77.651	90.777	51	55	1.780
THR	6.800	25.575	29.898	19	18	1.574
SER	5.649	21.184	24.765	16	15	1.548
GLU + GLN	14.945	56.078	65.558	34	40	1.928
PRO	9.286	34.823	40.710	26	24	1.566
GLY	15.666	58.748	68.679	43	42	1.597
ALA	19.818	74.318	86.881	53	53	1.640
VAL	9.244	34.655	40.513	30	25	1.350
MET	5.033	18.874	22.065	15	14	1.471
ILE	4.929	18.484	21.609	16	14	1.351
LEU	13.006	48.773	57.081	34	35	1.677
TYR	6.240	23.400	27.356	22	18	1.243
PHE	7.328	27.480	32.125	22	20	1.460
HIS	5.721	21.454	25.081	17	15	1.475
LYS	5.451	20.441	23.896	16	15	1.494
ARG	9.670	36.263	42.393	24	26	1.766
NORLEU	2.281	8.554	10.000	-	-	-

Average 1.560

[a] Total nmol detected in sample = $\frac{\text{nmol detected . total sample volume}}{\text{Injection volume}}$

[b] Loss correction = $\frac{\text{nmol of norleucine added to sample . total sample volume}}{\text{nmol of norleucine detected . injection volume}}$

[c] Number of residues = $\frac{\text{nmol of residue . number of ALA residues}}{\text{nmol of ALA residues}}$

[d] nmol of protein = $\frac{\text{nmol of residue}}{\text{number of residues (from DNA)}}$

METHOD B) 2,4-Dinitrophenolate burst: sodium phosphate buffer (50 mM, pH 6.8) containing pABG5 β-glucosidase was placed in a 1 cm pathlength glass cuvette and allowed to equilibrate to 37°C in the spectrophotometer. 2,4-Dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (twice the estimated enzyme concentration) was added and the optical density at 400 nm recorded. After corrections had been made for the added volume and the initial absorbance of the glucoside sample the enzyme concentration was calculated from the following equation.

$$\text{Concentration } (\mu \text{ mol}) = \frac{\text{A400 . Sample Vol.(mL)}}{11.3}$$

Where 11,300 M^{-1} cm^{-1} is the molar extinction coefficient of 2,4-dinitrophenolate at 37°C and pH 6.8.

Kinetic Experiments.

GENERAL PROCEDURES: all values of K_m and V_{max}, together with the errors associated with the scatter of the data, were calculated by fitting the data to the non-linear form of the Michaelis-Menten equation by the procedure of Wilkinson (1961). This procedure was carried out with the aid of a programme written for an Apple IIe computer (Street, I.P., M.Sc. Thesis 1985, University of British Columbia.) The data were plotted according to Lineweaver and Burke (1934), however because of the hazards associated with curve fitting to this type of double reciprocal plot (due to a non-linear error span) values for K_m and V_{max} were not determined by this method. These plots are included in this thesis because they have become a useful tool for recognizing deviations from linear behaviour caused by allostery or substrate inhibition as well as reversible inhibition type (competitive, non-competitive etc.). Approximate values for K_m and V_{max} (range finding experiments) were determined by measuring initial reaction rates over a wide range of substrate concentrations followed by treatment of the data using the computer-aided fitting procedure. Accurate values for these

constants were then determined by using 7 or 8 substrate concentrations usually in the range between 0.2 to 5 times the approximate K_m value.

Approximate values for inhibition constants (K_i) of reversible inhibitors were determined by measuring initial reaction rates at a single substrate concentration (usually equal to K_m) and 5 or 6 inhibitor concentrations and plotting the data according to Loeffler et al. (1979). The inhibition constants were then accurately determined by measuring initial reaction rates at 5 substrate concentrations in the absence of an inhibitor and at 4 inhibitor concentrations (25 data points in all). The data were then plotted according to Lineweaver and Burke in order to determine the inhibition pattern. The inhibition constant for competitive inhibitors was determined by plotting apparent K_m (determined by the computer-aided procedure) against inhibitor concentration. The best straight line was found by linear regression analysis for which the correlation coefficient (R) is reported. The standard error of estimate is reported as an absolute error of K_i. Kinetic experiments were performed by or with technical assistance from, Karen Rupitz.

K_m and V_{max} determination for deoxy- and deoxyfluoro-α-D-glucopyranosyl phosphates with glycogen phosphorylase *b*.

Initial reaction rates were determined in the direction of saccharide synthesis (Engers et al., 1970). Phosphate released was measured using the procedure of Baginski et al. (1967), but using 1.5 times the normal level of assay reagents. This ensured full colour development at high concentrations of phosphate. All substrates were used directly as their bis-cyclohexylammonium salts since K_m and V_{max} values for the bis-cyclohexylammonium and bis-potassium salts of α-D-glucopyranosyl phosphate were shown to be essentially identical. For stock solutions of these sugar phosphates concentrations were determined by quantifying the amount of inorganic phosphate released using the method of Fiske and SubbaRow (1925) after total hydrolysis in 5N hydrochloric acid (30 minutes at 100°C).

It was found that the reactivity of glycogen phosphorylase towards the different substrates varied greatly, and it was necessary to conduct initial range-finding experiments to determine optimum reaction time and enzyme concentration. During these experiments a single concentration of substrate (between 20 and 50 mM) was incubated with glycogen phosphorylase and at appropriate time intervals aliquots were withdrawn and assayed for phosphate released. The optimum reaction times and enzyme concentrations determined in this manner are given in Table 4-II.

All reactions were conducted in a buffer containing 20 mM glycerophosphate, 4mM DTT and 1 mM EDTA at pH 6.8 and 30 °C. The reaction mixtures also contained 1 mM AMP and 1% glycogen. both approximate and accurate values for K_m and V_{max} were then determined under these conditions. The range of substrate concentrations used in these experiments are also given in the legends to Figures 4-2 to 4-8. Control experiments were performed in which phosphorylase was incubated under standard assay conditions in the absence of substrate. These showed negligible losses in activity even over the longest incubation time.

pH Dependence of K_i for 1dαGlcCP and 2FαGlcP with glycogen phosphorylase *b*.

Approximate values for the inhibition constants of 2FαGlcP and 1dαGlcCP with glycogen phosphorylase *b* at different pH values were obtained by incubating enzyme (4.17 μg / mL) αGlcP (2.8 mM) and inhibitor at five different concentrations. For 2FαGlcP the following concentrations were used 1.10, 2.20, 3.30, 4.40 and 5.49 mM. For 1dαGlcCP the following concentrations were used 0.5, 0.99, 1.98, 2.97 and 4.95 mM. A sixth reaction was also run at each pH value, this contained substrate but no inhibitor. The reaction mixtures also contained 1 mM AMP and 1% glycogen and were incubated for 5 minutes at 37°C. The amount of phosphate released was quantified as described by Fiske and SubbaRow (1925).

A single buffer system was used to obtain pH values between 5.5 and 7.5. This buffer contained 50 mM triethanolamine, 100 mM potassium chloride, 1mM EDTA and 1 mM DTT and its initial pH value was obtained by addition of 2N potassium hydroxide. The final pH values of 5.40, 5.52, 5.73, 5.89, 6.09, 6.55, 6.83, 7.13 and 7.53 were recorded for mixtures made up in these buffers containing all of the reagents except glycogen phosphorylase. The following values of the equilibrium constants for the phosphorylase reaction in the direction of glycogen synthesis (XE) at various pH values were supplied by N.B. Madsen (personal communication) and were used in the calculation of initial reaction rates.

pH	XE
5.40	0.898
5.52	0.892
5.73	0.881
5.89	0.871
6.09	0.856
6.55	0.804
6.83	0.766
7.13	0.731
7.53	0.698

The values of K_m used in calculation of K_i were obtained from Withers et al. (1982b) and were used in calculation of the inhibitor constants.

Irreversible inhibition of glycosidases by 2-deoxy-2-fluoro-glycosides.

For irreversible inhibitors experiments were performed by incubating the enzyme in its buffer in the presence of inactivator. Aliquots (10 - 50 μL) were removed at appropriate time intervals and assayed for activity by dilution into a large volume (1 mL) of saturating concentrations of the corresponding substrate. For enzymes which exhibited a time dependent exponential decrease in activity, (pseudo first order inhibition) rate constants were determined by using a least squares regression analysis performed on an Apple IIe computer (Curve Fitter by P.K.Warne). Values for the dissociation constant of the non-covalent enzyme-inhibitor complex (K_d) and the rate constant for covalent bond formation (k_{on}) were

determined by computer fitting of the pseudo first order rate constants to the non-linear form of the Michaelis-Menten equation as for K_m and V_{max} determinations The data were also plotted according to Lineweaver and Burke.

The specific reaction conditions and inhibitor concentrations are detailed in the legends to the appropriate Figures in Chapter 2. Enzyme activity was determined in 50 mM sodium phosphate buffer pH 6.8 at 37°C containing 1.5 mM βGlcPNP and 1 mg mL^{-1} BSA. In the case of inactivation by 2FβGalF 3.0 mM βGalPNP was used as the assay substrate instead of the glucoside.

Reactivation of covalently inhibited glycosidases.

Inactivated enzyme was loaded onto a column of Sephadex G-25 (19.5 cm x 1 cm) and the column eluted with the appropriate buffer. Fractions containing protein were collected and assayed for the presence of any residual inhibitor by incubating small aliquots with fully active enzyme. The activity of these samples was checked at various times and provided they remained constant (or increased) the stock solution of enzyme was used for the following reactivation experiments. The activity of the fully reactivated enzyme sample was determined by a number of different methods; for rapid reactivation, the enzyme was incubated until a constant activity was attained over several assays. For experiments where reactivation was slow but large amounts of the inhibited enzyme were available, the end point activity was calculated from the ratio of the optical densities at 280 nm before and after gel permeation chromatography multiplied by the initial activity of the sample (prior to inhibition). For experiments where the reactivation was slow, and only small quantities of inhibited enzyme were available, or for samples that required BSA in their inhibition mix, the full activity was determined from the fraction of inhibition (prior to gel permeation chromatography) and the initial activity after chromatography according to the following equation.

$$\text{Full activity} = \frac{\text{Initial activity of sample}}{(1 - \text{Fraction inhibited})}$$

Samples of the inhibited enzyme were then incubated and assayed by incubating inhibited enzyme in 50 mM sodium phosphate buffer at pH 6.8 and 30°C in the presence of 1mg mL^{-1} BSA and any required transglycosylation acceptors. Aliquots were removed at appropriate time intervals and assayed for activity by addition to 1mL of 1.5mM βGlcPNP. Samples of 2FGal-pABG5 β-glucosidase were assayed by addition to 1mL of 3.0 mM βGalPNP. In all cases a time course for the return of activity followed simple first order (or pseudo first order) kinetics and rate constants for the reactivation process were calculated using the least squares regression analysis as detailed for the irreversible inhibition reactions. Where the reactivation experiments were conducted in the presence of a transglycosylation acceptors, values for the dissociation constant of the inhibited enzyme-substrate complex (Ks) and the rate constant for bond cleavage in the reactivation reaction (k_{trans}) were calculated by fitting the data to the non-linear form of the Michaelis-Menten equation using the procedure of Wilkinson (1961). The data were plotted according to Lineweaver and Burke. The reagent concentrations for these reactivation experiments are given in the legends of the appropriate Figures in Section 2. For enzyme intermediates with very slow reactivation rates control reactions to account for enzyme denaturation under the assay conditions were performed as follows: samples of the enzyme-intermediate were incubated in buffer and at appropriate time intervals aliqouts were removed and added to saturating concentrations of 1dβGlcϕ. The return of activity was monitored as described above. The amount of enzyme which had denatured under these incubation conditions was thus calculated as a function of the initial rate of reactivation and of the end-point activity of the sample taken at observed at the outset of the experiment.

K_m and k_{cat} determination for hydration of D-glucal by pABG5 β-glucosidase.

Determination of the kinetic constants for the hydration of D-glucal was performed by measuring the initial rates of 2-deoxy-D-glucose production using a hexokinase/glucose 6-phosphate dehydrogenase coupled assay system.

Preparation of standard curve: reaction mixtures contained 2-deoxy-D-glucose in 0.375 mL of 100mM triethanolamine buffer (pH 7.6) and 0.125 mL of the following assay mix (quantities are per mL fianl volume)

0.2 mL of 12 mM ATP (in water)
0.2 mL of 7.0 mM NADP (in water)
0.2 mL of 20 mM Magnesium chloride (in water)
0.3 mL of 100mM triethanolamine pH 7.6
60 units of Glucose 6-phosphate dehydrogenase
100 units of Hexokinase

The mixtures were incubated for 1 hour at 30°C, after this time the optical density at 340 nm had reached a steady value which did not change significantly in the next 20 minutes. The standard curve was linear within the range tested (R = 1.00) and calculation of 2-deoxy-D-glucose concentration using the molar extinction coefficient of NADPH at 340 nm (6220 M^{-1} cm^{-1}) indicated quantitative production of NADPH during the oxidation of 2-deoxy-D-glucose.

Initial rates for D-glucal hydration by pABG5 β-glucosidase were determined by incubating the enzyme (0.114 nmol) in 0.1 mL of 50 mM sodium phosphate buffer containing 1 mg mL^{-1} BSA and D-glucal (0.1-1.5 mM) for 2 hours at 37°C. The reactions were stopped by heating to 100°C for 1 minute. After they had cooled to room temperature, 0.275 mL of 100 mM triethanolamine buffer and 0.125 mL of assay mix were added and the mixture incubated for 1 hour at 30°C. The optical densities at 340 nm were recorded and values for k_{app} (the apparent rate constant for D-glucal hydration) calculated from the following equation:

$$k_{app} = \frac{[\text{2-deoxy-D-glucose}]}{t.\ e_o}$$

Where 2-deoxy-D-glucose concentration is given in μmole per assay volume; t is in minutes; e_o is the enzyme concentration in μmol per assay volume. Values for K_m and k_{cat} were determined by fitting the data to the non-linear form of the Michaelis Menten equation using the procedure of Wilkinson (1961).

Determination of the stereochemistry of D-glucal hydration by pABG5 β-glucosidase.

Deuterated 50 mM sodium phosphate buffer (pH 6.8) was prepared by lyophilization of 50 mL of normal (protiated) buffer and redissolving the remaining salts in the same volume of D_2O. Samples of pABG5 β-glucosidase in deuterated buffer were prepared by diluting 31 μL of the enzyme stock solution (8.55 mg mL^{-1}, 121 U mg^{-1}) into 2 mL of the deuterated phosphate buffer. This solution was concentrated using a Centricon micro-concentrator (30,000 Daltons molecular weight cut off) to the dead-stop volume (50μL), the volume was then made up to 100 μL giving a final enzyme concentration of 5.2 μM. The following samples were made up in deuterated phosphate buffer (final volume 0.5 mL) and left to incubate for 3 days at 30°C. After the incubation was complete the samples were lyophilized and then redissolved in 0.6 mL of D_2O. The proton NMR spectrum (400 MHz) of each sample was then recorded. The samples contained the following:

Sample A.
10μL D-glucal (100 mM in D_2O)
490μL Deuterated phosphate buffer

Sample B
50μL Enzyme
10μL 2-deoxy-D-glucose (100 mM in D_2O)
440μL Deuterated phosphate buffer.

Sample C.
50 μL Enzyme.
10μL D-glucal (100mM in D_2O)
440μL Deuterated phosphate buffer.

^{19}F-NMR determination of substrate activity for deoxyfluoro-α–D–glucopyranosyl phosphates with glycogen phosphorylase.

Spectra were recorded on a Bruker HXS-270 spectrometer operating at 254 MHz in the Fourier-transform mode with a 5 mm ^{19}F probe. A spectral width of 10,000 Hz was used , with a 50-70° pulse angle (7-10 μs) and a repetition time of 100ms. All experiments were conducted with broad band proton decoupling. Chemical shifts are quoted relative to $CFCl_3$ (δ =0.00 ppm) and were measured with reference to external hexafluorobenzene (δ = 161.90 ppm).

Reaction mixtures (0.7 mL) contained 5.0 mM substrate, 0.5% glycogen, 1mM AMP, 12.6 mg of glycogen phosphorylase *b* and 0.2 mL of D_2O. All reactions were conducted at pH 6.8 in a buffer containing 50 mM triethanolamine, 100 mM potassium chloride, 1mM EDTA and 1 mM DTT. For 2FαGlcP the reaction was run under similar conditions except that 4.5 mg of phosphorylase a and 36 mM maltopentaose were used in place of phosphorylase *b* and glycogen. For control reactions which were conducted in the absence of AMP, an AMP-free preparation of glycogen phosphorylase *b* was prepared by treatment with Norit A pharmaceutical grade decolourising carbon as detailed by Withers (1979). All reaction mixture were incubated for 12-48 hours prior to data acquisition.

^{19}F-NMR; reaction of 2-deoxyfluoro-β-glycosyl fluorides with pABG5 β-glucosidase.

Spectra were recorded on a Bruker HXS-270 spectrometer operating at 254 MHz in the Fourier-transform mode with a 5 mm ^{19}F probe. A spectral width of 40,000 Hz was used , with a 90° pulse angle (15μs). Experiments were performed using gated proton decoupling (decoupler on during acquisition) with a repetition delay of 2 seconds. Chemical shifts are quoted relative to $CFCl_3$ (δ = 0.00 ppm) and were measured with reference to an internal sample of 6-deoxyfluoro-α-D-glucopyranosyl phosphate (δ = 237.48 ppm). Exponential line broadening used prior to Fourier transform was generally 20 Hz, and all line width data have been corrected for this.

Protein samples were prepared by concentrating pABG5 β-glucosidase (S.A. 100 U mg^{-1}) in a Centricon micro-concentrator (30,000 Daltons molecular weight cut-off) until the sample volume had been reduced to approximately 1 mL. The sample was then diluted 2 fold with deuterated buffer and reconcentrated to a final volume of less than 0.4 mL. Paramagnetic impurities were removed from deuterated water by pre-treatment with Chelex. The final enzyme concentration was determined using the dinitrophenolate burst (Method B) after dilution of a small aliquot (5-10 μL) into 0.5 mL of 50 mM sodium phosphate buffer pH 6.8. Solutions of effectors (pH adjusted to 6.8) were added directly to make up the following samples (final volume 0.4 mL). Data acquisition was performed at a probe temperature of 20°C. The sample of 2FMan-pABG5 β-glucosidase was denatured by dialysis against 2 x 250 mL of 8M urea for 24 hours. The protein was then diluted 2 fold with 8 M urea in deuterated water and concentrated to a final volume of 0.5 mL using a Centricon micro-concentrator. The concentrations of substrates and enzymes are detailed in the legends to Figure 2-13, 2-14 and 2-15.

Purification of transfer product.

Sample containing the transfer product from 2FGlc-pABG5 and 1dαGlcϕ was transferred to a Centricon micro-concentrator (molecular weight cut-off 30,000 Daltons) and diluted to 2 mL with sodium phosphate buffer. The volume was reduced to 0.5 mL by centrifugation (3,000g), the fraction containing the low molecular weight components removed and the protein-containing fraction diluted to 2 mL. This process was repeated a further four times and then the low molecular weight fractions pooled and lyophilized. This sample was desalted and partially purified on a column of Biogel P-2 (1.5 cm x 84 cm) (see Figure 6-6). Fractions containing fluorinated glycoside were pooled, lyophilized and acetylated in 0.2 mL of dry pyridine by slow addition of 0.1 mL of acetic anhydride. The reaction was left overnight at room temperature and then quenched by addition of 1 mL of methanol. After 2 hours solvents were removed *in vacuo* and the residue examined by thin layer

chromatography (ethyl acetate, hexane 1:1). The chromatogram showed two components (Rf 0.63 and 0.42), the major component (Rf 0.63) co-migrated with an authentic sample of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl benzene. The remainder of the product mixture was chromatographed on a small column of silica gel (ethyl acetate, hexane 1:1) to give the pure minor component from the mixture.

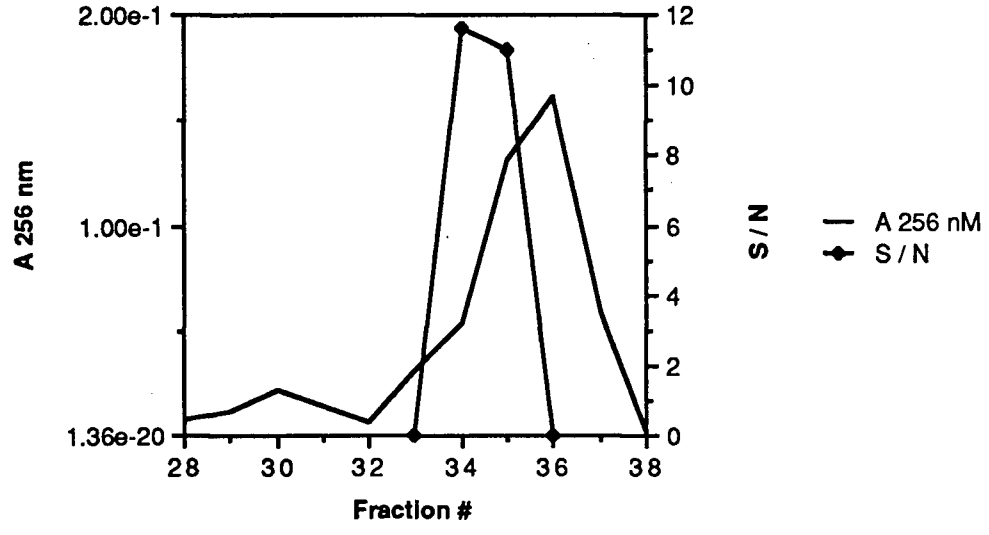


Figure 6-5. The elution profile of the low molecular weight fraction of Sample 2 from Biogel P-2 (mono and disaccharide region).

Sugars containing a glucosyl benzene moiety were located by monitoring the optical density of the column eluate at 256 nm. Fractions containing fluorinated saccharides were located by ^{19}F-NMR. S /N is the signal to noise ratio for 50,000 transients of a 0.5 mL sample from the appropriate fraction. The column was run in deionised water with a flow rate of 7.8 mL / hour, 4 mL fractions were collected.

^{19}F-NMR: 2FαGlcP with phosphorylase *b*.

Spectra were recorded on a Bruker HXS-270 spectrometer operating at 254 MHz in the Fourier-transform mode with a 5 mm ^{19}F probe. For these experiments a spectral width of 10,000 Hz was used. Experiments were conducted using either broad band proton decoupling or gated proton decoupling (decoupler on during acquisition). For continuous decoupling a pulse angle of 50-70° (7-10ms) was used with a repetition time of 100 ms. For

experiments using the gated decoupling sequence a 90° pulse angle (15ms) and a repetition delay of 3s were required. Chemical shifts are quoted relative to $CFCl_3$ (δ=0.00 ppm) and were measured with reference to external hexafluorobenzene (δ= 162.90 ppm). Deuterated water contained in the sample was used for the field / frequency lock and the external chemical shift reference was contained in a capillary tube which was inserted into the sample for referencing. Exponential line broadening used prior to Fourier transform was generally 20 Hz, and all line width data have been corrected for this.

Sample size was 0.5 mL in a 5 mm tube with enzyme concentrations between 0.6 and 0.68 mM calculated for the phosphorylase monomer molecular weight of 97,412. Glycogen phosphorylase *b* was prepared for these samples by dissolving a pelleted (10,000g, 10 min.) crystalline suspension of the enzyme in a buffer containing 50 mM triethanolamine, 100 mM potassium chloride,1 mM EDTA and 1 mM DTT. at pH 6.8. This solution was then dialysed against 1L of the same buffer (room temperature to 4°C) The enzyme was diluted by 2 fold with the same buffer made up in deuterated water and concentrated at room temperature using a Millipore ultra-filtration membrane (CX-30) to a final volume of 0.4 mL. The D_2O used for the preparation of the deuterated buffers had been freed from paramagnetic impurities by pre-treatment with Chelex. Solutions of effectors (pH adjusted to 6.8) were added directly to the enzyme solution to give the samples detailed in the legend to Figure 4-18. The stock solution of 2FGlcP was composed of approximately 80% of the α-anomer of the sugar phosphate, the remaining 20% being the β–anomer. All subsequent calculations were thus corrected for this anomeric composition.

^{31}P- NMR titration of 1dαGlcCP

Spectra were recorded on a Bruker HXS-270 spectrometer operating at 109.29 MHz in the Fourier-transform mode with a 10mm ^{31}P probe. A spectral width of 6,000 Hz was used, with a 50-70° pulse angle (15-20 ms) and a repetition time of 500ms. Chemical shifts are quoted relative to 85% phosphoric acid (δ= 0.00 ppm) and were measured with reference to a

100 mM sample of β–glycerophosphate (δ= 4.751 ppm) contained in a 1mm capillary tube which was inserted into the sample during acquisition.

A 1.5 mL sample of 20 mM 1dαGlcCP dissolved in 100 mM potassium chloride in 50% deuterated water was used for this experiment. This solution was titrated over a wide range of pH values (0.48 to 10.70) by adding small aliquots (1-2 μL) of 2 M potassium hydroxide, or 3M hydrochloric acid. The pH was recorded after each addition (by meter reading).and ^{31}P-NMR data acquired. A probe temperature of 28°C was used.

^{31}P-NMR: 1dαGlcCP with glycogen phosphorylase *b*.

These experiments were performed on a Bruker HXS-270 spectrometer operating at 109.29 MHz in the Fourier-transform mode with a 10mm ^{31}P probe. A spectral width of 6,000 Hz was used , with a 50-70° pulse angle (15-20 ms) and a repetition time of 2s. Chemical shifts are quoted relative to 85% phosphoric acid (δ= 0.00 ppm) and were measured with reference to this compound contained in a 1mm capillary tube which was inserted into the sample The D_2O present in the sample was used for the field / frequency lock during acquisition. Exponential line broadening used prior to Fourier transform was 20 Hz, and all line width data have been corrected for this.

Phosphorylase *b* was freed from AMP as described by Withers et al. (1979) and was prepared in a 50% deuterated buffer containing 50 mM triethanolamine, 100 mM potassium chloride, 1 mM EDTA and 1mM DTT at pH 6.8. Sample volume was 1.4 mL to 1.6 mL and where necessary enzyme preparations were concentrated using a Millipore CX-30 immersible concentrator. Solutions of effectors (pH adjusted to 6.8) were added directly to the enzyme solution to give the samples detailed in the legend to Figure 4-15 and in Table 4-III.

β-Glucosides as Anti-Fungal agents.

All media were sterilized in an autoclave at 120°C for 20 minutes. Solutions of βGlcPNP (obtained from Sigma chemical Co.) were sterilized by passage though a 0.22 μm Millipore filter.

The mineral salts mixture contained the following per litre final volume.

Ammonium sulphate	0.5g
Potassium dihydrogen phosphate	1.0g
Potassium chloride	0.5g
Magnesium sulphate (crystalline)	0.2g
Calcium chloride (hydrate)	0.1g
L-Asparagine	0.5g
Difco yeast extract	0.5g
Difco Agar	20.0g

The volume of the mineral salts was made up to 900 mL with distilled water and the pH adjusted to 6.2 prior to addition of the carbon source. Cellulose suspension was prepared by grinding 100 g of Whatman cellulose powder in a ball mill for 3 days. The powder was suspended in 1L of distilled water and stored at 4°C.The cellulose medium was prepared by adding 100 mL of this suspension to 900 mL of the mineral salts mixture prior to sterilization. Maltose media contained 2% by weight of maltose per litre of mineral salts mixture.

Experiments were conducted in specially modified Petri dishes and all manipulations were performed under sterile conditions. The agar medium was poured into the centre of the dish to a depth of approximately 1-2 mm below the lip of the wells and allowed to cool. A sterile βGlcPNP stock solution was then diluted appropriately with molten (≈ 45°C) agar medium to a final volume of 1 mL and quickly transferred to a well where it was allowed to solidify. These plates were inoculated by introducing a plug from an inoculum culture into the centre of the plate. Cultures used to inoculate these experiments were grown on 1.5% malt agar and were used before sporulation had occurred.

Toxicity limits for βGlcPNP: a sterile stock solution βGlcPNP (10.0 mg mL^{-1}) was diluted to give the following concentrations: 1000, 500, 250, 100 μg mL^{-1}. A control containing no βGlcPNP was also provided in every dish. After inoculation with fungal cultures, these samples were incubated at 20°C for 14 to 25 days.The following organisms were used:*T. reesei, C. versicolor, C. globosum, P. chrysosporium* and *C. puteana.*

Weight loss from doweling: samples of ponderosa pine and aspen doweling were numbered for future identification and heated to 110°C for 18 hours. Dry weights were recorded after samples had cooled to room temperature in a desiccator. These samples were sterilized by gamma radiation (27 Kgy) and sterile conditions maintained while they were soaked for 2 minutes in sterile stock solutions of βGlcPNP at the following concentrations: 1,500, 500, 170, 50 μg mL^{-1}. Control samples were soaked in water and all samples were dried for 24 hours at room temperature prior to introduction into the modified petri dishes. The average uptake of βGlcPNP per doweling sample was calculated from the average volume of solution absorbed per sample multiplied by the concentration of the stock solution. Cellulose / agar medium was used in the remainder of the dish and the experiments inoculated with the appropriate fungal culture after the plates had solidified and cooled. All experiments were performed in triplicate and the following organisms were used: *T. reesei, T. harzianum, C. versicolor, C. globosum, P. chrysosporium* and *C. puteana.* After 6 and 10 weeks incubation, excess fungi were removed from the doweling samples and dry weight recorded after drying for 18 hours at 110°C as described before. The weight losses recorded for these samples are presented in Table 6-III.

Table 6-III. Weight losses produced by various cellulolytic organism.

Organism	Sample	βGlcPNP (µg / sample)	Initial Dry weight (g)	Final Dry weight 6weeks	Final Dry weight 10 weeks
C. puteana	P.Pine	0	0.339	0.265	0.159
		20.8	0.322	0.256	0.145
		70.8	0.338	0.630	0.157
		208.0	0.301	0.175	0.129
		625.0	0.350	0.283	0.202
P.chrysosporium	P.Pine	0	0.302	0.295	0.247
		20.8	0.328	0.300	0.293
		70.8	0.339	0.344	0.282
		208.0	0.375	0.366	0.283
		625.0	0.315	0.344	0.290
P.chrysosporium	Aspen	0	0.336	0.245	0.248
		20.8	0.356	0.312	0.267
		70.8	0.340	0.273	0.259
		208.0	0.337	0.337	0.235
		635.0	0.346	0.299	0.248
C. globosum	Aspen	0	0.339	0.337	0.108
		20.8	0.315	0.326	0.276
		70.8	0.354	0.346	0.304
		208.0	0.359	0.344	0.273
		635.0	0.332	0.298	0.300
T. harzianum	P.Pine	0	0.356	0.364	0.353
		20.8	0.331	0.302	0.341
		70.8	0.310	0.285	0.333
		208.0	0.338	0.355	0.324
		625.0	0.364	0.378	0.354
T.reesei	P.Pine	0	0.375	0.378	0.364
		20.8	0.315	0.284	0.321
		70.8	0.360	0.353	0.357
		208.0	0.368	0.366	0.355
		635.0	0.311	0.304	0.300

Initial dry weight is average of three samples per concentration per organism. Final dry weight: at 6 weeks is from 1 sample per concentration, at 10 weeks average of 2 samples per concentration per organism.Average uptake of βGlcPNP per sample as described in text. All incubations were at 20°C.

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