MECHANISTIC STUDIES OF FAMILY 4 GLYCOSIDASES GlvA AND BGlu

By

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We accept this thesis as conforming
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ABSTRACT

The glycosidases are a group of enzymes that can hydrolyze the O-glycosidic bond between two or more carbohydrates or between a carbohydrate moiety and a non-carbohydrate moiety. Based on their substrate specificities, this group of enzymes was classified into the enzyme classification scheme as EC 3.2.1.-. In an alternative enzyme scheme based on sequence similarity, the glycosidases have been assigned to 87 families at the most recent count. The family 4 glycoside hydrolases is a unique family, not only because the enzymes in this family all require NAD and divalent metal ion as cofactors, but also because this family contains a wide range of substrate specificities. As a consequence the catalytic mechanisms and different substrate specificities are of considerable interest.

Two enzymes in this family, GlvA from *Bacillus subtilis* 168 and Bglu from *Bacillus halodurans* are the focus of this study. The substrates of GlvA are 6-phospho-α-glucosides and the substrates of Bglu are 6-phospho-β-glucosides. NMR analysis of the cleavage of 4'-nitrophenyl β-D-glucopyranoside-6-phosphate by Bglu in the presence of 3 M methanol revealed that the product formed is the methyl-β-glucoside; indicating that Bglu is a retaining glycosidase. This is the first determination of stereochemical outcome for an enzyme in family 4.

Hydrolysis of 4'-nitrophenyl β-D-glucopyranoside-6-phosphate by Bglu in deuterium oxide exclusively yields [2-²H]-glucose-6-phosphate, thus the hydrogen at the 2 position is substituted by deuterium during cleavage. Further, reaction of Bglu with 2-deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride did not result in trapping of a glycosyl-enzyme intermediate. Based on these results, a novel mechanism was proposed. In order
to further study the proposed mechanism, some isotopically labeled substrates for GlvA were synthesized for kinetic isotope effect (KIE) measurements, along with samples of the natural substrate, maltose-6'-phosphate.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Abg</td>
<td><em>Agrobacterium</em> sp. β-glucosidase</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>Asp, D</td>
<td>aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethyl formamide</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethyl sulfide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxy ribonucleic acid</td>
</tr>
<tr>
<td>DNP</td>
<td>dinitrophenol</td>
</tr>
<tr>
<td>E+</td>
<td>electrophile</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine</td>
</tr>
<tr>
<td></td>
<td>tetraacetic acid</td>
</tr>
<tr>
<td>FDNB</td>
<td>fluoro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>Glu, E</td>
<td>glutamate</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>KIE</td>
<td>kinetic isotope effects</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>Nu</td>
<td>nucleophile</td>
</tr>
<tr>
<td>ORFs</td>
<td>open reading frames</td>
</tr>
<tr>
<td>PDCA</td>
<td>pyridinium dichromate-acetic anhydride</td>
</tr>
<tr>
<td>PEP-PTS</td>
<td>phosphoenol pyruvate-dependent sugar</td>
</tr>
<tr>
<td>PNP</td>
<td>4-nitrophenol</td>
</tr>
<tr>
<td>Py</td>
<td>pyridine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate/polyacrylamide-gel electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetimidate</td>
</tr>
<tr>
<td>tlc</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultra-violet, visible</td>
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</table>
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For my parents
Chapter I Introduction

1.1 Glycosidases

Carbohydrates have long been known as the major energy reserve of most cells and also play an important role in the cellular structure of organisms. However, another subtler role is now becoming apparent. Because of the variety of stereoisomeric forms of monosaccharide moieties and glycosidic combinations possible, carbohydrates can contain much more information than protein and nucleotide molecules. Indeed, carbohydrates are known to be involved in a wide range of biological processes such as cell to cell recognition, fertilization, embryogenesis, neuron development, hormone activities, the proliferation of cells and their organization into specific tissues, viral and bacterial infection and tumor cell metastasis.

In light of the importance of the biological role of carbohydrates, it is not surprising that enzymes that degrade, modify, or create glycosidic bonds are becoming important subjects of research. Among all the carbohydrate active enzymes, the glycoside hydrolases or glycosidases are of particular interest to biochemists.

\( O \)-Glycoside hydrolases (EC 3.2.1.-) are enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. (Figure 1.1)

Glycosidases can be classified functionally according to three different criteria:

i) Stereochemistry of the substrate at the anomeric center: \( \alpha \)-glycosidases hydrolyze glycosides with \( \alpha \)-glycosidic linkages and \( \beta \)-glycosidases hydrolyze substrates having \( \beta \)-glycosidic linkages;
Figure 1.1 Reaction catalyzed by glycosidases.

ii) Stereochemical outcome of the reaction: if the stereochemistry at the anomeric center of the product is the same as in the substrate, the glycosidase is said to be retaining; if not, the glycosidase is said to be inverting;

iii) Glycone specificity: because of the variety of stereochemistries of the glycone, glycosidases will have different interactions between the enzyme and the glycone portion of the substrate. Each glycosidase will have a preferred substrate with which they exhibit optimum activity. For example, a 6-phospho-glucosidase will prefer a 6-phospho-glucoside as the substrate, although a 6-phospho-galactoside may also be a substrate.

In an alternative approach, Henrissat and co-workers classified glycosidases into families based on amino acid sequence similarities. This classification method reflects the structural features of these enzymes and makes a connection between enzyme structure and function. Within each family, the catalytic mechanism will be similar due to similar structure, and the study of a representative enzyme member in each family will provide more general information applicable to other members of the family. Over the years, the number of families of glycosidase has grown steadily and the most updated
information about the glycosidase families and other carbohydrate active enzymes can be found in an excellent web site: http://afmb.cnrs-mrs.fr/CAZY/index.html. In the most recent count (June 2002) from this website, the number of glycosidase families had already increased to 87. Because the 3D structure of a protein may be more conserved than its amino acid sequence, some of the sequence-based families have related structures, thus, those families are grouped together. This grouping of the structures has been denoted “clans”5. So far, 12 such clans have been described in the webpage listed above.

1.1.1 General mechanism for glycosidases

There are two possible stereochemical outcomes of the glucosidase catalyzed hydrolysis reaction: retention or inversion of the anomeric configuration. In inverting glycosidases, the two catalytic carboxylic acids are approximately 10 Å apart 6,7. The reaction involves a single displacement mechanism with one of the carboxylic acids acting as the general base and the other as the general acid (Figure 1.2a). In retaining glycosidases, the two catalytic carboxylic acids are located approximately 5.5 Å apart 6,7. The reaction catalyzed by retaining glycosidases involves a double displacement mechanism in which the stereochemistry at the anomeric center is inverted twice, resulting in net retention of stereochemistry. In the first glycosylation step, a nucleophilic group in the enzyme active site (an Asp or Glu residue), attacks the anomeric center
Figure 1.2 General glycosidase mechanisms for (a) an inverting \( \beta \)-glycosidase and (b) a retaining \( \beta \)-glycosidase.
displacing the aglycone and forming a covalent glycosyl-enzyme intermediate. In the second deglycosylation step, nucleophilic water attacks the anomeric center displacing the enzyme nucleophile to regenerate the enzyme and produce the free sugar (Figure 1.2b). In general, both mechanisms involve transition states with oxocarbenium ion-like character and a pair of catalytic carboxylic acids at the active site.

1.1.2 Approaches for probing the mechanism of glycosidases

In studying the catalytic mechanisms of glycosidases, some general approaches have been successfully used in previous research.

1.1.2.1 Rate-Determining steps

In the double displacement mechanism of the retaining glycosidase, in order to fully characterize each step, a series of aryl glycoside substrates of differing phenol leaving group can be used to determine which step in the mechanism is the rate-determining step. This method was successfully used in the study of almond β-glucosidase\(^8\), \textit{Escherichia coli} β-galactosidase\(^9\), and \textit{Agrobacterium sp} β-glucosidase (Abg)\(^10\). In the case of Abg, a plot of log \(k_{\text{cat}}\) values for the hydrolysis of a series of aryl glucoside substrates vs. pK\(_a\) of the aglycone phenol produced a concave-downward, biphasic Brønsted relationship (Table 1.1 and Figure 1.3). This curve indicates that there is a change of rate determining step when the aglycone leaving group ability increases.
Table 1.1 The $k_{cat}$ values for the hydrolysis of aryl glucosides by *Agrobacterium sp* β-glucosidase (Abg)\(^\text{10}\).

<table>
<thead>
<tr>
<th>Phenol substituent</th>
<th>pKa</th>
<th>$k_{cat}$ (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dinitro</td>
<td>3.96</td>
<td>87.9</td>
</tr>
<tr>
<td>2,5-dinitro</td>
<td>5.15</td>
<td>120</td>
</tr>
<tr>
<td>3,4-dinitro</td>
<td>5.36</td>
<td>185</td>
</tr>
<tr>
<td>2,4,6-trichloro</td>
<td>6.39</td>
<td>240</td>
</tr>
<tr>
<td>4-chloro-2-nitro</td>
<td>6.45</td>
<td>144</td>
</tr>
<tr>
<td>4-nitro</td>
<td>7.18</td>
<td>169</td>
</tr>
<tr>
<td>2-nitro</td>
<td>7.22</td>
<td>111</td>
</tr>
<tr>
<td>3,5-dichloro</td>
<td>8.19</td>
<td>159</td>
</tr>
<tr>
<td>3-nitro</td>
<td>8.39</td>
<td>108</td>
</tr>
<tr>
<td>4-cyano</td>
<td>8.49</td>
<td>129</td>
</tr>
<tr>
<td>4-bromo</td>
<td>9.34</td>
<td>28.8</td>
</tr>
<tr>
<td>4-chloro</td>
<td>9.38</td>
<td>29.6</td>
</tr>
<tr>
<td>2-naphthyl</td>
<td>9.51</td>
<td>25.3</td>
</tr>
<tr>
<td>H</td>
<td>9.99</td>
<td>5.44</td>
</tr>
<tr>
<td>4-tert-butyl</td>
<td>10.37</td>
<td>5.13</td>
</tr>
</tbody>
</table>

Figure 1.3 Bronsted relationship for hydrolysis of a series of aryl β-glucosides with *Agrobacterium sp* β-glucosidase\(^\text{10}\).
For substrates to the right of the break, the glycosylation step is rate-determining since the k\textsubscript{cat} of the hydrolysis reaction is dependent on the p\textsubscript{Ka} of the aglycone leaving group. To the left of the break, the k\textsubscript{cat} value is independent of the structure of the leaving group, indicating that the rate-determining step is some other step.

1.1.2.2 Non-covalent interactions

Non-covalent enzyme/substrate interactions play very important roles in catalysis by most enzymes. Because the substrates of glycosidases are multi-hydroxylated carbohydrates, hydrogen–bonding interactions likely contribute most of the noncovalent interactions. To probe the importance of the hydrogen-bonding interactions at each of the sugar hydroxyl groups along each step of the reaction coordinate, a series of substrate analogues in which the individual hydroxyl groups on the glycone have been replaced by hydrogen or by fluorine was synthesized and kinetic parameters for each step of the mechanism determined. The basis of this approach is that replacement of a sugar hydroxyl by hydrogen will completely remove all of the hydrogen-bonding at that position while the replacement of hydroxyl by fluorine will only allow hydrogen bonds in which the fluorine acts as an acceptor. In the case of Abg, substrate analogues with the very good 2,4-dinitrophenol leaving group were used in detailed kinetic studies. The use of a good leaving group makes it probable that the deglycosylation step will be the rate-limiting step in each case, thus pre-steady state and steady-state kinetic parameters for each substrate allowed the contribution of each hydroxyl group to be evaluated at both the glycosylation and deglycosylation transition states by interpreting the change in rate constants in terms of free energy changes ($\Delta G^* = -RT \ln \frac{k_1}{k_2}$).
1.1.2.3 Kinetic isotope effects

Measurements of reaction rates for substrates in which deuterium has been incorporated at the reaction center have proven to be very valuable in the study of reaction mechanisms. Isotopic substitution does not change the qualitative chemical reactivity of the substrate, but it can often change the rate of the reaction. The zero-point energy of the C-D bond is less than that of the C-H bond due to the greater mass of deuterium. Therefore, the substitution of protium by deuterium will lower the zero-point energy of a molecule. In a reaction that involves cleavage of a C-H or C-D bond, the transition state will have approximately the same energy for the protonated and the deuterated species, thus the deuterated molecule will have higher activation energy to reach the transition state. The reaction rate of the deuterated molecule will therefore be lower than that of the protium counterpart. This is known as a primary kinetic isotope effect (primary KIE). The theoretical maximum for a primary kinetic isotope effect $k_{H}/k_{D}$ is about 7 at room temperature$^{12,13}$.

![Diagram showing differing zero-point energies of protium- and deuterium-substituted molecules.](image)

**Figure 1.4** Differing zero-point energies of protium- and deuterium-substituted molecules.
Isotope effects may be observed even when the substituted hydrogen atom is not directly involved in the reaction. Such effects are called secondary kinetic isotope effects. They are the result of a tightening or loosening of the C-H bond at the transition state. As reaction occurs, the strength of the bond may change because of a hybridization change or a change in the extent of hyperconjugation. If $sp^3$ hybridized carbon is converted to $sp^2$, a hydrogen bound to the carbon will be more free to C-H bending, since the C-H bond is slightly longer than the C-D bond, the freeing is larger for C-H bond than C-D bond thus makes the difference in vibrational energy between the C-H bond and the C-D bond in the transition state less than in the ground state and then $\Delta G^e_H < \Delta G^e_D$. Secondary kinetic isotope effects are smaller than primary ones and lie in the range of $k_H/k_D = 0.7-1.5$. They are also classified as $\alpha$, $\beta$, etc., depending on whether the isotopic substitution is on the reaction carbon or further away. Secondary isotope effects have been used thoroughly in the study of nucleophilic substitution reactions$^{14-17}$ in general and glycosidases in particular$^{18-20}$.

1.1.2.4 Trapping the covalent intermediate

As shown in § 1.1.1, the general mechanisms of retaining glycosidases involve the formation of an enzyme/substrate covalent intermediate. Direct trapping of the covalent intermediate can be achieved by introducing a fluorine substituent into the 2- or 5-position of the substrate to slow the reaction by destabilizing the positive charge at the transition state and also to remove the important 2-position hydrogen-bonding interactions at the transition state$^{21-24}$. Since the fluorine substitution slows both the glycosylation and deglycosylation steps, a very good leaving group (a fluoride or
dinitrophenolate) must be incorporated into the analogues to ensure that the glycosylation step is faster than the deglycosylation step, thus the intermediate will accumulate.

Figure 1.5 Mechanism-based inactivation of a retaining glycosidase by fluorosugars

The accumulated enzyme/substrate covalent intermediate can be analyzed by mass spectrometry and the covalent intermediate will have a higher mass equal to that of the carbohydrate moiety plus the mass of the enzyme itself. To identify the nucleophilic amino acid, this labeled protein can be proteolytically cleaved by a number of proteases, followed by isolation and sequencing of the glycosylated peptide. This process can be greatly simplified by use of LC/MS/MS. This approach has proven to be a powerful tool to identify the active site nucleophilic amino acid and was successfully applied to many glycosidases.

1.1.2.5 Site-directed mutagenesis of glycosidases

By detailed kinetic study of mutants in which conserved amino acids are replaced useful information about the catalytic mechanism can be obtained. As shown above, the
key active-site residues of glycosidases are a pair of carboxylic acids in both retaining and inverting enzymes. In the case of retaining glycosidases, one of the carboxylic acids serves as the nucleophile and the other serves as the acid/base catalyst. If the acid/base catalyst carboxylic acid side chain is replaced by a methyl group (Ala), the glycosylation step is greatly slowed for substrates with poor leaving groups but affected relatively little for substrates having a good leaving group such as fluoride or dinitrophenolate. However, since the base catalyst is missing, the second deglycosylation step is drastically slowed by the same amount for both types of substrate. Thus, when using substrates (glycoside) with good leaving group for such mutants, the deglycosylation step is rate limiting and the acid/base mutant can be trapped as its covalent intermediate. This covalent intermediate can also be analyzed by the methods described in the above section.

![Figure 1.6 Formation of covalent intermediate with acid/base mutant](image)

However, the deglycosylation step can be accelerated by the addition of nucleophilic anions such as azide, formate, or acetate. This is accompanied by the formation of a new product with retained anomeric configuration.
Enzyme

Figure 1.7 Azide rescue of activity of an acid/base catalyst mutant.

This rescue methodology has been further developed as tool for the identification of both the nucleophile and acid/base catalyst of retaining glycosidases. To identify the catalytic nucleophile, the nucleophilic amino acid residue is replaced by Ala. This mutant is completely inactive as a glycosidase, but can cleave activated substrates such as dinitrophenyl glycosides in the presence of azide, formate, or acetate via the mechanism shown below. Again, a new product is formed, but the anomeric configuration is inverted.

Figure 1.8 Azide rescue of activity of a nucleophile mutant.

In order to identify the acid/base residue and the catalytic nucleophilic residue, it is first necessary to generate mutants in which, individually, all the conserved Glu and
Asp are converted to Ala. These enzymes are then screened with an activated substrate (glycoside with good leaving group such as fluoride or dinitrophenolate) in the presence and absence of azide ion. Any mutants showing greatly increased activity will be the candidates for either the acid/base catalyst mutant or the catalytic nucleophile mutant. The differentiation of those two roles can be achieved by characterizing the isolated product. As shown above, the acid/base catalyst mutant will make an azide product with the same anomeric configuration as the substrate and the catalytic nucleophile mutant will yield a product that has inverted anomeric configuration. Up to now, this approach has only been used with retaining glycosidases.

1.1.3 Family 4 glycosidases

Among all the sequence-based glycosidase families, the family 4 glycosidases have attracted attention recently. In the most recent count (June 2002), there are already 55 members of this family. However, only one publication has reported mechanistic studies on enzymes from this family. The Family 4 glycosidases exhibit a very wide range of substrate specificity, and include maltose-6-phosphate glucosidase or 6-phospho-α-glucosidase (EC 3.2.1.122); α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22); 6-phospho-β-glucosidase (EC 3.2.1.86) and α-glucuronidase (EC 3.2.1.139). The enzymes in this family require NAD\(^+\) (or NADH) and divalent metals (Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) or Fe\(^{2+}\)) as cofactors for activity. Some very interesting mechanistic problems were posed to us. For example, the roles of NAD and Mn\(^{2+}\) were unclear and the stereochemistry of the product was unknown (i.e. is the enzyme retaining or inverting?). As mentioned above, family 4 contains both 6-phospho-α-glucosidase (EC
3.2.1.122) and 6-phospho-\(\beta\)-glucosidase (EC 3.2.1.86). Therefore, how members in this family tolerate both axial (\(\alpha\)-linked) and equatorial (\(\beta\)-linked) substrates in the active site was also an interesting problem.

1.1.3.1 6-Phospho-\(\alpha\)-glucosidase: GlvA and its initial investigation

The phosphoenolpyruvate-dependent sugar phosphotransferase system (PEP-PTS) allows simultaneous transport and phosphorylation of sugar (both monosaccharides and disaccharides) across the bacterial membrane. It was first discovered by Roseman and colleagues in 1964 and represents a landmark in our understanding of the carbohydrate transport system by microorganisms.

This system is found in both Gram-positive and Gram-negative bacteria and has been established as the primary mechanism by which bacteria accumulate sugars in their cytoplasm. The multi-component (PEP-PTS) system mainly has two general cytoplasmic enzymes, which are Enzyme I and HPr. They associate with sugar-specific proteins (IIA, IIB,) to permit the transfer of high energy phosphoryl moieties from the PEP to the incoming sugar (Figure 1.9) and simultaneously translocate the sugar (monosaccharides or disaccharides) across the membrane.

In order to enter the energy-generating pathways of bacteria, the phosphorylated disaccharide must be cleaved to the appropriate free and phosphorylated sugar moieties. This function is performed by a series of phosphoglycoside hydrolases including 6-phospho-\(\beta\)-glucosidase and 6-phospho-\(\beta\)-galactosidase.
Figure 1.9 Schematic representation of the phosphotransferase (PTS) system. (A) The glucose phosphotransferase system in Salmonella typhimurium. (B) The lactose phosphotransferase system in Staphylococcus aureus.

In the Bacillus subtilis genome, gene glvA is encoded within the maltose PEP-PTS operon\textsuperscript{37,38}, which is common for phosphoglycoside hydrolases. This gene was cloned and expressed by Thompson and co-workers in Escherichia coli\textsuperscript{34}. The purified protein GlvA is a 449 amino acid 6-phospho-\(\alpha\)-glucosidase, which is involved in the
hydrolysis of the phosphorylated α-glucoside accumulated via the phosphoenolpyruvate-dependent sugar phosphotransferase system (PEP-PTS) in *Bacillus subtilis*.

Thompson and colleagues conducted an initial investigation with the enzyme GlvA\(^{34}\). By sequence alignment, the enzyme GlvA has 74% and 78% identity with the 6-phospho-α-glucosidase MalH from *Fusobacterium mortiferum* and GlvG from *Escherichia coli*, respectively, and was therefore assigned to family 4 of the glycosidases. Sequence alignment study revealed that, like all the other members in the glycosidase family 4, GlvA also contains a region with a conserved Gly-XXX-Gly-Ser (GXGS) motif near its N-termini that is reminiscent of the fingerprint region of Rossmann fold, a conserved structure for binding NAD or NADH. The protein has a molecular weight of 50,513 Da and a theoretical pI of 4.77. The extinction coefficient was determined by them\(^{34}\) to be \(A_{260nm}^{\text{em}} = 1.25 \pm 0.01 \text{ ml/mg}\).

The natural substrate of GlvA is maltose-6'-phosphate, which is hydrolysed to glucose and glucose-6-phosphate. The enzyme requires both NAD\(^+\) (or NADH) and a divalent metal ion for activity. However, the NAD\(^+\) (or NADH) is not consumed in the hydrolysis reaction. Enzyme activity is greatest between 35 and 38 °C in buffer of pH 7.5-8.0. Consequently, enzyme activity was measured in 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM Mn\(^{2+}\) and 0.1 mM NAD at 37 °C. The enzyme shows Michaelis-Menten saturation kinetics with 4'-nitrophenyl α-glucoside-6-phosphate (\(K_m = 0.09 \text{ mM, } k_{\text{cat}} = 1.8 \text{s}^{-1}\))\(^{34}\).
Interestingly, Thompson et al. detected two additional products (X and Y) in the reaction mixture when GlvA was used to catalyze the hydrolysis of maltose-6'-phosphate, the natural substrate of GlvA. They suggested that the formation of these two additional products might result from a secondary function of GlvA.

Although GlvA has broad substrate specificity, the glycone moiety must satisfy the following requirements: (a) an O-α glycosidic bond, (b) a phosphate group at the 6 position of the non-reducing sugar ring, and (c) hydroxyl groups at C2 and C4 in the equatorial configuration. For the aglycone moiety, there are fewer requirements. This can be another glucosyl moiety or an aromatic group.

Varrot and Davies have already crystallized GlvA and have completed a preliminary X-ray analysis. However, due to difficulties in resolving the data, the X-ray structure of GlvA has not yet been solved. Knowledge of the structure will provide
further information on family 4 glycosidases and help in the elucidation of the mechanism of GlvA.

1.1.3.2 6-Phospho-β-glucosidase: Bglu

Takami and coworkers reported the complete genome sequence of the alkalophilic bacterium *Bacillus halodurans* and compared it with the genome sequence of *Bacillus subtilis*. They found several open reading frames (ORFs) in the genome of *Bacillus halodurans* showing significant similarities to those of *Bacillus halodurans*. Among those ORFs, ORF LicH (BH0183) (NCBI GenBank/GenPept number BAB03902) may encode a protein which is a new member of the family 4 glycosidase. Our collaborator, Dr. Annabelle Varrot (Department of Chemistry, University of York, UK), subcloned this gene (named *bglu* by her) from the bacterium *Bacillus halodurans*. The gene was expressed in *Escherichia coli* and the purified protein (Bglu) was a 6-phospho-β-glucosidase with a histidine tag at the C-termini. By sequence alignment, it has 51% identity with CelF, another 6-phospho-β-glucosidase belonging to family 4, and 30% identity with two other 6-phospho-α-glucosidases, GlvA and MalH, which belong to family 4 as well. The results of sequence alignment will be described in chapter II.

Bglu catalyses the hydrolysis of 6-phospho-β-glucosides, and requires both NAD (or NADH) and a divalent metal for activity. This enzyme may be assigned to family 4 of glycosidases based on the above knowledge of Bglu.
1.1.4 Novel possible catalytic mechanisms for the family 4 glycosidases

With very little knowledge of the catalytic mechanism for either GlvA or Bglu, several possibilities exist. In family 4 glycosidases it is even more complicated because:

- The role of the two cofactors, NAD(H) and divalent metal, is not known.
- Family 4 contains both α-glucosidases (GlvA) and β-glucosidases (Bglu) and they may share a similar mechanism or have very different mechanisms.

Therefore, in the following sections, I will only use the 6-phospho-β-glucosidase (Bglu) as the representative enzyme to propose the possible catalytic mechanisms. If evidence for a proposed mechanism is obtained, it may be applied to the other members of family 4 glycosidases, including GlvA.

1.1.4.1 A novel possible mechanism for the family 4 glycosidase

Thompson et al. found a very interesting phenomenon\(^\text{41}\) when they used GlvA to catalyze the hydrolysis of 4′-nitrophenyl glucoside-6-phosphate in D\(_2\)O. Product NMR analysis suggested that H-2 was exchanged with a deuterium from the solvent.

![Figure 1.12](image)

**Figure 1.12** Bglu catalyzed hydrolysis of 4′-nitrophenyl β-glucoside-6-phosphate.
Figure 1.13 H-2 was exchanged with deuterium by GlvA when the enzyme-catalyzed reaction was performed in D\textsubscript{2}O.

This phenomenon cannot be explained by the general mechanism of glycosidases since protons at position 2 are not usually acidic enough to spontaneously exchange with deuterium from the solvent. Therefore it was suggested that, in the catalytic mechanism of reaction, it was involved elimination occurred between C1 and C2 followed by addition of water. (Figure 1.14)

Figure 1.14 Mechanism to incorporate deuterium into the sugar by Bglu in the D\textsubscript{2}O buffer.

In this mechanism (Figure 1.14), because the NAD\textsuperscript{+} is required but not consumed, it may oxidize the C-3 hydroxyl group to a carbonyl group, making H-2 more acidic and assisting the elimination between C-1 and C-2. After that, the water (D\textsubscript{2}O) would be added to the double bond between C-1 and C-2 and the product of the oxidation step (NADH) then reduces the C-3 carbonyl back to a hydroxyl. Assuming that Bglu can also
exchange H-2 with deuterium, an oxidation-elimination-addition-reduction mechanism is shown below (Figure 1.15) using the 6-phospho-β-glucosidase (Bglu) as an example. In this mechanism, NAD$^+$ is not consumed in the reaction and the addition step should be a 1,2-cis addition if the enzyme is a retaining glycosidase. If the enzyme is an inverting enzyme, the addition step should be a 1,2-trans addition.

Figure 1.15 A possible novel oxidation-elimination-addition-reduction mechanism for Bglu.

Thompson at al. found that if NADH was substituted for NAD$^+$ as the cofactor, the enzyme still maintained 61% of its activity $^{34}$. Therefore, the mechanism described above is not able to explain why NADH can also be a cofactor unless NADH is somehow oxidized to NAD$^+$ in the reaction mixture. Consequently, alternative mechanisms should be considered.
1.1.4.2 Alternative possible mechanisms

The mechanistic study results of $\alpha$-1,4-glucan lyase$^{42}$ give us the clues to propose an alternative possible mechanism. Because in the catalytic mechanism of this enzyme, it is also involved an elimination step between C-1 and C-2 but without the assistance of NAD to oxidize C-3 to make the H-2 more acidic for elimination. Thus, it is suggested in this alternative possible mechanism, NAD$^+$ is not involved in the reaction and only serves as a cofactor to assist the enzyme in folding into the proper conformation. The first step of the reaction is a glycosylation step which results in the formation of a covalent glycosyl-enzyme intermediate. Subsequently, an elimination occurs between C1 and C2 to form a double bond. Hydration of this hydroxyglucal intermediate results in the formation of the final product. If the enzyme is a retaining glycosidase, the addition step should be a 1,2-cis addition, and if the enzyme is an inverting glycosidase, the addition step should be a 1,2-trans addition. In this possible alternative mechanism, NAD(H) is not involved in the reaction.
Figure 1.16 A novel possible catalytic mechanism of Bglu without involvement of NAD(H) in the reaction.

1.1.4.3 Third possible mechanism

The catalytic mechanisms illustrated above involve a covalent glycosyl-enzyme intermediate. The elimination reaction then occurs between C1 and C2 to form a double bond. In a further alternative mechanism, this elimination might occur as an E1-type syn-
elimination without formation of a covalent glycosyl-enzyme intermediate, as shown below. (Figure 1.16) Again, this mechanism proposes no role for NAD(H).

Figure 1.17 Third possible mechanism

1.2 Substrate Synthesis

In order to investigate the catalytic mechanisms of these two family 4 glycosidases, a variety of substrates and analogues was needed. The substrates for enzyme GlvA are 6-phospho-α-glucosides (including maltose-6’-phosphate) and the
substrates for Bglu are 6-phospho-β-glucosides. However, there are no commercially available substrates for these enzymes, therefore all substrates had to be chemically or enzymatically synthesized. From the general structural feature of all the substrates needed, there are two very important steps. One of them is the synthesis of the aryl glucoside (α or β) and the other is the selective phosphorylation on the 6 position of the glucosides. Therefore, the design of a good synthetic pathway to realize these two steps in the synthesis became very important for the entire synthesis work.

1.2.1 The formation of the glycosidic linkage

![Diagram of glycosidic linkage](image)

Figure 1.18 Formation of the glycosidic linkage with two possible anomeric configurations.

In general, the glycosidic linkage is formed from a glycosyl donor and a glycosyl acceptor. (Figure 1.17) A glycosyl donor, generally of α or β configuration at C1, condenses with the glycosyl acceptor, eliminating the HX to form two products which (α and β) have different configuration (α or β) at the anomeric center C1.
In this process, there are two major problems that need to be carefully considered. One of them is control of stereoselectivity, and the other is control of regioselectivity, i.e. reaction with the desired hydroxyl group of the glycosyl acceptor. Carbohydrate chemists have developed a number of excellent methods for formation of the glycosidic linkage.

1.2.1.1 Synthesis of glycosides by direct alkylation of the hemiacetal hydroxyl group

Under basic conditions, the free sugar equilibrates with the anomic mixture of the anions derived from the deprotonation of the anomic hydroxyl group. Alkylation of the oxyanion from the β anomer yields the β-glycoside.

![Mechanism of direct glycosylation under the strong basic condition](image)

Figure 1.19 Mechanism of direct glycosylation under the strong basic condition

This method was very useful in the synthesis of DNP-β-glucoside and DNP-β-xyloside\textsuperscript{43,44}. 

26
1.2.1.2 The Koenigs-Knorr Reaction\textsuperscript{45,46}(1,2-trans)

As early as the year 1901, Koenigs and Knorr developed an approach by treating "acetobromglucose" with alcohols in the presence of silver(I) carbonate (promoter) to form the 1,2-trans glycosidic bond.

Since this method was first developed, much work has been done to improve the process including using a co-solvent (for acceptors more complex than simple alcohols), adding powdered molecular sieves to absorb the liberated hydrogen halide and using
soluble promoters such as mercury(II) cyanide (used in acetonitrile or nitromethane) and silver(I) triflate\(^{47,48}\),

![Mechanism of heterogeneous Koenigs-Knorr Reaction]

**Figure 1.22** Mechanism of heterogeneous Koenigs-Knorr Reaction

A substantial body of work has been done to elucidate the mechanism of the Koenigs-Knorr reaction. For the heterogeneous process (insoluble silver(I) and mercury(II) compounds), a “push-pull” mechanism has been suggested, resulting in configuration inversion at the anomeric center. (Figure 1.21)

For the homogeneous process (soluble silver(I) and mercury(II) salts), it is suggested that the C1-halogen bond is first broken to generate an anomeric carbocation, stabilized by resonance and undergoing reaction with the neighboring ester group at C-2 to form a new cyclic carbocation, because the “concentration” of the C-2 ester group is relatively much higher than that of the added nucleophile in the system. The newly formed cyclic carbocation has two fates: one is that the alcohol attacks the anomeric carbon to generate the trans-glycoside and the other one is attack on the side chain carbocation, resulting in the formation of an orthoester. Therefore, orthoesters are often
significant by-products. However, in the acidic conditions usually used in a Koenigs-Knorr reaction, it is probable that any orthoester would rearrange to the glycoside.

Figure 1.23 Reaction mechanisms of heterogeneous Koenigs-Knorr reaction
1.2.1.3 The Trichloroacetimidate Method \(^{50,51}\) (1,2-trans and 1,2-cis)

With a proper base, the treatment of sugar derivatives that have a free Cl hydroxyl group with trichloroacetonitrile in methylene chloride will yield stereochemically pure, stable trichloroacetimidates (TCA), which are useful glycosyl donors.

**Figure 1.24** Synthesis of trichloroacetimidates (TCA) under basic conditions.

The use of potassium carbonate favors the formation of the \(\beta\) anomer and sodium hydride favors the \(\alpha\)-anomer; prolonged reaction time also will favor the more stable \(\alpha\) anomer\(^{52}\).

**Figure 1.25** Glycoside synthesis via TCA as a precursor.
The stereochemical outcome is determined by the presence of the C-2 participating group, the choice of promoter and the choice of solvent. If there is a participating group (esters) at C-2, 1,2-trans glycosylation will occur regardless of the C-1 configuration of the starting TCA. When TCA donors have a non-participating group at C-2, a mild promoter (like Et₂OBF₃) in the presence of the alcohol acceptor will result in the formation of the glycoside with inversion of the configuration at the anomeric center. Use of a stronger promoter (trimethylsilyl triflate) will leave the stereochemical control to the choice of solvent. If the solvent is ether or dichloromethane, an α-glycoside is formed and if the solvent is acetonitrile, the β-glycoside is favored.

![Figure 1.26 Formation of a β-glycosidic linkage via use of a TCA donor with a C-2 participating group](image)

**Figure 1.26** Formation of a β-glycosidic linkage via use of a TCA donor with a C-2 participating group.

### 1.2.1.4 Difficulty and methods of synthesis of aryl-α-glucosides (1,2-cis glycosylation)

Twenty seven years ago, Lemieux developed a method to synthesize 1,2-cis glycosides, termed as "halide ion catalysis". Under catalysis by tetraalkylammonium bromide, the 2-O-benzyl protected α-glycosyl bromide rapidly equilibrates with its β anomer. This highly reactive β anomer then reacts rapidly with the acceptor to form the α-glycoside. Many 1,2-cis glycosides were synthesized by this method and this method is still commonly used today. Since most β-glycosyl halides are too unstable to permit their
puriﬁcation, this approach provides a method to perform "in situ" anomerization to generate the β-halide and then react with the acceptor.

However, in the synthesis of pNP α-glucoside, a benzyl group cannot be used to protect the C-2 hydroxyl because the method (catalytic hydrogenation) for deprotection of the benzyl ether protecting group will reduce the nitro group, therefore forcing us to use an ester group (acetyl ester) to protect the C-2 hydroxyl. Unfortunately, this protecting group is a participating group that will favor 1,2-trans glycosylation. So, all the methods for the synthesis of pNP α-glucoside or pNP α-galactoside always yield significant β-glucoside as side-product. Considerable work has been done to ﬁnd conditions for better yields and higher ratios of α anomer to β anomer.

As early as the 1960’s, Trevelyan and Kucsmán synthesized the pNP tetra-O-acetyl-α-glucoside by condensing the pNP with penta-O-acetyl-α-D-glucopyranose in the presence of ZnCl₂ (as a catalyst). They got yields of 23% and 26% respectively but also got substantial amounts of the β anomer.

![Figure 1.27 Synthesis of pNP tetra-O-acetyl-α-glucoside](image)

Ingle et al developed a method involving use of tin (IV) chloride as a catalyst to synthesis pNP tetra-O-acetyl-α-glucoside as well as other aryl α-glucosides. Under their optimum conditions, equimolar quantities of reactants were kept at 30-35 °C for 60 hr and the formation of α anomers was favored. However, they used benzene as
the solvent in both reaction and workup procedure, which is now considered a carcinogenic reagent. Apparut^{58} et al. used methylene chloride as a solvent, and reacted p-nitrophenol with penta-O-acetyl-\(\beta\)-D-galactopyranose in the presence of SnCl\(_4\) to give p-nitrophenyl \(\alpha\)-D-galactoside in fair yield but this method failed when o-nitrophenol was used. Furthermore, some other catalysts such as anhydrous copper(II) sulfate^{59}, phosphorus oxychloride^{60} and zinc chloride-phosphorus oxychloride^{61} can also be used to synthesize aryl \(\alpha\)-glucosides starting from penta-O-acetyl-\(\beta\)-glucose.

![Figure 1.28 Synthesis of p-nitrophenyl \(\alpha\)-D-glucoside catalyzed by SnCl\(_4\)](image)

Yamaguchi^{62} developed a method to synthesize aryl 2,3,4,6-tetra-O-acetyl-\(\alpha\)-D-glucopyranosides starting from 2,3,4,6-tetra-O-acetyl-\(\alpha\)-D-glucopyranosyl fluoride in the presence of BF\(_3\)/Et\(_2\)O.
Since the synthesis of 2,4-dinitrophenyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside could not be achieved by the method used above, it was converted from its β anomer with K$_2$CO$_3$ in DMSO$^{63,64}$. The β-glycoside was synthesized by the method described before in §1.2.1.1.

### 1.2.2 Selective phosphorylation of the primary hydroxyl group of sugars

There are a variety of methods and reagents that can be used to phosphorylate biological molecules$^{65}$. The most useful reagent is diphenyl chlorophosphate which can be selectively installed on the primary hydroxyl due to the steric hindrance generated by the two big phenyl groups.

Figure 1.30 Selective phosphorylation of the primary hydroxyl group using diphenyl chlorophosphate
However, this reagent cannot be used in the phosphorylation of some of the aryl glycosides because the method (catalytic hydrogenation) for removal of the phenyl ether protecting groups will reduce the nitro group on the aglycone. Therefore to selectively phosphorylate the 6 position of an aryl glucoside, some other phosphorylating agent is needed. Because carbohydrates are polyhydroxylated, a bulky phosphorylating agent is needed to selectively react with the primary hydroxyl. Otherwise, protecting groups are needed on the other secondary hydroxyls before phosphorylating.

![Synthetic pathway for the synthesis of aryl glucoside-6-phosphate](image)

**Figure 1.31** Synthetic pathway for the synthesis of aryl glucoside-6-phosphate

Although this method solves the problem of selective phosphorylation of the 6 position of the glucoside, it needs many steps, which is time and labor consuming.

Morse et al developed a one step phosphorylating method. In this reaction, phosphorous oxychloride in trimethyl phosphate solvent was used to phosphorylate unprotected sugars at position 6. Interestingly, a trace of water was added to the reaction mixture to enhance the selectivity of phosphorylation. The mechanism of this reaction is not clearly known yet, and due to the acidic conditions of this reaction, the glycosidic
bond is easily broken. Therefore, the yield of this reaction ranges from 25% to 40%. However, since this is a single step reaction, which is more efficient than a multi-step reaction, it could be a good method for synthesis of aryl glucoside-6-phosphates.

Some other interesting reagents may also be able to phosphorylate the unprotected glucoside. 2,2,2-Tribromoethyl phosphoromorpholinochloridate was successfully used to synthesize the ribonucleoside monophosphates\textsuperscript{66}, and it may be applied to aryl glucosides.

Figure 1.32 Synthesis of ribonucleoside monophosphate

An alternative approach for the phosphorylation is an enzymatic one. Hexokinase is an enzyme that selectively phosphorylates glucose on its 6 position using ATP as a phosphate source. Thus, hexokinase may be a potential enzyme to enzymatically phosphorylate the glucoside or glucosyl fluoride, if it will tolerate an aglycone. Most recently, Thompson and Lichtenthaler\textsuperscript{67} discovered that \textit{Klebsiella pneumoniae} utilizes not only sucrose, but also its five linkage-isomeric \(\alpha\)-D-glucosyl-D-fructose as an energy
source for growth. This process involves phosphorylation by a phosphoenolpyruvate-dependent phosphotransferase system at the O-6 of the non-reducing glucosyl moiety of this disaccharide. They successfully used the strain ATCC 23357 of Klebsiella pneumoniae to phosphorylate a number of disaccharides at their non-reducing glucosyl moiety at position-6. It is expected that other glucosides, including both aryl glucosides and disaccharides might be similarly phosphorylated by this bacterium.

1.3 Objectives

Since only a limited number of enzymological studies have been done for GlvA and none for Bglu, many mechanistic questions need to be addressed regarding these unique enzymes. These questions include:

- What is the stereochemical outcome of the enzyme-catalyzed reaction? Is the enzyme a “retaining” glycosidase or an “inverting” glycosidase?
- What are the roles of the two cofactors, NAD(H) and divalent metal, in the catalytic mechanism?
- What is the identity of the two additional products X and Y from the GlvA catalyzed hydrolysis reaction? The identity of these two products will provide information that may be useful in the elucidation of the catalytic mechanism.
- Why does family 4 include both α-glucosidases and β-glucosidases as its members? Do they share a similar mechanism and the same stereochemical outcome?
Since time is limited for a M.Sc. project, it is impossible to answer all the questions addressed above. So, based on the basic approaches for the glycosidase mechanism studies, the goal of this project will be to focus on the following tasks.

1. Synthesis of both \( pNP \alpha\text{-glucoside-6-phosphate} \) (substrate for GlvA) and \( pNP \beta\text{-glucoside-6-phosphate} \) (substrate for Bglu) and use of these substrates to do basic kinetic measurements.

2. Synthesis of isotopically labeled substrate analogues for GlvA and Bglu, and attempt to measure kinetic isotope effects (KIEs).

3. Determine the stereochemical outcome of the reaction catalyzed by Bglu.

4. Look for deuterium incorporation into substrates at C-2 in D\(_2\)O buffer.

5. Chemical synthesis of maltose-6'-phosphate, the natural substrate of GlvA, and use of this compound to further isolate and characterize the two additional products X and Y detected from the product mixture of GlvA-catalyzed hydrolysis of maltose-6'-phosphate.

6. Synthesis of the potential mechanism-based inhibitor 2-deoxy-2-fluoro-6-phospho-\( \beta\)-\( D\)-glucosyl fluoride and attempted use of this compound to trap any covalent glycosyl-enzyme intermediates.
Chapter II Results and Discussion

2.1. Synthesis

2.1.1 Introduction

Because none of the substrates for either GivA or Bglu were commercially available, all substrates were chemically or enzymatically synthesized. In the process of the substrate synthesis, most of the reactions used followed published literature procedures and some were slightly modified in order to improve yield, save time and work.

2.1.2 Chemical synthesis of natural substrate: maltose-6'-phosphate

Maltose 6'-phosphate was chemically synthesized because of the substantial amount needed for the analysis of the products formed in the enzyme catalyzed hydrolysis reaction. Because the maltose has many hydroxyl groups, it is necessary to protect other hydroxyl groups and leave the 6' hydroxyl free for phosphorylation.

The synthesis started from maltose, on which the 4' and 6' hydroxyls were protected by a benzylidene group. After the remaining hydroxyls were protected by acetyl groups, the benzylidene group was removed by hydrogenation. The 6' hydroxyl group was then phosphorylated using diphenyl chlorophosphate. After deprotection, the maltose-6'-phosphate was produced.
In designing this synthetic strategy, we initially wanted to regioselectively open the 4',6'-benzylidene ring to leave only the 6' hydroxyl free. The most commonly used method to open the benzylidene ring leaving the 6 position free is to cleave the ring by using LiAlH₄-AlCl₃ in diethyl ether. 

**Figure 2.1** Synthetic method for maltose-6'-phosphate.

![Chemical structures](image)

**Figure 2.2** Regioselective opening of the 4,6-benzylidene ring of a glucoside.
Unfortunately, in the synthetic strategy employed here, the ester protecting groups would be reduced by LiAlH₄. For this reason, the benzylidene protecting group was fully removed by hydrogenation to free both the 4' and 6' hydroxyls at the same time. Because the 6' hydroxyl is a primary hydroxyl and the 4' is a secondary hydroxyl, the difference in reactivity between them is significant and this difference is used to selectively phosphorylate the 6' position.

![Figure 2.3 Selective phosphorylation of the primary hydroxyl by diphenyl chlorophosphate in pyridine.](image)

Results show that under room temperature conditions and a 1:1 molar ratio of 1,2,3,6,2',3'-hexa-O-acetyl-maltose and diphenyl chlorophosphate with the pyridine as solvent, the diphenyl chlorophosphate reacts first with the 6' hydroxyl: only a small amount of the diphosphorylated compound was formed, as detected by TLC. The two bulky phenyl groups also contribute to the selectivity for the 4 position primary hydroxyl due to steric hindrance effects.
2.1.3 Synthesis of pNP α-glucoside-6-phosphate and pNP β-glucoside-6-phosphate: Selective phosphorylation at 6 position.

pNP α-Glucoside-6-phosphate is the substrate of GlvA and pNP β-glucoside-6-phosphate is the substrate of Bglu. To synthesize these two substrates, selective phosphorylation of the commercially available pNP α-glucoside or pNP β-glucoside at their 6 position was necessary using an approach that does not require subsequent hydrogenation. The most commonly used reagent is phosphorous oxychloride using a procedure developed by Morse et al.69 for the phosphorylation of primary hydroxyl groups in unprotected nucleosides70. This approaches was therefore tested on pNPGlc via reaction with POCl₃ in trimethyl phosphate in the presence of water in an ice bath for 3 hours. Unfortunately, the acidic reaction conditions result in some degradation of the glycosidic bond, making the yield low (25%-40%). Nonetheless, the desired product was obtained. The neutralized reaction mixtures contained large amounts of inorganic phosphate and chloride. The products were therefore purified by selectively absorbing the phosphorylated and non-phosphorylated glucoside onto activated charcoal then eluting them with an aqueous pyridine solution. These products were converted to their dicyclohexyl ammonium salts by passing through Amberlite IR-120 ion-exchange resin (hydrogen form) followed by adjusting the pH to 9 with cyclohexylamine. The dicyclohexyl ammonium salt of the glycoside-6-phosphate was recrystallized from ethanol-diethyl ether. This method was used to synthesize all the 4'-nitrophenyl glucopyranoside-6-phosphates because only one step was required to selectively phosphorylate the unprotected glycosides.
2.1.4 Synthesis of isotopically labeled substrates for GlvA

In order to measure kinetic isotope effects (KIEs) for GlvA, the isotopically labeled substrate analogues for GlvA were synthesized. As reviewed in chapter I, a number of methods for the synthesis of aryl-α-glucosides had been reported starting from penta-O-acetyl-α-glucose or penta-O-acetyl-β-glucose. After testing some of those methods starting with penta-O-acetyl glucose, one was chosen for the synthesis of pNp α-D-[2-²H] glucopyranoside and pNp α-D-[3-²H] glucopyranoside. In this method, the starting material penta-O-acetyl-β-glucose was reacted with 4-nitrophenol in dichloromethane under reflux with SnCl₄ as catalyst. Because tin (IV) chloride is a harsh reagent, temperature control was a crucial factor and the oil bath temperature was kept below 60 °C during the reaction. Also, the dichloromethane needed to be replenished during the reaction. The overall yield of the glucoside was 80% with anomeric ratio of α:β = 6:4. The α anomer was separated by flash chromatography on silica using 2:1 diethyl ether:petrol ether. After deprotection of the acetyl group and selective phosphorylation of the hydroxyl at the 6 position by the method discussed in §2.1.3, the substrate analogues for GlvA were obtained. The overall synthetic strategies can be summarized in the following figure:
Three compounds: 1:R1=H, R2=H, 2:R1=H, R2=D, 3:R1=D, R2=H.

a) Ac2O, NaOAc, reflux, 83%.
b) β-nitrophenol, SnCl4, CH2Cl2, reflux, 5 h, 40%.
c) NaOMe, MeOH, 2 h, 95%.
d) POCl3, (CH3O)3PO, H2O, 0°C, 3 h, 30%.

**Figure 2.4** Synthetic strategies for substrates.

As shown above, the starting molecule is the glucose or isotopically labeled glucose (at position 2 or 3). The [2-2H] glucose can be obtained commercially but the [3-2H] glucose was chemically synthesized, as discussed in the following section.

### 2.1.5 [3-2H] glucose

Although the title compound is commercially available, it is very expensive, thus synthesis of this compound became necessary because a large amount was required. As early as 1970, Koch and Perlin synthesized the 3-position deuterium-labeled glucose.
They started from 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose and oxidized the 3-hydroxyl with dimethyl sulfoxide and acetic anhydride.

![Chemical Structures](image)

**Figure 2.5** Oxidation of 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose with DMSO and acetic anhydride.

This method not only requires 24 to 48 hours to complete, but also produces several side products. The work up procedure also requires special equipment to absorb the dimethyl sulfide formed as the reduction product of DMSO, and removal of the solvent DMSO is difficult. Another method was reported by Andersson and Samuelsson, in which they oxidized the 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose with pyridium dichromate-acetic anhydride (PDCA). This method has nearly 100% yield and the work up procedure is relatively simple. Besides the two aforementioned oxidation methods, several other methods were also reported that utilized the following oxidation reagents: DMSO-phosphorus pentoxide, ruthenium tetroxide and DMSO-carbodiimide.

Reduction of compound 6 with sodium borodeuteride will introduce deuterium at position 3. This reaction is stereospecific and leads to the formation of 1,2:5,6-di-O-isopropylidene-α-D-[3-^2^H]-allofuranose. An equilibrium between the hydrated and
non-hydrated forms of compound 6 exists\textsuperscript{77}, requiring use of excess of sodium borodeuteride.

![Chemical Structure]

Figure 2.6 Reduction of 1,2:5,6-di-O-isopropylidene-α-D-ribo-hexofuranose-3-ulose with excess sodium borodeuteride to move the equilibrium between hydrate and keto forms.

The stereochemistry at carbon-3 was then inverted by tosylation followed by displacement of the tosyl group by benzoate. Deprotection yielded glucose isotopically labeled at the 3-position.

2.1.6 Synthesis of 2-Deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride

The synthesis of 2-Deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride started from 3,4,6,-tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucosyl bromide (synthesized by Dr. Yasushi Todoroki). This compound was stirred with silver(I) fluoride in acetonitrile for 48 h to get the 3,4,6,-tri-O-acetyl-2-deoxy-2-fluoro-β-D-glucosyl fluoride. The latter compound was then deprotected and selectively phosphorylated using diphenyl chlorophosphate in pyridine using the procedure discussed in §2.1.2, as shown below:
Figure 2.7 Synthesis of 2-deoxy-2-fluoro-6-phospho-\(\beta\)-D-glucosyl fluoride

2.2. Enzymology

Two recombinant enzymes, GlvA and Bglu, were expressed in \textit{E. coli} and purified to study the catalytic mechanism of family 4 glycosidases. Basic kinetic studies of these two enzymes were performed and the stereochemical outcome of the Bglu catalyzed reaction was determined. The reaction of Bglu in deuterium oxide was also investigated and kinetic isotope effects (KIE) on Bglu were measured as follows.
2.2.1 Expression, purification, and basic kinetics of GlvA and Bglu

The high expression vector pET22b containing the GlvA gene provided from Dr. Annabelle Varrot (Department of Chemistry, University of York, UK) was transformed in E. coli BL21(DE3) cells. The recombinant GlvA enzyme was overexpressed after induction by IPTG. The whole cell SDS-PAGE analysis revealed one major band at about 50 kDa corresponding to the molecular weight of GlvA (50,510 Da). Since the enzyme has a 6-histidine tag at the C-terminus, it was then easily purified by using metal chelate chromatography.

Kinetic parameters for the hydrolysis of 4'-nitrophenyl α-D-glucoside-6-phosphate were determined for the recombinant GlvA enzyme ($K_m = 84 \mu M$, $k_{cat} = 5.69 \times 10^{-3} s^{-1}$). The $K_m$ value was similar to the previously published result of 90 μM; however, the $k_{cat}$ value was 300-fold lower than the published result of 1.8 s$^{-1}$. It seemed possible that excess Ni$^{2+}$ from the purification procedure might bind to the protein in place of the proper cofactor Mn$^{2+}$ lowering $k_{cat}$ values. Therefore, the enzyme was dialyzed with 20 mM EDTA several times and the kinetic measurements were repeated in the presence of Mn$^{2+}$. The $K_m$ of this protein sample was determined to be 82 μM and the $k_{cat}$ was 0.0440 s$^{-1}$, 8 times higher than before, but still 40-fold lower than the published result. Because the activity of the enzyme was too low to be trustworthy, further mechanistic investigations could not be carried out using this sample of enzyme and our attention shifted to Bglu.

The constructed pET28Bglu expression vector provided by Dr. Annabelle Varrot (Department of Chemistry, University of York, UK) was also used to transform electrocompetent Escherichia coli BL21(DE3) cells. The enzyme Bglu was
overexpressed in *E. coli* BL21(DE3) and also has a 6-histidine tag at the C-terminus making purification simple using metal chelate chromatography. Kinetic parameters for the hydrolysis of 4′-nitrophenyl β-D-glucopyranoside-6-phosphate were obtained for Bglu (*K_m* = 16 μM and *k_cat* = 0.0664 s⁻¹). The stock solution of enzyme was assayed to determine whether the kinetic parameters remained constant over time. In this way it was established that the enzyme was stable for at least 5 months when stored at 4 °C in 50 mM Tris-HCl (pH 7.5) buffer containing: 1 mM Mn²⁺, 0.1 mM NAD⁺ and 500 mM NaCl.

As the Bglu requires a divalent metal ion for activity, different divalent metal ions were tested with the enzyme. The results show that Mn²⁺ will be the best cofactor for enzyme activity and the relative activities for the other metal ions are shown below.

- Mn²⁺ 100%
- Mg²⁺ 1.9%
- Ca²⁺ 3.0%
- Co²⁺ 25.8%
- Ni²⁺ 15.2%
- Cu²⁺ <2%
- Zn²⁺ enzyme precipitated

The results obtained indicate that, besides Mn²⁺, both Co²⁺ and Ni²⁺ can serve as cofactors for Bglu. This is similar to what was found for GlvA³⁴.

The substrate specificity of Bglu was also tested using pNP α-glucoside-6P, pNP β-glucuronide, and pNP β-glucoside as possible substrates. None of those compounds were detectably cleaved by Bglu, indicating that the substrate of Bglu requires a β
configuration at the anomeric center and a phosphate group at the 6 position which cannot be substituted by a carboxylic acid group.

2.2.2 Sequence alignment of Bglu

To verify that the newly expressed enzyme Bglu is a member of family 4, the sequence of Bglu was analyzed using the BLAST 2.0 (the Basic Local Alignment Search Tool) program for similarity search in the SwissProt protein database. The top five proteins, which have greatest sequence similarity to Bglu, are all enzymes that belong to glycosidase Family 4. (Table 2.1 and Figure 2.8)

Table 2.1 Results of similarity search for Bglu (6-phospho-beta-glucosidase from *Bacillus halodurans*)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
<th>Identities</th>
<th>Similarities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LICH_BACSU (LicH)</td>
<td>Probable 6-phospho-beta-glucosidase</td>
<td>78%</td>
<td>89%</td>
<td>78</td>
</tr>
<tr>
<td>CELF_ECOLI (CelF)</td>
<td>6-phospho-beta-glucosidase</td>
<td>51%</td>
<td>71%</td>
<td>79</td>
</tr>
<tr>
<td>GLVA_BACSU (GlvA)</td>
<td>Maltose-6'-phosphate glucosidase</td>
<td>30%</td>
<td>51%</td>
<td>34</td>
</tr>
<tr>
<td>AGLB_KLEPN (AglB)</td>
<td>6-phospho-alpha-glucosidase</td>
<td>30%</td>
<td>51%</td>
<td>67</td>
</tr>
<tr>
<td>MALH_FUSMR (MalH)</td>
<td>Maltose-6'-phosphate glucosidase</td>
<td>30%</td>
<td>53%</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 2.8 Results of similarity search for Bglu — Sequence similarity to Bglu decreases from the top protein to the bottom protein.

To further study the sequence of Bglu, the sequences of the above six enzymes were input into the program Clustal W for sequence alignment. The result obtained is shown below:

CLUSTAL W (1.82) multiple sequence alignment

Ma1H Fusmr  
Ag1B Klepn  
GlvA Bacsu  
Bglu Bacha  
LicH Bacou  
CelF Ecoll  

---MKQFSILAIAGGSTFTPGIIILMLDLDDKFPQRIKMFDND---ABRAQA 46

---MKKFSVIAAGGSTFTPGVLMILLANQDRPRLKFDN---GARQE 46

---MKKFSVIAAGGSTFTPGVLMILLADHLKFLKFDN---GARQE 46

---MICIKIATGGGSSYTPFLVEGFIKRYDELVRMLEGDFIEGKED 48

---MICIKIATGGGSSYTPFLVEFVIKRYDELVRMLEGDFIEGKED 49

---MSQKLKVTVGGSSYTPFLVEFVIKRYHELPLVSLLVDVSEGKFDL 49
MalH Fusmr
KIGBACAILLKEKAPQIKFSYTNPEAFTDIFVMAMIRVGKYPMRELD 96
AglB Klepn
TTABACKVILKEQEPFIEEEFTTPQAARTDVDVFVMAHRVGKYPMRQD 96
Glva Bacsu
RTAGACDFVIFPADFEAAPTFDTDVFVMAHRVGKYAMRDL 98
Bglu Bacha
IVONLAKRQVQKALGIDTHLDRREAOKQADTFPQPRVOLLQRAKDK 98
LicH Bacsu
IVGTLAKRKEVGVPIDTHLDRKALKDADFTTVFQPRVOLLQRAKDK 99
CelF Ecoli
IIIFDLGCQRDINHAPVMPNLCYKDLTDRREALOAIVVQQLstell 149

MalH Fusmr
EKIPLRHGIGEVLGQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 147
AglB Klepn
EKIPLRHGIGEVLGQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 147
Glva Bacsu
EQIPLKYGIGEVLGQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 148
Bglu Bacha
ERIPLKYVQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 149
LicH Bacsu
ERIPLKYVQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 149
CelF Ecoli
ERIPLSHYQETCGPGGIAYGMRSIGGVLEILDYMEKYPDMLNYS 149

MalH Fusmr
NPAAIVAEABTLLRNPSKVLNICDPMGIEVRMAEILGLESRKDMIYK 191
AglB Klepn
NPAAIVAEABTLLRNPSKVLNICDPMGIEVRMAEILGLESRKDMIYK 191
Glva Bacsu
NPAAIVAEABTLLRNPSKVLNICDPMGIEVRMAEILGLESRKDMIYK 191
Bglu Bacha
NPAGMVTEAYRIYQSVKVLNICDPMGIEVRMAEILGLESRKDMIYK 193
LicH Bacsu
NPAGMVTEAYRIYQSVKVLNICDPMGIEVRMAEILGLESRKDMIYK 193
CelF Ecoli
NPAGMVTEAYRIYQSVKVLNICDPMGIEVRMAEILGLESRKDMIYK 193

MalH Fusmr
GLNHFGWWKSVRDKQGNDLMPKLREHVSQYVQVPGDKQDNHTEASWNMTD 245
AglB Klepn
GLNHFGWWTSIEDLDGNDLMPKLREYVAKYGYVPPSDN9HTEASWNMTD 245
Glva Bacsu
GLNHFGWWTSIQDQEGNDLMPKLREYVAKYGYVPPSDN9HTEASWNMTD 245
Bglu Bacha
GLNHMWGLDW-LDGVSVKKEQVIEAMGD---EPNKATMKNISGAEWEPDF 245
LicH Bacsu
GLNHMVFIKDVL-INGKSRFAELLDGVASG-QLKASSVKNIFDLFPSGL 247
CelF Ecoli
GLNHMVFIKDVL-INGKSRFAELLDGVASG-QLKASSVKNIFDLFPSGL 247

MalH Fusmr
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349
AglB Klepn
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349
Glva Bacsu
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349
Bglu Bacha
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349
LicH Bacsu
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349
CelF Ecoli
SACRA1IAQKSTAGDLDEBSASNYDVTLATAOFTQMRMLVFLPMN 349

MalH Fusmr
EAWIEGSYQKLMQATMSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441
AglB Klepn
DABQWSYHKLMQATLSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441
Glva Bacsu
DABQWSYHKLMQATLSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441
Bglu Bacha
DABQWSYHKLMQATLSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441
LicH Bacsu
DABQWSYHKLMQATLSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441
CelF Ecoli
DABQWSYHKLMQATLSKVTSPASVAKDILDLIDIEANKWEYVLK---- 441

MalH Fusmr
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
AglB Klepn
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
Glva Bacsu
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
Bglu Bacha
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
LicH Bacsu
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
CelF Ecoli
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394

MalH Fusmr
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
AglB Klepn
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
Glva Bacsu
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
Bglu Bacha
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
LicH Bacsu
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
CelF Ecoli
IADNIDPAWAVETMCKLGRDTAPTHHFDVNMOLGIHITKGFIAAS 394
Figure 2.9 Sequence alignment of Bglu

From the results obtained through site-directed mutagenesis of GlvA\textsuperscript{34}, Glu\textsuperscript{111} and Glu\textsuperscript{359} are the probable proton donor and nucleophile or base, respectively of a 'normal' glycosidase mechanism is followed. These two amino acids are conserved in Bglu and other family 4 glycosidases listed above (Figure 2.9, see amino acid in red color) and will be candidates for site-directed mutagenesis studies in the future.

Similar to all the other members of family 4 glycosidase, Bglu also contains the Rossmann fold, a conserved structure motif for binding the NAD or NADH, near its N-termini. (Figure 2.9, see amino acid in blue color for the conserved GXGS motif)

2.2.3 Product analysis of Bglu-catalyzed hydrolysis in deuterium oxide

Because 6-phospho-α-glucosidase GlvA apparently exchanges H-2 with deuterium when it catalyzes the hydrolysis of 4'-nitrophenyl glucoside-6-phosphate in deuterium oxide, it was interesting to find out whether such exchange is general to the entire family 4 or is a unique character of GlvA. For this study, another family 4 glycosidase, 6-phospho-β-glucosidase Bglu, was used to hydrolyze its substrate 4'-nitrophenyl β-glucoside-6-phosphate in deuterium oxide. The products were analyzed by \textsuperscript{1}H NMR. (Figure 2.10)
Figure 2.10 $^1$H NMR analysis of the Bglu-catalyzed hydrolysis of pNPGlc-6P in D$_2$O (bottom) and $^1$H NMR spectrum of glucose-6-phosphate (top).

To fully ensure that glucose-6-phosphate does not spontaneously incorporate deuterium into position 2, a sample of glucose-6-phosphate was incubated in D$_2$O without enzyme under identical conditions to those of the Bglu-catalyzed hydrolysis reaction as a control. The most important peaks are those at $\delta$ 5.08 ($J = 3.8$) and 4.50 ($J = 7.7$) which are from the anomeric proton coupled to H-2. The product of the Bglu-catalyzed hydrolysis reaction in D$_2$O is shown in the bottom spectrum of Figure 2.10. The peaks corresponding to the hydrogen at the anomeric position became singlets, but the chemical shift remained the same. This indicates that the neighbouring hydrogen was substituted by deuterium.
Because the free sugar was difficult to purify from buffer, it was difficult to fully assign all the peaks in the $^1$H NMR spectrum. The product was therefore further treated with alkaline phosphatase to remove the phosphate and all the free hydroxyls were protected with acetyl groups by reacting with acetic anhydraide in pyridine. The final products were purified and NMR analysis revealed that they are penta-O-acetyl-β-D-[2-$^2$H]-glucopyranose (red) and penta-O-acetyl-α.-D-[2-$^2$H]-glucopyranose (blue).

**Figure 2.11** $^1$H NMR analysis of the final product after dephosphorylation and full acetylation.

This NMR analysis further supports the idea that the product of Bglu catalyzed hydrolysis is [2-$^2$H] glucose-6-phosphate and the whole process can be described as follows:
2.2.4 Stereochemical outcome determination of Bglu

The stereochemical outcome of the glycosidase-catalyzed hydrolysis reaction is one of the key features for the classification of glycosidases. There are no previously published results reporting the stereochemical outcome for any enzymes in family 4. The determination of the stereochemical outcome therefore became a key step before any further mechanistic investigations.

The best method for determining the stereochemical outcome of the reaction of glycosidases involves the use of NMR to monitor the reaction system. In this experiment, the NMR spectrum of the substrate in the proper buffer is measured first, and then an aliquot of enzyme is injected into the solution. The whole reaction mixture is then monitored by taking a series of spectra at specific time points during the reaction. By observing the chemical shift and relative intensity of the anomeric proton and its coupling constant with H-2 in real time, the stereochemical outcome can be determined.
However, a limitation of this approach is that free sugars undergo mutarotation in aqueous solution. This gradually changes the stereochemistry of the anomeric center of the product from its original configuration to its counterpart until equilibrium is reached. However, by usually adding enough enzyme, the rate of the enzyme-catalyzed hydrolysis can be made greater than the rate of mutarotation. Thus, the initial products from the enzyme-catalyzed reaction will accumulate to levels detectable by NMR analysis. Unfortunately, in the case of Bglu, there was an additional problem. Bglu requires the paramagnetic Mn\(^{2+}\) as a cofactor for activity, thus in the presence of this metal, the peaks of the NMR spectrum will be broadened. Therefore, it was difficult to assign accurate chemical shifts and impossible to measure coupling constants. Unfortunately, it was not possible to remove the Mn\(^{2+}\) prior to analysis since during that time, mutarotation would occur. This experiment was therefore deemed inappropriate for Bglu.

![Mutarotation of glucose-6-phosphate in aqueous solution.](image)

**Figure 2.13** Mutarotation of glucose-6-phosphate in aqueous solution.
An alternative experiment for the determination of the stereochemical outcome of the glycosidase-catalyzed reaction involves analysis of the product of enzyme-catalyzed methanolysis. In this experiment, assuming the presence of methanol will not change the catalytic mechanism, the enzyme-catalyzed hydrolysis is performed in the presence of an appropriate concentration of methanol. Thus, in addition to the normal hydrolysis product, a methyl glucoside will be formed as the product of the methanolysis reaction. The methyl glucoside will not undergo mutarotation, so determination of the stereochemistry at the anomeric center for this product will provide the stereochemical outcome for the enzymatic reaction.

Methanolysis was first done on a small scale. TLC analysis revealed that at a concentration of methanol of 3 M, Bglu was still quite active and gave a good yield of methyl glucoside. The Bglu catalyzed methanolysis was then performed on a larger scale in H₂O and the products were analyzed by NMR after lyophilization. At the same time, a parallel experiment was done in a mixture of D₂O and CD₃OD. The spectra obtained are shown below. (Figure 2.14)

The bottom spectrum (c) is the control experiment in which the glucose-6-phosphate was incubated without enzyme in buffer containing 3 M methanol. The middle spectrum (b) shows the product of the methanolysis experiment performed in H₂O. The spectrum shows two peaks corresponding to H-1 in α-glucose-6-phosphate ($\delta = 5.08, J < 2$ Hz) and β-glucose-6-phosphate ($\delta = 4.50, J = 6.6$ Hz). Another doublet peak ($\delta = 4.24, J = 7.7$ Hz) arises from H-1 of the methyl-glucoside-6-phosphate. The coupling constant of about 8 Hz suggests that this peak corresponds to methyl-β-glucoside-6-phosphate. The methanolysis performed in D₂O reveals that coupling with
H-2 is now missing conforming that proton exchange also occurs in the methanolysis reaction.

**Figure 2.14** $^1$H NMR analysis of products of Bglu catalyzed methanolysis reactions performed in D$_2$O and in H$_2$O. a) $^1$H NMR spectrum of the products of Bglu-catalyzed methanolysis performed in D$_2$O. b) $^1$H NMR spectrum of the products of Bglu-catalyzed methanolysis performed in H$_2$O. c) $^1$H NMR spectrum of the product in the control experiment.

In order to fully assign the peaks of the product, the mixture of the products were enzymatically dephosphorylated and fully acetylated as described in section 2.2.2. The final product mixture was analyzed by NMR. (Figure 2.15)
From the spectrum, the three products from the methanolysis reaction in $\text{H}_2\text{O}$ are penta-O-acetyl-$\beta$-D-glucopyranose, penta-O-acetyl-$\alpha$-D-glucopyranose and methyl 2,3,4,6-tetra-O-acetyl-$\beta$-D-glucoside. The three products from the methanolysis reaction in $\text{D}_2\text{O}$ are penta-O-acetyl-$\beta$-D-[2-$^2\text{H}$]-glucopyranose, penta-O-acetyl-$\alpha$-D-[2-$^2\text{H}$]-glucopyranose and [1',1',1'-2$^2\text{H}$] methyl 2,3,4,6-tetra-O-acetyl-$\beta$-D-[2-$^2\text{H}$]-glucoside.

Figure 2.15 $^1\text{H}$ NMR of the final products after the dephosphorylation and full acetylation of the Bglu catalyzed methanolysis reaction products. Bottom spectrum: products of methanolysis reaction in 3 M methanol; Top spectrum: products of methanolysis reaction in 3 M methanol-$d_4$ in $\text{D}_2\text{O}$ buffer.
From the above results, the 6-phospho-β-glucosidase Bglu is a retaining glycosidase. This work represents the first stereochemical outcome determination for any family 4 glycosidase.

2.2.5 Attempt of trapping the covalent glycosyl-enzyme intermediates

Since Bglu was determined to be a retaining glycosidase, the use of potential mechanism based inhibitor 2-deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride to trap any covalent glycosyl-enzyme intermediates was conducted by incubating 1 mg above compound with 100 μL (1.7 mg/mL) Bglu in 37 °C overnight. However, the LC/MS analysis of the product mixture did not detect any glycosyl-enzyme intermediates. It reveals that the Bglu does not involve the general mechanism of retaining glycosidase and very possible to have a unique catalytic mechanism.

2.2.6 Conclusions and future work

After 3 months of working on the expression and purification of GlvA, a high activity enzyme was not obtained. The study was therefore switched to another family 4 glycosidase, Bglu. The 6-phospho-β-glucosidase Bglu has 50% similarity and 30% identity with GlvA, but it has different substrate specificity than GlvA. With p-nitrophenyl β-glucoside-6-phosphate as substrate, the $K_m$ of Bglu was 16 μM and $k_{cat}$ was 0.0664 s$^{-1}$. The enzyme was determined to be a retaining glycosidase through NMR analysis of methanolysis products. Interestingly, the enzyme-catalyzed hydrolysis reaction in D$_2$O involves substitution of the H-2 with a deuterium, suggesting that the catalytic mechanism most likely involves an elimination and addition step.
Based on the results of this study, further mechanistic investigations are needed. After the synthesis of isotopically labeled substrates, the KIE experiments of Bglu may give important information for the mechanism illustration. The linear free energy relationships can also be investigated by using a series of aryl β-glucoside which can be chemically synthesized.
Chapter III Materials and Methods

3.1 Synthesis

3.1.1 General methods and materials

All $^1$H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 300 instrument. Chemical shifts given are on the $\delta$ scale and referenced as follows: CDCl$_3$: 7.24, D$_2$O 4.63. $^{31}$P NMR and $^{19}$F NMR spectra were recorded on the same instrument without proton decoupling. $^{31}$P NMR spectra were referenced to H$_3$PO$_4$ ($\delta = 0$) in D$_2$O and $^{19}$F NMR spectra were referenced to TFA ($\delta = 0$) in D$_2$O. Melting points were measured on a Laboratory Devices Mel-Temp II melting point apparatus, and are uncorrected. Micro-analyses were performed by Mr. P. Borda, micro-analytical laboratory, University of British Columbia, Vancouver.

Solvents and reagents were either reagent grade, certified, or spectral grade. Solvents were dried as follows: methanol was distilled from magnesium and iodine; dichloromethane, pyridine and acetonitrile were distilled from calcium hydride; dry N,N-dimethylformamide was obtained by sequential drying over 4 Å molecular sieves. Dried solvents were used for all reactions, unless otherwise stated. Thin layer chromatography (TLC) was used to follow all reactions, unless otherwise stated. TLC separations were performed using Macherey-Nagel Alugram Sil G/UV 254 aluminum-backed analytical plates. Compounds were visualised with ultra violet light (where possible) and/or charred with 10% sulfuric acid in methanol. Sili-cycle silica gel, 230-400 mesh, was used for all column chromatography.
Mass spectral analysis of the synthesized compounds was performed at the UBC Mass Spectrometry Facility. Low resolution desorption chemical ionization mass spectrometry (DCI-LRMS) was performed on a Delsi Nermag R10-10C single quadrupole mass spectrometer using ammonia as a reagent gas. Low and high resolution secondary ion mass spectrometry (LSIMS-LRMS and LSIMS-HRMS) were performed on a Kratos-Concept IIH mass spectrometer equipped with a cesium-ion gun using glycerol and methanol as the matrix. Low and high resolution fast atom bombardment mass spectrometry (FAB-LRMS and FAB-HRMS) were performed using a Kratos Concept IIHQ mass spectrometer.

3.1.2 General procedures

3.1.2.1 Deacetylation with MeOH/NaOMe

The acetylated carbohydrate derivatives (2 mmol) were dissolved in 50 mL anhydrous methanol, and several small pieces of sodium were added into the solution. The reaction mixture was stirred for 3 hours and quenched by adding Amberlite IR-120 ion-exchange resin. After stirring the resin suspension for another 1 hour, the resin was immediately filtered and the solvent was evaporated in vacuo. The product was then further purified by recrystallization or column chromatography.

3.1.2.2 Acetylation with pyridine/acetic anhydride

The carbohydrate derivatives (5 mmol) were dissolved in dry pyridine (30 mL) and cooled in an ice-water bath. Acetic anhydride (20 mL) was added dropwise into the solution. Depending on the reactivity of the carbohydrate derivatives, the reaction was
stirred from 3 hours to overnight. The pyridine was evaporated under high vacuum at 40 °C. The residue was then transferred with acetyl acetate and water into a separation funnel and the organic layer was washed successfully with 1 M HCl, saturated sodium bicarbonate, water and brine. The organic layer was then dried over calcium chloride and evaporated to dryness. The product was further purified by column chromatography or recrystallization.

3.1.3 4'-Nitrophenyl glucopyranoside-6-phosphate

4'-Nitrophenyl β-D-glucopyranoside-6-phosphate (2)

4'-Nitrophenyl β-D-glucopyranoside (3.01 g, 10 mmol, obtained from Aldrich) was dissolved in trimethyl phosphate (11 mL). Water (180 μL, 10 mmol) was added and phosphorus oxychloride (2.8 mL, 11.1 mmol), pre-dissolved in trimethyl phosphate (11 mL), was slowly dropped into the solution. The mixture was kept in an ice-water bath and stirred for 3 hours. The reaction mixture was diluted with water to 200 mL and the pH was adjusted to 6 with concentrated NH₃/H₂O. Activated charcoal (20 g), which had been washed with 1 M HCl and dried by suction, was then added. After the suspension had been stirred for 1 hour at room temperature, the charcoal was filtered and first washed with 100 mL 0.2 M HCl, followed by 600 mL of pyridine:water (1:4, v/v). After evaporating the pyridine, the solution was passed through Amberlite IR-120 ion-exchange resin (hydrogen form) and the pH was adjusted to 9 with cyclohexylamine. The basic solution was evaporated to dryness to yield product 1. Product 1 was recrystallized from EtOH and diethyl ether as white crystals (2.03 g, 3.5 mmol, 35%) which were stored at -20 °C. Product 1 was transformed to the sodium form by passing
an aqueous solution of it through Amberlite IR-120 ion-exchange resin (sodium form). After evaporating the water, a yellow powder 2 was formed. $^1$H NMR (D$_2$O) $\delta$ 8.12 (d, 2 H, $J_{2',3'}$ 9, $J_{5',6'}$ 9 Hz, H3', H5'), 7.12 (d, 2 H, $J_{2',3'}$ 9, $J_{5',6'}$ 9 Hz, H2', H6'), 5.13 (d, 1 H, $J_{1,2}$ 7 Hz, H1), 3.98-3.83 (m, 2 H, H6a, H6b), 3.58-3.48 (m, 4 H, H2, H3, H4, H5), $^{31}$P NMR (D$_2$O) $\delta$ 4.95 (t, $J_{P,H6a,H6b}$ 6 Hz); $^1$H-$^{31}$P 2D NMR HSQC confirms the coupling between P and H6a,H6b; Exact mass calc'd for C$_{12}$H$_{15}$O$_{11}$NP: 380.0383; found: 380.0384; Exact mass calc'd for C$_{12}$H$_{14}$O$_{11}$NPNa: 402.0202; found: 402.0204.

4'-Nitrophenyl $\alpha$-D-glucopyranoside-6-phosphate (4)

4'-Nitrophenyl $\alpha$-D-glucopyranoside (3.01 g, 10 mmol, obtained from Aldrich) was dissolved in trimethyl phosphate (11 mL) and water (180 $\mu$L, 10 mmol) was added. Phosphorus oxychloride (2.8 mL, 11.1 mmol), pre-dissolved in trimethyl phosphate (11 mL), was then slowly dropped into the solution. After the reaction mixture was kept in an ice-water bath and stirred for 3 hours, it was diluted with water to 200 mL and the pH was adjusted to 6 with concentrated NH$_3$/H$_2$O. After that, the activated charcoal (20 g), which had been washed with 1 M HCl and dried by suction, was added. The suspension was then stirred for 1 hour at room temperature, and the charcoal was filtered and first washed with 100 mL 0.2 M HCl, followed by 600 mL of pyridine-water (1:4, v/v). After evaporating the pyridine, the solution was passed through an Amberlite IR-120 ion-exchange resin (hydrogen form) and the pH was adjusted to 9 with cyclohexylamine. The basic solution was evaporated to dryness to get product 3. Product 3 was recrystallized from EtOH and diethyl ether as white crystals (1.51 g, 2.6 mmol, 26%) which were stored at -20 °C. Product 3 was transformed to the sodium form by passing
an aqueous solution of it through Amberlite IR-120 ion-exchange resin (sodium form). After evaporating the water, a yellowish white powder was obtained 4. $^1$H NMR (D$_2$O) $\delta$ 8.08 (d, 2 H, J$_{2',3'}$ 9, J$_{3',6'}$ 9 Hz, H3', H5'), 7.13 (d, 2 H, J$_{2',3'}$ 9, J$_{5',6'}$ 9 Hz, H2', H6'), 5.64 (d, 1 H, J$_{1,2}$ 3 Hz, H1), 3.92-3.78 (m, 2 H, H6a, H6b), 3.70-3.59 (m, 4 H, H2, H3, H4, H5), $^{31}$P NMR (D$_2$O) $\delta$ 3.96 (t, J$_{P,H6a,H6b}$ 6 Hz); $^1$H-$^{31}$P 2D NMR HSQC confirms the coupling between P and H6a,H6b; Exact mass calc'd for C$_{12}$H$_{15}$O$_{11}$NP: 380.0383 found: 380.0385; Exact mass calc'd for C$_{12}$H$_{14}$O$_{11}$NPNa: 402.0202; found: 402.0202.

3.1.4 4'-Nitrophenyl $\alpha$-D-[3-$^2$H]-glucopyranoside-6-phosphate

1,2:5,6-Di-O-isopropylidene-$\alpha$-D-glucofuranose (5)

Anhydrous $\alpha$-D-glucose (50 g, 0.28 mol), powered in a Waring blender, was stirred vigorously with 1 L of acetone in an ice bath. Sulfuric acid (96%, 40 mL) was added in 5 mL portions at 10-15 min intervals, while maintaining the temperature between 5-10 °C. After the additions of sulfuric acid, vigorous stirring was continued for 5 hours, allowing the temperature to rise gradually to 20-25 °C. The solution was cooled again (ice bath), and 50% sodium hydroxide solution (61.2 g of NaOH in 75 mL water) was added with stirring to bring the pH to near neutrality. The addition was made slowly to avoid heating. A small amount of sodium hydrogen carbonate was added to maintain the solution near neutrality. After standing overnight, the salts were removed by filtration, and the acetone solution was concentrated under reduced pressure to a thick syrup which was then dissolved in chloroform and extracted with water. The water solution was then washed with chloroform and the combined chloroform fractions were concentrated to dryness. The syrup was recrystallized from cyclohexane to yield 30 g of
compound 5 (0.115 mol, 41%), mp 110-111 °C; lit. \(^1\) mp 110-111 °C, \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 5.90 (d, 1 H, J\(_{1,2}\) 4 Hz, H1), 4.48 (d, 1 H, J\(_{1,2}\) 4 Hz, H2), 4.31-4.26 (m, 2 H, H3, H5), 4.12 (dd, 1 H, H6a), 4.02 (dd, 1 H, H4), 3.95 (dd, 1 H, H6b), 2.75 (bs, 1 H, OH), 1.46, 1.40, 1.32, 1.28 (4 x s, 12 H, 4 x OAc).

1,2:5,6-Di-O-isopropylidene-\(\alpha\)-D-[3-\(\text{\textsuperscript{2}}\)H]-allofuranose (7)

Pyridinium dichromate (22.57 g, 60 mmol) and 28.3 mL acetic anhydride (300 mmol) were added to 250 mL of dry CH\(_2\)Cl\(_2\) and the mixture stirred for 5 min at room temperature. A solution of compound 5 (26 g, 100 mmol) in CH\(_2\)Cl\(_2\) (50 mL) was added dropwise into the mixture with stirring and then boiled under reflux for 5 hours. After boiling, the mixture was applied to the top of a short column of silica gel in ethyl acetate, at which point the chromium compounds precipitated in the layer of ethyl acetate on top of the gel. The desired product was eluted with ethyl acetate and the eluate was concentrated to near dryness. Toluene was distilled several times from the residue in order to remove acetic acid and pyridine to yield 25 g (97 mmol, 97%) of compound 6. Compound 6 was used in the next reaction without characterization.

Compound 6 (13 g, 50 mmol) was dissolved in 50% aqueous methanol (200 mL) and reacted with NaBD\(_4\) (5 g) over 1 hour. The solution was diluted with water (100 mL) and then extracted with chloroform. The extract was dried and evaporated to a colorless syrup (13 g, 100%) and recrystallized from cyclohexane as fine white crystals 7 (9 g, 34.5 mmol, 69%), mp 76-77 °C. \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 5.78 (d, 1 H, J\(_{1,2}\) 4 Hz, H1), 4.58 (d, 1 H, J\(_{1,2}\) 4 Hz, H2), 4.28 (m, 1 H, H5), 4.01 (m, 2 H, H6, H6'), 3.79 (d, 1 H, H4), 2.50 (bs, 1 H, OH), 1.55,1.44,1.36,1.34 (4 x s, 12 H, 4 x OAc).
1,2:5,6-Di-O-isopropylidene-3-O-toluene-p-sulfonyl-α-D-[3-²H]-allofuranose (8)

Toluene-p-sulfonyl chloride (10.5 g) in dry pyridine (100 mL) was added to a solution of compound (7) (8.4 g, 32 mmol) in dry pyridine (100 mL) and the solution was kept at room temperature for 48 hours. Most of the pyridine was then evaporated under vacuum at 40°C. The resulting residue was transferred with chloroform into a separation funnel. The chloroform layer was washed with 1 M HCl, water, NaHCO₃ and brine respectively, dried over CaCl₂, and evaporated to dryness. The compound was purified by flash chromatography to get product 8 (10.3 g, 24.8 mmol, 77.5%). ¹H NMR (CDCl₃) 7.79 (d, 2 H, J 8 Hz, H₂', H₆'), 7.30 (d, 2 H, J 8 Hz, H₃', H₅'), 5.89 (d, 1 H, J 3.5 Hz, H1), 4.79 (d, 1 H, J 3.5 Hz, H2), 4.02-3.85 (m, 4 H, H₄, H₅, H₆a, H₆b), 2.42 (s, 3 H, CH₃), 1.44, 1.27, 1.16, 1.12 (4 x s, 12 H, 4 x OAc).

3-O-Benzoyl-1,2:5,6-Di-O-isopropylidene-α-D-[3-²H]-glucofuranose (9)

Compound 8 (8 g, 19.2 mmol) was dissolved in dry N,N-dimethylformamide (400 mL) and sodium benzoate (30 g) was added. The suspension was heated under reflux for 24 hours, water (500 mL) was added to dissolve the sodium benzoate, and the solution was extracted with chloroform and washed 3 times with water. The chloroform extract was first evaporated at room temperature and then at 50°C under high vacuum to near dryness in order to remove most of the DMF. The residue was then dissolved in chloroform and washed 5 times with equal volumes of water. After drying and filtering, the solution was concentrated to give a colorless syrup, which was purified by flash chromatography to yield product 9 (5.6 g, 15.4 mmol, 80%). ¹H NMR (CDCl₃) 8.02-7.41
Compound 9 (5 g, 13.7 mmol) was dissolved in dry methanol (200 mL) and several small pieces of Na were added into the solution. The mixture was stirred at room temperature for 1 hour and Amberlite IR-120 ion-exchange resin (hydrogen form) was added and stirred for another 1 hour to neutralize the solution. The solution was then filtered and evaporated to dryness. The residue was extracted with chloroform and was washed with 1 M HCl, water, NaHCO₃, and brine, respectively. After drying and filtering, the extract was evaporated to give a light yellow mass, which was then recrystallized from cyclohexane as white crystals of 10 (2.9 g, 11.1 mmol, 81%). ¹H NMR (CDCl₃) δ 5.90 (d, 1 H, J₁₂ 4 Hz, H1), 4.48 (d, 1 H, J₁₂ 4 Hz, H2), 4.31-4.26 (m, 1 H, H5), 4.12 (dd, 1 H, H6a), 4.02 (d, 1 H, H4), 3.95 (dd, 1 H, H6b), 2.75 (bs, 1 H, OH), 1.46, 1.40, 1.32, 1.28 (4 x s, 12 H, 4 x OAc).

D-[3-²H]-Glucose (11)

Compound 10 (2.9 g, 11.1 mmol) was dissolved in water (200 mL) containing Amberlite IR-120 ion-exchange resin (50 mL), and the suspension was stirred and heated at 80°C for 5 hours. The resin was then filtered off and the filtrate evaporated to a syrup to give compound (11) (1.75 g, 9.72 mmol, 87%). ¹H NMR (α only) (D₂O) δ 4.50 (d, 1 H, J 4 Hz, H1), 3.75-3.60 (m, 2 H, H6a, H6b), 3.34-3.12 (m, 3 H, H2, H4, H5).
Penta-O-acetyl-β-D-[3-2H]-glucopyranose (12)

A suspension of sodium acetate (0.875 g, 10.7 mmol) in acetic anhydride (15 mL) was heated to boiling in a 250 mL three-neck flask equipped with a condensor. Compound 11 (1.75 g, 9.7 mmol), which was previously dissolved in a minimum amount of DMF, was slowly added. The mixture was cooled down to room temperature and then transferred with water (100 mL) and ethyl acetate (100 mL) to a separation funnel. The organic layer was thoroughly washed with water, NaHCO₃, 1 M HCl and water, respectively. The ethyl acetate layer was dried and evaporated to dryness to give a white solid. The white solid, a mixture of anomers, was further recrystallized from ethyl alcohol as pure β anomers (12) (2.9 g, 7.42 mmol, 76%). ¹H NMR (CDCl₃) δ 5.64 (d, 1 H, J 8 Hz, H1), 5.04 (m, 2 H, H2, H4), 4.21 (dd, 1 H, J 6a, 5 5, J 6a,6b 13 Hz, H6), 4.02 (dd, 1 H, J 6b, 2, J 6a,6b 13 Hz, H6’), 3.75 (m, 1 H, H5), 2.03, 2.00, 1.95, 1.93 (4 x s, 15 H, 5 x OAc).

4'-Nitrophenyl 2,3,4,6-tetra-O-acetyl-α-D-[3-2H]-glucopyranoside (13)

Tin(IV) chloride (1.2 mL, 11 mmol) was added to a solution of compound 12 (2 g, 5.1 mmol) and p-nitrophenol (1.04 g, 7.4 mmol) in CH₂Cl₂ (10 mL). The mixture was kept at 60°C for 5 hours after which 10 mL CH₂Cl₂ was added. The solution was then poured into an ice-cold saturated aqueous bicarbonate solution and filtered on a Celite bed. The solution was extracted with CH₂Cl₂ and the organic phase was washed again with saturated aqueous bicarbonate solution and water until the water phase was colorless. The organic layer was then dried over CaCl₂, filtered, and the solvent was evaporated to dryness. The product was then further purified by chromatography to yield
compound 13 (1.4 g, 2.98 mmol, 58%). $^1$H NMR (CDCl$_3$) $\delta$ 8.18 (d, 2 H, J 9 Hz, H3', H5'), 7.16 (d, 2 H, J 9 Hz, H2', H6'), 5.81 (d, 1 H, J 3.5 Hz, H1), 4.90-5.24 (m, 2 H, H2, H4), 4.10 (m, 2 H, H6a, H6b), 3.92 (m, 1 H, H5), 2.03, 2.00, 1.99, 1.98 (4 x s, 12 H, 4 x OAc).

4'-Nitrophenyl $\alpha$-D-[3-$^2$H]-glucopyranoside (14)

Compound 13 was deacetylated as described in §3.1.2.1. 4'-Nitrophenyl 2,3,4,6-tetra-O-acetyl-$\alpha$-D-[3-$^2$H]-glucopyranoside (13) (1.2 g, 2.55 mmol) was dissolved in dry methanol (50 mL) and several small pieces of sodium were added to the solution. The reaction was allowed to proceed for 30 minutes and was then neutralized with IR-120 resin (H$^+$ form) and worked up as previously described to yield syrup of compound 14 (0.74 g, 2.45 mmol, 96%). Compound 14 was further purified by column chromatography. $^1$H NMR (D$_2$O) $\delta$ 8.11 (d, 2 H, J 9 Hz, H3', H5'), 7.17 (d, 2 H, J 9 Hz, H2', H6'), 5.66 (d, 1 H, J 4 Hz, H1), 3.64 (d, 1 H, J 4 Hz, H2), 3.60-3.51 (m, 3 H, H5, H6a, H6b), 3.39 (d, 1 H, J 9 Hz, H4).

4'-Nitrophenyl $\alpha$-D-[3-$^2$H]-glucopyranoside-6-phosphate (15)

4'-Nitrophenyl $\alpha$-D-[3-$^2$H]-glucopyranoside (0.3 g, 1 mmol) was dissolved in trimethyl phosphate (1.1 mL). Water (18 µL, 1 mmol) was added and phosphorus oxychloride (0.28 mL, 1.11 mmol) pre-dissolved in trimethyl phosphate (1.1 mL), was slowly dropped into the solution. The mixture was kept in an ice-water bath and stirred for 3 hours. The reaction mixture was diluted to 20 mL with water, pH was adjusted to 6 with concentrated NH$_4$OH and charcoal (2 g), which had been washed with 1 M HCl and
dried by suction, was added. After the suspension had been stirred for 1 hour at room temperature, the charcoal was filtered and first washed with 0.2 M HCl 10 mL, followed by 60 mL of pyridine:water (1:4, v/v). After evaporating the pyridine, the solution was passed through Amberlite IR-120 ion-exchange resin (hydrogen form) and the pH was adjusted to 9 with cyclohexylamine. This basic solution was evaporated to dryness (0.16 g, 0.28 mmol, 28%) to give product 14, which was recrystallized from EtOH and diethyl ether as white crystals and stored at -20 °C. Compound 14 was transformed to the sodium form by passing it through Amberlite IR-120 ion-exchange resin (sodium form). After evaporating the water, a yellowish white powder was formed (15). \( ^1 \text{H NMR} \) (D\( _2 \)O) \( \delta \) 8.05 (d, 2 H, J 9 Hz, H3', H5'), 7.10 (d, 2 H, J 9 Hz, H2', H6'), 5.62 (d, 1 H, J1,2 3 Hz, H1), 3.90-3.80 (m, 2 H, H6a, H6b), 3.68-3.56 (m, 3 H, H2, H4, H5), \( ^{31} \text{P NMR} \) (D\( _2 \)O) \( \delta \) 3.98 (t, \( J_{P,H6a,H6b} \) 6 Hz); \( ^1 \text{H-}^{31} \text{P HSQC} \) 2D NMR confirms the coupling between P and H6a,H6b; Exact mass calc'd for C\( _{12} \)H\( _{14} \)DO\( _{11} \)NP: 381.0445; found: 381.0446; Exact mass calc'd for C\( _{12} \)H\( _{14} \)DO\( _{11} \)NPNa: 403.0264; found: 403.0266.

3.1.5 4'-Nitrophenyl \( \alpha \)-D-[2-\( ^2 \text{H} \)]-glucopyranoside-6-phosphate

Penta-O-acetyl-\( \beta \)-D-[2-\( ^2 \text{H} \)]-glucopyranose (16)

A suspension of sodium acetate (0.5 g, 6.1 mmol) in acetic anhydride (7 mL) was heated to boiling in a 100 mL three-neck flask equipped with a condenser. D-[2-\( ^2 \text{H} \)]-Glucose (obtained from Cambridge Isotopes) (1 g, 5.52 mmol) was added slowly in portions. The mixture was cooled to room temperature and then transferred to a separation funnel with water (50 mL) and ethyl acetate (50 mL). The organic layer was thoroughly washed with water, NaHCO\( _3 \), 1 M HCl, water and brine, respectively. The
ethyl acetate layer was dried and evaporated to dryness to yield a white solid. The white solid, a mixture of anomers, was further recrystallized from ethyl alcohol as the pure β anomer 16 (1.78 g, 4.55 mmol, 75%). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 5.69 (s, 1 H, H1), 5.22 (d, 1 H, J 9 Hz, H3), 5.10 (t, 1 H, J 9 Hz, H4), 4.26 (dd, 1 H, J\(_{6a,5}\) 5, J\(_{6a,6b}\) 13 Hz, H6a), 4.08 (dd, 1 H, J\(_{6b,5}\) 2, J\(_{6a,6b}\) 13 Hz, H6b, 3.81 (m, 1 H, H5), 2.09, 2.06, 2.00, 1.98 (4 x s, 15 H, 5 x OAc).

4'-Nitrophenvl 2,3,4,6-tetra-O-acetyl-α-D-[2-\(^2\)H]-glucopyranoside (17)

Tin(IV) chloride (0.9 mL, 8.3 mmol) was added to a solution of compound 16 (1.5 g, 3.8 mmol) and p-nitrophenol (0.78 g, 5.55 mmol) in CH\(_2\)Cl\(_2\) (7.5 mL). The mixture was stirred at 60°C for 5 hours after which 7.5 mL CH\(_2\)Cl\(_2\) was added. The solution was then poured into an ice-cold saturated aqueous bicarbonate solution and filtered on a Celite bed. The solution was extracted with CH\(_2\)Cl\(_2\) and the organic phase was washed again with saturated aqueous bicarbonate solution and water until the water phase was colorless. The organic layer was then dried over CaCl\(_2\) and the solvent was evaporated to dryness. The product was further purified by chromatography to yield compound 17 (0.89 g, 1.9 mmol, 50%). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.20 (d, 2 H, J 9 Hz, H3'), 7.18 (d, 2 H, J 9 Hz, H2', H6'), 5.82 (s, 1 H, H1), 5.66 (d, 1 H, J 9 Hz, H3), 5.15 (t, 1 H, J 9 Hz, H4), 4.12 (m, 2 H, H6a, H6b), 3.94 (m, 1 H, H5), 2.03, 2.00, 1.99, 1.98 (4 x s, 12 H, 4 x OAc).
4'-Nitrophenyl α-D-[2-2H]-glucopyranoside (18)

The per-acetylated derivative (17) was deacetylated as described in §3.1.2.1. 4'-Nitrophenyl 2,3,4,6-tetra-O-acetyl-α-D-[2-H]-glucopyranoside (0.8 g, 1.7 mmol) was dissolved in dry methanol (50 mL) and several small pieces of sodium were added to the solution. The reaction mixture was stirred for 30 minutes and then neutralized with IR-120 resin (H⁺ form) and worked up as previously described to form a syrup of compound 18, which was further purified by column chromatography to yield 4'-Nitrophenyl α-D-[2-2H]-glucopyranoside (18) (0.36 g, 1.19 mmol, 70%). ¹H NMR (D₂O) δ 8.10 (d, 2 H, J 9 Hz, H3', H5'), 7.16 (d, 2 H, J 9 Hz, H2', H6'), 5.67 (s, 1 H, H1), 3.81 (d, 1 H, J 9 Hz, H3), 3.61-3.51 (m, 3 H, H5, H6a, H6b), 3.39 (t, 1 H, J 9 Hz, H4).

4'-Nitrophenyl α-D-[2-2H]-glucopyranoside-6-phosphate (20)

4'-Nitrophenyl α-D-[2-2H]-glucopyranoside (18) (0.3 g, 1 mmol) was dissolved in trimethyl phosphate (1.1 mL). Water (18 μL, 1 mmol) was added and phosphorus oxychloride (0.28 mL, 1.11 mmol) pre-dissolved in trimethyl phosphate (1.1 mL), was slowly dropped into the solution. The mixture was kept in an ice-water bath, stirred for 3 hours, then diluted to 20 mL with water and the pH was adjusted to 6 with concentrated NH₄OH. Activated charcoal (2 g), which had been washed with 1 M HCl and dried by suction, was then added. After the suspension had been stirred for 1 hour at room temperature, the charcoal was filtered and first washed with 10 mL 0.2 M HCl, then with 60 mL of pyridine-water (1:4, v/v). After evaporating the pyridine, the solution was passed through Amberlite IR-120 ion-exchange resin (hydrogen form) and the pH was adjusted to 9 with cyclohexylamine. The basic solution was evaporated to dryness (0.16
(g, 0.28 mmol, 28%) to give product 19 which was recrystallized from EtOH and diethyl ether as white crystals and stored at -20 °C. Product 19 was transformed to the sodium form by passing an aqueous solution through Amberlite IR-120 ion-exchange resin (sodium form). After evaporating the water, a yellowish white powder of the sodium salt (20) was obtained. $^1$H NMR (D$_2$O) $\delta$ 8.08 (d, 2 H, J 9 Hz, H3', H5'), 7.13 (d, 2 H, J 9 Hz, H2', H6'), 5.64 (s, 1 H, H1), 3.92-3.78 (m, 2 H, H6a, H6b), 3.70-3.59 (m, 4 H, H3, H4, H5), $^{31}$P NMR (D$_2$O) $\delta$ 3.96 (t, $J_{P,H6a,b}$ 6 Hz); $^1$H-$^{31}$P 2D NMR HSQC confirms the coupling between P and H6a,H6b; Exact mass calc'd for C$_{12}$H$_{14}$DO$_{11}$NP: 381.0445; found: 381.0447; Exact mass calc'd for C$_{12}$H$_{13}$DO$_{11}$NPNa: 403.0264; found: 403.0268.

3.1.6 Maltose-6'-phosphate

1,2,3,6,2',3'-Hexa-O-acetyl-4',6'-O-benzylidene maltose (22)(α/β anomer mixture)

Maltose (4.00 g, 11.1 mmol) was dissolved in dry DMF (100 mL) at 50°C over 30 minutes. Benzaldehyde dimethylacetal (1.8 mL, 12 mmol) and p-toluenesulfonic acid (~10 mg) were added, and the mixture was rotated at 60-70°C on a rotary evaporator under vacuum to remove methanol that formed. The reaction was allowed to continue for 8 hours, during which time DMF was occasionally added to keep the solution at a constant volume. The mixture was then neutralized with a basic ion-exchange resin (Dowex-1, OH$^-$ form) and then filtered. The DMF was evaporated at 40°C under high vacuum to yield a syrup which was then transferred into a separation funnel with ethyl acetate and water. The organic phase was extracted twice with water and the combined water phase fractions were washed three times with ethyl acetate. The aqueous phase was concentrated by evaporation and purified by flash chromatography. The 4',6'-O-
benzylidene compound 21 (2.37 g, 5.51 mmol, 50%) obtained was then dissolved in dry pyridine (30 mL) and acetic anhydride (20 mL) was added and the mixture kept at room temperature with stirring overnight. After the acetylation reaction was complete, the mixture was evaporated at 40°C under high vacuum to remove most of the pyridine and acetic acid. The remaining residue in the flask was transferred with EtOAc and water to a separation funnel. The organic phase was washed with 1 M HCl, saturated sodium bicarbonate, water and brine, respectively. The organic phase was then dried, filtered, and evaporated to dryness to yield a white solid of compound 22 (3.71 g, 5.45 mmol, 49% from maltose), which was further purified by column chromatography. 

\[ \text{H NMR (CDCl}_3\text{) (}\alpha\text{ anomer only) } \delta 7.41-7.31 (m, 5 H, Ar), 6.21 (d, 1 H, J 4 Hz, H1), 5.50-5.43 (m, 3 H, H3, H3', H1''), 5.35 (d, 1 H, J 4 Hz, H1''), 4.95 (dd, 1 H, J1,2 4 Hz, J2,3 10 Hz, H2), 4.88 (dd, 1 H, J1,2 4 Hz, J2,3 10 Hz, H2), 4.12-4.08 (m, 1 H, H5), 3.86-3.80 (m, 1 H, H5'), 3.70 (t, 1 H, J 9 Hz, H4), 3.62 (t, 1 H, J 10 Hz, H4'), 2.20, 2.09, 2.06, 2.03, 2.01,1.97 (6 x s, 18 H, 6 x OAc).

\[ \text{1,2,3,6,2',3'-Hexa-O-acetvl-maltose (23) (}\alpha/\beta\text{ anomer mixture) } \]

Compound 22 (3.41 g, 5.00 mmol) was dissolved in ethanol (40 mL) and cyclohexene (20 mL), 20% palladium hydroxide on carbon (0.85 g) was added and the suspension was stirred under reflux for 5 hours. The catalyst was removed by filtration and the filtrate was evaporated to dryness to yield compound 23 (2.82 g, 4.74 mmol) which was then purified by chromatography. 

\[ \text{H NMR (CDCl}_3\text{) (}\alpha\text{ anomer only) } \delta 6.17 (d, 1 H, J 4 Hz, H1), 5.44 (t, 1 H, J 8 Hz, H3), 5.32-5.12 (m, 2 H, H1', H3''), 4.94-4.88 \]
(m, 1 H, H2), 4.72-4.66 (m, 1 H, H2'), 4.44-4.37 (m, 1 H, H6a), 4.18-4.13 (m, 1 H, H6b),
4.00-3.94 (m, 1 H, H4), 3.81-3.57 (m, 5 H, H4', H5, H5', H6'a, H6'b), 2.73 (bs, 2 H, 2 x
OH), 2.16, 2.06, 2.03, 2.02, 2.00, 1.98 (6 x s, 18 H, 6 x OAc).

1,2,3,6,2',3'Hexa-O-acetyl-6'-diphenylphosphoryl-maltose (24) (α/β anomer mixture)

Compound 23 (2.08 g, 3.50 mmol) was dissolved in dry pyridine (100 mL),
diphenyl chlorophosphate (0.75 mL, 3.60 mmol) was added and the mixture was stirred
at room temperature for 24 hours. When the reaction was complete, as judged by TLC
analysis, water (10 mL) was added and the mixture stirred for 1 hour. The solution was
then evaporated at 40 °C under high vacuum to remove most of the pyridine, then the
remaining residue in the flask was transferred with EtOAc and water to a separation
funnel. The organic phase was washed with 1 M HCl, saturated sodium bicarbonate,
water and brine, respectively. The organic phase was dried, filtered, evaporated to
dryness, and then purified by column chromatography to yield product 24 (2.15 g, 2.60
mmol). ¹H NMR (CDCl₃) (α anomer only) δ 6.20 (d, 1 H, J 4 Hz, H1), 5.45 (t, 1 H, J 10
Hz, H3), 5.30 (d, 1 H, J 4 Hz, H1'), 5.20 (t, 1 H, J 10 Hz, H3'), 4.89 (m, 1 H, H2), 4.61-
4.58 (m, 2 H, H2', H6a'), 4.38 (m, 1 H, H6a), 4.26 (m, 1 H, H6b'), 4.10 (m, 1 H, H6b),
4.04 (m, 1 H, H5), 3.93 (m, 1 H, H4), 3.71 (m, 1 H, H5'), 3.61 (bs, 1 H, OH), 3.45 (m, 1
H, H4'), 2.07, 2.04, 2.03, 1.99, 1.98, 1.97 (6 s, 18 H, 6 OAc).

Maltose-6'-phosphate (27)

Compound 24 (2.07 g, 2.50 mmol) was dissolved in methanol and deacetylated
with sodium methoxide using the method described in §3.1.2.1 to produce compound 25.
This compound, without characterization, was dissolved in dry methanol and hydrogenolysed with PtO₂ (200 mg) as a catalyst to produce compound 26. The reaction was monitored by TLC (ethyl acetate:methanol:water = 7:2:1). Upon completion of the reaction, the catalyst was filtered and the solvent was evaporated to dryness. The residue was then dissolved in water, washed with ethyl acetate, passed through Amberlite IR-120 ion-exchange resin (H⁺ form) and the pH then adjusted to 9 with cyclohexylamine. The solution was then evaporated to dryness and the product crystallized from ethanol/ether. Conversion to the sodium form was achieved by passing it through a cation exchange resin (sodium form) to yield compound 27 (0.49 g, 1.05 mmol). ¹H NMR (D₂O) δ 5.35 (d, 1 H, J 3 Hz, H1'), 4.65 (d, 1 H, J 8 Hz, H1), 4.03-3.93 (m, 3 H, H6'a, H6'b, H6b), 3.78-3.75 (m, 3 H, H6a, H5, H5'), 3.69-3.59 (m, 5 H, H2', H3, H3', H4, H4'), 3.29 (t, 1 H, J 8 Hz, H2). Exact mass calc'd for C₁₂H₂₂O₁₄P: 421.0747; found: 421.0748.

3.1.7 2-Deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride

2-Deoxy-2-fluoro-β-D-glucosyl fluoride (29)

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucosyl bromide (synthesized by Dr. Yasushi Todoroki) (0.504 g, 1.36 mmol) was dissolved in 8 mL acetonitrile and silver fluoride (1.2 g, 9.46 mmol) was added. The suspension was stirred for 48 h at room temperature and then filtered. The solution part was then evaporated to dryness to yield white solid, which was further purified by column chromatography to yield product 28. (0.390 g, 1.26 mmol, 93%) The ¹H NMR was consistent with literature data. This compound (28)(0.31 g, 1 mmol) was then deacylated by following the method outlined
in the §3.1.2.1 to yield product 29 (0.170 g, 0.92 mmol, 92%) The $^1$H NMR was consistent with the literature data.82

2-Deoxy-2-fluoro-6-(diphenylphospho)-β-D-glucosyl fluoride (30)

Compound 29 (0.170 g, 0.92 mmol) was dissolved in a mixture of pyridine (1.14 mL) and 1,4-dioxane (3.8 mL) at 0 °C. Diphenyl chlorophosphate (190 μL, 0.92 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The mixture was then evaporated and the residual oil was dissolved in chloroform. The organic phase was washed with 1 M HCl, 0.1 M sodium bicarbonate, water and dried by calcium chloride. It was then concentrated to dryness to yield a clear syrup which was further purified by column chromatography to get product 30 (0.280 g, 0.67 mmol, 73%). $^1$H NMR (CDCl$_3$) δ 7.33-7.14 (m, 10 H, Ar), 5.13 (ddd, 1 H, J$_{1,F1}$ 53, J$_{1,F2}$ 3.8, J$_{1,2}$ 6.6 Hz, H1), 4.65 (d, 1 H, J$_{4,OH}$ 4.6 Hz, OH4), 4.50-4.35 (m, 3 H, H6, H6',OH3), 4.13 (m, 1 H, H2), 3.63 (m, 1 H, H3), 3.50 (m, 1 H, H5), 3.39 (m, 1 H, H4), $^{31}$P NMR (CDCl$_3$) δ -10.2 (t, J$_{P,H6a,6b}$ 9 Hz), $^{19}$F NMR (CDCl$_3$) δ -65.7 (m, J$_{F1,H1}$ 53, J$_{F1,F2}$ 16, J$_{F1,H2}$ 16 Hz, F1), δ = 124.9 (m, J$_{F1,F2}$ 16, J$_{F2,H3}$ 16, J$_{F2,H2}$ 50 Hz, F2).

2-Deoxy-2-fluoro-6-phospho-β-D-glucosyl fluoride (31)

Compound 30 (0.270 g, 0.65 mmol) was dissolved in dry methanol (50 mL) and PtO$_2$ (100 mg) was added into the solution. The suspension was then stirred under 1 atm. pressure of hydrogen at room temperature overnight. The solution was then filtered, concentrated and the residue dissolved in 10 mL of water. The aqueous solution was then passed through Amberlite IR-120 ion-exchange resin (sodium form) and lyophilized to
yield product 31 (0.191 g, 0.62 mmol, 95%). $^1$H NMR (D$_2$O) δ 5.38 (ddd, 1 H, J$_{1,1}$ 53, J$_{1,2}$ 3.5, J$_{1,2}$ 6.9 Hz, H1), 4.24 (m, 1 H, H2), 4.03-3.89 (m, 2 H, H6a, H6b), 3.77-3.66 (m, 1 H, H3), 3.60-3.42 (m, 2 H, H4, H5), $^{31}$P NMR (D$_2$O) δ 2.36, $^{19}$F NMR (D$_2$O) δ –67.5 (m, J$_{F1,H1}$ 53, J$_{F1,F2}$ 16, J$_{F1,H2}$ 16 Hz, F1), δ –125.9 (m, J$_{F1,F2}$ 16, J$_{F2,H3}$ 16, J$_{F2,H2}$ 50 Hz, F2).

3.2. Enzymology

3.2.1 General Procedures

A plasmid containing the gene $Bglu$ in the pET28 vector was generously provided by Dr. Annabelle Varrot of the Department of Chemistry, University of York, York, United Kingdom. The plasmid was stored at 4°C.

All absorbance measurements were recorded on a UNICAM UV/Vis UV4 spectrometer equipped with a circulating water bath. Acryl-cuvettes were used to measure the absorbance at wavelengths greater than 350 nm and quartz cuvettes were used at wavelengths less than 350 nm. Both types of cuvettes had a 1 cm path length.

All the substrates were obtained synthetically, as outlined in § 3.1.

$K_m$ and $V_{max}$ parameters were determined by non-linear regression by using Grafit 4.0.

3.2.2 Enzyme expression and purification

The constructed pET28B$glu$ expression vector was transformed into $Escherichia coli$ BL21(DE3) cells via electroporation. The $E. coli$ transformants were selected on LB$_{kan}$ (50 µg/mL) agar plates. Several single colonies were picked and grown overnight
in 25 mL of LB<sub>kan</sub> at 37 °C. These cultures were subsequently used to inoculate 4 x 500 mL LB<sub>kan</sub>. After the culture grew to an OD<sub>595</sub> of 0.6 at 37 °C, 0.15 mM isopropyl β-D-thiogalactoside (IPTG) was added to induce protein expression overnight at 16 °C. Induced cells were then harvested by centrifugation (10 min at 5000 rpm) and half of the pellet was resuspended in 25 mL of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). The cell suspension was passed through a French press twice and centrifuged at 15000 rpm for 30 min at 4 °C to yield soluble cell extract.

The soluble cell extract was applied to a 30 mL chelating fast flow column charged with nickel. The column was washed with 10 volumes of binding buffer (20 mM Tris-HCl pH 7.5, 5 mM imidazole and 500 mM NaCl) and then washed with 6 volumes of wash buffer (20 mM Tris-HCl pH 7.5, 60 mM imidazole and 500 mM NaCl) to wash away any non-specifically bonded protein. The desired protein was then eluted from the Ni<sup>2+</sup>-column with elution buffer (20 mM Tris-HCl pH 7.5, 250 mM imidazole and 500 mM NaCl). Fractions were analyzed using SDS-polyacrylamide gel electrophoresis and assayed for activity with pNP-β-Glc6P (2 mM) as the substrate in buffer composed of 25 mM Tris HCl pH 8, 0.1 mM NAD and 1 mM Mn<sup>2+</sup>. Fractions containing pure protein were pooled and concentrated to 10 mL. The protein was dialysed in steps as follows: 50 mM Tris HCl pH 8 buffer containing 20 mM EDTA to remove any metals which may bonded to the desired protein (Bglu); 50 mM Tris HCl pH 8 to remove the EDTA; and 50 mM Tris HCl pH 8 containing 1 mM Mn<sup>2+</sup> and 0.1 mM NAD to finally leave the prepared protein (Bglu) in optimal condition for storage. The protein was stored at 4 °C.
3.2.3 Basic enzyme kinetics

Prior to kinetic analysis, the protein was dialyzed against 20 mM EDTA in 50 mM Tris HCl pH 8 to remove Mn$^{2+}$ and NAD and then 50 mM Tris HCl pH 8 to remove the EDTA. This procedure yields metal-free and NAD-free protein samples for kinetic studies.

Measurement of enzyme activity

The rate of hydrolysis of nitrophenyl glycoside substrate was determined by the continuous spectrometric monitoring of the release of pNP at 400 nm ($\Delta A_{400}/\text{min}$) over 0.5 to 5 minutes. Spectrometer cuvettes contained 0.1% BSA (w/v) in 50 mM Tris-HCl, pH 7.5, 0.1 mM NAD$^+$ and 1 mM Mn$^{2+}$. The enzyme was pre-incubated in the above buffer in cuvettes at 37 °C for 5 minutes and assays were initiated by the addition of the substrate solution. The activity of Bglu was determined with pNP-β-Glc6P using at least 7 different concentrations from 0.005 mM – 0.150 mM and the activity of GlvA was determined with pNP-α-Glc6P using at least 7 different concentrations from 0.02 mM – 0.650 mM.

Requirement of divalent metal for activity

The activity of Bglu was determined as described above in the presence of different divalent metals (1 mM): Mg$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$ to substitute Mn$^{2+}$ as the cofactor. The concentration of substrate pNP-β-Glc6P was fixed at 0.2 mM.

Activity testing for other potential substrates for Bglu

The activity of Bglu was also determined as described above by using pNP-α-Glc6P and pNP-β-glucuronide as the substrate at 1 mM concentrations.
3.2.4 Bglu catalyzed substrate hydrolysis in D$_2$O: product analysis

The substrate 4'-nitrophenyl β-D-glucopyranoside-6-phosphate (20 mg, 0.047 mmol) was lyophilized 4 times with 99.96% D$_2$O. The assay buffer (5 mL), containing 50 mM Tris HCl pH 8, 1 mM Mn$^{2+}$, 0.1 mM NAD$^+$, was also lyophilized 4 times with 99.9% D$_2$O. The 6-phospho-β-glucosidase Bglu (2 mg) was dialysed with 99.96% D$_2$O by repeated concentration and dilution using a 30 kDa molecular mass cut off centrifugal concentrator. After dissolving the substrate in deuterated assay buffer, the enzyme was added and the mixture was incubated at 37 °C overnight. The reaction was monitored by TLC and the enzyme was removed using a 30 kDa molecular mass cut off centrifugal concentrator after the reaction had finished. The solution was then adjusted to pH 7.0, Chelex-100 (100 mg) was added and the mixture stirred overnight. The Chelex-100 resin was removed by filtration, the solution was lyophilized to dryness and redissolved in 500 µL 99.96% D$_2$O for NMR experiments. The solution in the NMR tube was then transferred into a 1.5 mL eppendorf tube and 100 units of alkaline phosphatase were added. The solution was incubated at 37 °C overnight and monitored by TLC. After the reaction was complete, the enzyme was removed as described above and the solution was lyophilized to dryness.

The residue was dissolved in 1.5 mL dry pyridine and 1 mL acetic anhydride was added. The mixture was stirred at room temperature overnight and worked up as outlined in § 3.1.2.2. The final product was then analyzed by $^1$H NMR.
3.2.5 Stereochemistry experiments

Substrate methanolysis was first tested on a small scale. Reaction was monitored by TLC using aluminum-backed silica plates and a solvent of 3:1:1 butanol:acetic acid:water. The plates were dried and visualized first with UV light and then by charring with 10% sulphuric acid/methanol. Once the optimum concentration of methanol was selected, the Bglu catalyzed methanolysis reactions were performed on a large scale. The substrate 4'-nitrophenyl \( \beta \)-D-glucopyranoside-6-phosphate (20 mg, 0.047 mmol) was dissolved in 5 mL assay buffer containing 50 mM Tris HCl pH 8, 1 mM Mn\(^{2+}\), 0.1 mM NAD\(^+\) and 3 M methanol. The 6-phospho-\( \beta \)-glucosidase Bglu (5 mg) was added and the mixture was incubated at 37 °C overnight. The reaction was monitored by TLC and the enzyme Bglu was removed by using a 30 kDa molecular mass cut off centrifugal concentrator after the reaction was complete. The solution was then adjusted to pH 7, and Chelex-100 (100 mg) was added and stirred overnight. The Chelex-100 resin was then removed by filtration, and the solution was lyophilized to dryness and redissolved in 500 \( \mu \)L 99.96% D\(_2\)O for NMR analysis. After the NMR experiment, the sample solution in the NMR tube was transferred into a 1.5 mL eppendorf tube and 100 units of alkaline phosphatase were added. The solution was incubated at 37 °C overnight and monitored by TLC. After the reaction was finished, the alkaline phosphatase was removed as described above and the solution was lyophilized to dryness.

The residue was dissolved in 1.5 mL dry pyridine, 1 mL acetic anhydride was added and the mixture was stirred at room temperature overnight and worked up as outlined in § 3.1.2.2. The final products were subjected to NMR analysis.
Parallel experiments were carried out for methanolysis, except that the reaction was performed in D₂O and CD₃OD instead of water and methanol and all the substrates and buffer were lyophilized with D₂O and the enzyme was dialyzed with D₂O as outlined in §3.2.4.

3.2.6 Sequence similarity search and alignment

Sequence similarity search for Bglu was performed using the BLAST 2.0 program (found at http://www.ch.embnet.org/software/BottomBLAST.html?), sponsored by the Swiss Institute of Bioinformatics. The SwissProt protein database was used for searching for similar protein sequences.

Sequence alignments for the family 4 members were performed using the Clustal W alignment program (found at http://www.ebi.ac.uk/clustalw/), sponsored by the European Bioinformatics Institute.
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