MECHANISTIC STUDIES ON HUMAN PANCREATIC \( \alpha \)-AMYLASE

by

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 2000

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Department of **Biochemistry & Molecular Biology**

The University of British Columbia
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Date **August 3, 2000**
Abstract

Human pancreatic α-amylase (HPA) is known to hydrolyze malto-oligosaccharides with retention of anomeric configuration utilizing at least two active site carboxylic acids as nucleophile and acid/base catalyst in a double displacement reaction. The roles of three conserved active site carboxylic acids (D197, E233, and D300) in the catalytic mechanism of HPA were studied by utilizing site-directed mutagenesis in combination with structural and kinetic analyses of the recombinant enzymes. All three residues were altered to both alanine and the corresponding amide, in addition several other variants were generated (E233A/D300A, E233K, D300H, D300Y).

The ten mutants, and the wild-type HPA, were successfully expressed in *Pichia pastoris*, and were secreted at modest levels (10 mg/L). Isolation of the mutant proteins required development of a new purification strategy since the affinity precipitation used to purify wild-type HPA was demonstrated to be ineffective on the low activity α-amylases. Hydrophobic interaction chromatography followed by anion exchange chromatography provided the desired mutant enzymes in highly purified form. In addition, the recombinant enzymes were shown, by electrospray ionization mass spectrometry (ESI-MS) in combination with endoglycosidase F treatment, to be heterogeneously glycosylated at a single asparagine (N461). Partial deglycosylation of the recombinant enzymes allowed for their successful crystallization and subsequent structural characterization to 1.8 Å resolution.

Kinetic analyses were performed on the mutants, utilizing a range of substrates. All results suggested that D197 was the nucleophile, since virtually all activity (>10⁵ fold decrease in $k_{cat}$ values) was lost for the enzymes mutated at this position when assayed
with each substrate. The significantly greater second order rate constant of E233 mutants on "activated" substrates \( (k_{\text{cat}}/K_m \text{ value for } \alpha\text{-maltotriosyl fluoride} = 15 \text{ s}^{-1}\text{mM}^{-1}) \) compared with "unactivated" substrates \( (k_{\text{cat}}/K_m \text{ value for maltopentaose} = 0.0030 \text{ s}^{-1}\text{mM}^{-1}) \) strongly suggested that E233 is the general acid catalyst. Data for the D300 mutants will be presented, although the role of D300 remains unclear.

In conclusion, structural and kinetic analyses of wild-type and mutant HPAs have provided strong evidence for the roles of two conserved active site carboxylic acids (D197 and E233) in its double displacement mechanism, while the role of D300 remains elusive.
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<td>2Cl2FαGluCl</td>
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<td>HF-pyridine</td>
<td>Hydrogen fluoride-pyridine (70 % hydrogen fluoride)</td>
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Amino Acid Abbreviations

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<td>Met</td>
<td>(M) Methionine</td>
</tr>
<tr>
<td>Phe</td>
<td>(F) Phenylalanine</td>
</tr>
<tr>
<td>Pro</td>
<td>(P) Proline</td>
</tr>
<tr>
<td>Ser</td>
<td>(S) Serine</td>
</tr>
<tr>
<td>Thr</td>
<td>(T) Threonine</td>
</tr>
<tr>
<td>Typ</td>
<td>(W) Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>(Y) Tyrosine</td>
</tr>
<tr>
<td>Val</td>
<td>(V) Valine</td>
</tr>
</tbody>
</table>

Kinetic Constants

- $k_{\text{cat}}$: Catalytic rate constant
- $K_i$: Dissociation constant for the enzyme-inhibitor complex
- $K_m$: Michaelis-Menten constant
- $rFK_i$: "Range finder" $K_i$
- $V_{\text{max}}$: Maximal rate of an enzyme-catalyzed reaction
Acknowledgements

This project would not have been possible without the help of many people, to all of whom I owe a debt of gratitude. First, I would like to thank Dr. R.A.J. Warren for his generous gift of the endoglycosidase F-cellulose binding domain fusion protein and Mr. Shouming He for obtaining the mass spectra. Their valuable resources saved much time and heartache, and continue to do so. Second, my gratitude goes to Drs. Hung Vo and Hélène Côté who were responsible for the initial cloning of HPA, and the early stages of mutagenesis, and to Dr. Ross MacGillivray for his help on my first manuscript and for providing lab space for me in my first term at UBC. Dr. Gary Brayer’s continuous enthusiasm in this project, and the unselfish sharing of his resources were greatly appreciated. Thanks also to Dr. Robert Maurus, and Mr. Gary Sidhu for their work on solving the various amylase structures, and for many thought provoking conversations regarding science and life. The many interesting discussions, and helpful advice of Mrs. Yili Wang were an enormous aid through the early stages of this thesis, and continue to keep me grounded in reality. Thanks to Mr. Angus McQuibbin, Dr. Bjorn Steffenson, and the rest of the Overall lab for much useful advice in molecular biology and protein purification. My sincerest gratitude to Dr. Chris Overall for taking me into his lab through much of this project, and providing me with space and resources when both were very limited. Thanks especially go to members of the Withers lab, past and present, who have provided me with invaluable support and insight, as well as friendship. Never have I had the chance to work with a better group of people.
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In addition, I would like to acknowledge the loving support of my family. I would like to express my thanks to my sister Teresa for, although we have grown apart, as sibling often do, I still cherish our friendship. Finally, words cannot express my gratitude to my parents, Elvin and Marsha Rydberg. Although I am travelling ever farther from you, I will always appreciate everything you have done for me.
Chapter 1

General Introduction
Carbohydrates have been a topic of scientific study for almost two hundred years. For much of that time the primary interest has been in the industrial uses of carbohydrates such as their roles in fermentation and pulp and paper manufacturing. As the study of carbohydrates has increased over the last several decades so too has the understanding of the role that these molecules play in biology. Complex carbohydrates are used in several forms for storage of the convenient energy source glucose (as glycogen in mammals, starch in plants, trehalose in insects, and cyclodextrins in some bacteria). They are crucial for the formation of rigid biological structures such as cell walls (as peptidoglycan) and on a macroscopic scale they provide the structure for plant stems and trunks (as cellulose), and are the primary component in the exoskeletons (as chitin) of various organisms (see (Voet, D. and Voet, J.G. 1990) for an overview). They have been found to be ubiquitously associated with mammalian cells and perform roles in cellular recognition, both by the organism’s immune system as well as by pathogens, and cellular mobilization. Glycoproteins, molecules primarily composed of protein with some carbohydrate, are often rendered more stable to thermal denaturation or proteolytic cleavage by the carbohydrate groups while proteoglycans, molecules that are primarily carbohydrate with some protein, have a wide range of functions including shock absorption, inhibition of blood clotting, and modulation of growth factor effects, to name a few (see (Voet, D. and Voet, J.G. 1990) for an overview). With this broad spectrum of effects, it is not difficult to see why carbohydrate research has increased in prominence in the last few decades. Apart from the basic knowledge to be gained, there are many medical and industrial applications that will benefit from research in this field.
Polysaccharides are metabolized by two large groups of enzymes. Glycosyl transferases are responsible for the anabolism of polysaccharides and glycosyl hydrolases (glycosidases) perform the catabolic steps. In order to fully exploit these enzymes for industrial (fermentation, pulp and paper) or medicinal purposes (as drug targets) their catalytic mechanisms must be understood in detail. Such understanding comes from the identification and characterization of crucial catalytic residues as well as the identification of intermediates in the reactions. Knowledge of these mechanistic facets will allow for the development of engineered enzymes with desired properties as well as the production of new inhibitors for use in controlling carbohydrate-related medical conditions.

This thesis addresses unresolved questions regarding the mechanism of human pancreatic α-amylase (HPA). It attempts to build a foundation of kinetic and structural evidence, aided by site-directed mutagenesis, to facilitate the elucidation of the catalytic roles of three conserved active-site carboxylic acids. Resolution of the mechanistic details of HPA could have ramifications regarding drug design for control of some dietary carbohydrate disorders (such as diabetes or obesity), development of \textit{in vivo} imaging agents for use in diagnostic tests, as well as enhancing our understanding of glycosidase mechanisms.
1.1 Hydrolysis of α(1,4) Glycosidic Linkages

1.1.1 Glycosyl Hydrolases

As mentioned earlier, catabolism of carbohydrates occurs via the action of a large group of enzymes known as glycosyl hydrolases or glycosidases. Glycosidases are ubiquitous in nature being found in all organisms from archaebacteria to humans. They are largely responsible for generating dietary monosaccharides although they play roles in other processes, such as the remodeling of glycoprotein carbohydrates in the protein secretory pathways of mammals and in infectious pathways by pathogens. The immense diversity of substrate specificities and sequences in this group of enzymes, and even between enzymes of the same function, initially hampered studies in this field. Even with the broad grouping of glycosidases into inverting and retaining enzymes (discussed later), which aided mechanistic studies greatly, the incredible numbers and differences within the class was still a significant hindrance to their study.

Fortunately, like many complex problems, a few important generalizations were observed that allowed for the development of a systematic, meaningful method of characterization for glycosidases. With an increasing number of three-dimensional protein structures being solved by x-ray diffraction and NMR studies there has come the realization that, although the sequences of the members of a protein family might be very different, there appear to be only a small number of different global folds. In other words, although there may be very little sequence similarity between different enzymes of similar function, there is often a very high degree of structural similarity. At the same time as these structural insights were being made, Henrisatt and Davies (Henrissat, B.
1991; Henrissat, B. et al. 1995; Henrissat, B. and Davies, G. 1997) made the discovery that, despite having low overall sequence similarities, many glycosidases of similar function shared several small regions of very high sequence similarity. Sequence alignment of these regions, which have been shown to be crucial active-site loops in many cases, has been used to classify almost all of the glycosidases into families (more than 80 families at last count). The similarities in overall global fold have been used to categorize these families into clans (10 at last count - Table 1). This information is compiled in the “Carbohydrate Active enZYmes” (CAZY) database, which is accessible on the world wide web at the URL http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html (Coutinho, P.M. and Henrissat, B. 1999; Coutinho, P.M. and Henrissat, B. 1999).

<table>
<thead>
<tr>
<th>GH-Clan</th>
<th>Families</th>
<th>Enzyme Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 51, 53</td>
<td>β-glucosidase, β-galactosidase, 6-phospho-β-galactosidase, 6-phospho-β-glucosidase, lactase-phlorizin hydrolase, β-mannosidase, myrosinase, β-glucuronidase, endoglucanase, β-mannanase, endo-1,3-glucanase, endo-1,6-β-glucanase, xylanase, endoglucoceramidase, endo-1,3-β-xylanase, cellobiohydrolase, lichenase, α-L-iduronidase, α-L-arabinofuranosidase, endo-1,4-β-galactanase</td>
</tr>
<tr>
<td>B</td>
<td>7, 16</td>
<td>β-endoglucanase, cellobiohydrolase</td>
</tr>
<tr>
<td>C</td>
<td>11, 12</td>
<td>xylanase</td>
</tr>
<tr>
<td>D</td>
<td>27, 36</td>
<td>α-galactosidase, α-N-acetylgalactosaminidase, isomalto-dextranase</td>
</tr>
<tr>
<td>E</td>
<td>33, 34</td>
<td>sialidase or neuraminidase, trans-sialidase</td>
</tr>
<tr>
<td>F</td>
<td>43, 62</td>
<td>β-xylosidase, α-L-arabinofuranosidase, arabinanase, xylanase</td>
</tr>
<tr>
<td>G</td>
<td>37, 63</td>
<td>trehalase</td>
</tr>
</tbody>
</table>
Because the above insights have revealed a direct relationship between structure and sequence, the Henrissat and Davies categorization has several advantages over the traditional Enzyme Commission classification, which is based largely on substrate specificity. First, it better reflects the structural features of these enzymes. Second, it may help reveal evolutionary relationships between glycosidases. Third, and most important for this thesis, this method of classification may prove useful for gaining insights into the mechanistic details of the glycosidases (Henrissat, B. 1991; Henrissat, B. and Bairoch, A. 1993).

1.1.2 Inverting vs Retaining

Glycosyl hydrolases have been broadly classified as either inverting or retaining based on the stereochemistry of their products with respect to the substrates (Figure 1). An inverting glycosidase yields a product whose anomeric stereochemistry is opposite to that of the substrate. Conversely, if the product has the same anomeric configuration as the substrate the enzyme is a retaining glycosidase. This classification is useful
mechanistically since the mechanisms of inverting and retaining glycosidases differ significantly.

![Inverting and Retaining Reactions Diagram](image)

**Figure 1** Anomeric configurations of substrates and products for inverting and retaining reactions. Also shown are the relative orientations of the catalytic carboxylic acids.

Inverting glycosidases contain two active-site carboxylic acids separated by about 9-10 Å that are crucial for enzymatic function (reviewed in (McCarter, J. and Withers, S.G. 1994)). These residues play the roles of general acid and general base catalysts in a single displacement reaction utilizing water as the nucleophile.

Retaining glycosidases also contain at least two active-site carboxylic acids that are crucial for enzymatic activity. In the case of retainers, however, these residues are separated by only 4.5 Å – 5.5 Å. Two possible mechanisms have been proposed for the retaining enzymes.

1) The first mechanism, proposed in 1953 by Koshland (Koshland, D.E. 1953) involves a double displacement reaction (Figure 2). In this mechanism one of the
active-site carboxylic acids acts as a nucleophile while the other takes on the role of general acid/base catalyst. The first residue, the enzymic nucleophile, attacks the sugar anomeric center while the second residue, acting as general acid, protonates the aglycone leaving group thus forming a glycosyl-enzyme intermediate. An incoming water molecule is then deprotonated by the second residue, now acting as a general base, and attacks the anomeric center of the glycosyl-enzyme displacing the enzyme and generating the hydrolyzed product with net retention of anomeric stereochemistry.

2) In the second proposed mechanism (Blake, C.C.F. et al. 1967) the first carboxylic acid is thought to play the role of general acid and aids in an $S_n1$-like departure of the aglycone to generate an enzyme-stabilized oxacarbenium ion intermediate. The second residue, as general base, again deprotonates an incoming water molecule which then attacks the intermediate, generating the hydrolyzed product.

Although many researchers still favour the ion pair mechanism, there is a large and growing body of evidence to support the double displacement mechanism.

1.1.3 The Double Displacement Mechanism

Strong evidence favouring the double displacement mechanism is available for both $\alpha$- and $\beta$- retaining glycosidases. These take the form of both kinetic and structural studies.

Currently, most of the detailed kinetic investigations regarding the nature of the intermediate in the reaction of retaining glycosidases have been performed with $\beta$-glycosidases. In general, the reactions for $\alpha$- and $\beta$-glycosidases are expected to be highly analogous therefore these studies on $\beta$-glycosidases will be presented here, along
with evidence for similarities and differences between the mechanisms of the two types of glycosidases.

![Double displacement mechanism for a retaining α-glycosidase.](image)

**Figure 2** Double displacement mechanism for a retaining α-glycosidase. The mechanism involves two active site carboxylic acids, one acting as a nucleophile and the other as a general acid/base. Both steps of the reaction proceed via oxacarbenium-like transition states.

Evidence for substantial oxacarbenium ion character at the transition states in the hydrolytic mechanism has come largely in the form of α-deuterium kinetic isotope effects (αDKIE). Values for αDKIEs have ranged from 1.05 to 1.25 providing evidence of significant development of positive charge in the transition state. The use of
glycosylation and deglycosylation rate-limited substrates in αDKIE studies with Agrobacterium sp. β-glucosidase has allowed the relative amounts of positive charge in each transition state to be compared. The average αDKIE values for substrates rate-limiting in enzyme deglycosylation ($k_H/k_D = 1.11$) were larger than those for substrates rate-limiting in glycosylation ($k_H/k_D = 1.06$) suggesting that the deglycosylation step has more oxacarbenium ion character and hence is more dissociative. For β-glycosidases, the axial orientation of the aglycone (the enzyme carboxylate) in the deglycosylation step may allow for stereoelectronic assistance from the endocyclic oxygen during departure of this leaving group. In the glycosylation step, a large value of $\beta_{lg} = -0.7^*$ indicates that there is significant bond cleavage in the transition state, therefore the lower αDKIE value (1.06) suggests there is more preassociation by the nucleophile. This preassociation would be beneficial in aiding the departure of the equatorial aglycone, which does not benefit from stereoelectronic assistance from the ring oxygen (Kempton, J. and Withers, S. 1992).

Kinetic evidence for the nature of the transition state in yeast α-glucosidase has also been derived from KIEs using α-aryl and α-pyridinium glycosides (Hosie, L. and Sinnott, M. 1985). The small $\beta_{lg}$ values observed for these isotope effects led the authors to suggest a boat conformation of the sugar in the transition state, since this conformation would maximize any stereo-electronic factors which may be required for hydrolysis.

* $\beta_{lg}$ is the Breatsted catalysis coefficient for the leaving group in the hydrolysis of phenyl glycosides. It is a proportionality constant that relates the free energy of activation for the reaction in question and the free-energy change associated with the transfer of a proton from an acid to a base. Experimentally, it is the slope of a plot of log k vs pKa. A value of 1.0 represents a fully developed charge on the leaving atom during the transition state, while a value near 0 represents very little charge development.
Recent crystallographic studies ((Uitdehaag, J. et al. 1999), discussed in §1.3.5), however, have demonstrated that the most likely conformation of the sugar in the transition state is a half chair. Thus, stereo-electronic assistance is probably not a significant factor in the catalytic mechanism of family 13 enzymes. In contrast, crystallographic analyses of the Michaelis complexes and glycosyl-enzyme intermediates of β-glycosidases suggest that some of these enzymes may require stereoelectronic assistance during hydrolysis (Davies, G. et al. 1998).

1.1.4 Mechanism-based Inactivation of Retaining Glycosidases

One consequence of the double displacement mechanism, which proposes hydrolysis via a covalent glycosyl-enzyme intermediate, would be the ability to trap the intermediate using mechanism-based inactivators. Since both the glycosylation and deglycosylation steps of the double displacement mechanism are postulated to proceed through oxacarbenium-like transition states, destabilization of the charge development on the anomeric carbon or endocyclic oxygen should dramatically reduce the rates of both steps. Positioning a highly electronegative substituent such as fluorine near either atom (for example, at C2 (McCarter, J. et al. 1993) or C5 (McCarter, J.D. and Withers, S.G. 1996) of a pento- or hexo-pyranose) should inductively destabilize charge development in the transition states sufficiently to realize substantial rate reduction. Furthermore, the introduction of a fluorine substituent should have little effect on the binding of the molecule since a fluorine atom is similar in size to a hydroxyl group. Selective enhancement of the glycosylation rate, necessary to allow for accumulation of the intermediate, can be accomplished using aglycones with much better leaving group
ability than the sugar aglycone in the natural substrate. Typically 2,4-dinitrophenol or fluoride have been used successfully, although for sugars with severely deactivated anomeric centers – such as 2-deoxy-2,2-difluoroglucose, a leaving group such as 2,4,6-trinitrophenol is required (Braun, C. et al. 1995). Some binding energy may be lost due to the inability of the 2-fluoro group to act as a hydrogen bond donor. This should be overcome, however, by the relatively greater destabilization of the second transition state. The good leaving groups result in selectively enhanced glycosylation rates, and therefore greater lifetimes of the intermediates. Thus, it is to be expected that the enzyme would have comparable affinities for both the “normal” substrate and the deoxyfluoro version.

The use of fluoro substituents on sugars with activated aglycones has proven to be the most generally applicable means of labeling glycosidase nucleophiles to date, and this strategy has been successfully employed to study a number of α- and β-glycosidases (Table 2). For β-glycosidases, 2,4-dinitrophenyl-2-deoxy-2-fluoro β-glycosides have proven to be highly successful for trapping the covalent intermediate, while the intermediate of α-glycosidases has been rendered accessible with either 5-fluoro glycosyl fluorides (of the opposite stereochemistry at C5! (Howard, S. et al. 1998; McCarter, J.D. and Withers, S.G. 1996)), 2,4,6-trinitrophenyl-2-deoxy-2,2-difluoro α-glycosides (Braun, C. et al. 1995), or even utilizing the non-fluorinated sugar with an active site mutant (Mosi, R. et al. 1997). In addition to providing evidence for covalent intermediates through ESI-MS studies, these labeling studies have provided the strongest support for the double displacement mechanism to date – the 3-dimensional structures of both β- and α-glycosidase covalent intermediates (for C. fimi xylanase Cex (White, A. et al. 1996), Humicola insolens cellulase Cel7b (Mackenzie, L.F. et al. 1997), Sinapis alba
myrosinase (Burmeister, W.P. et al. 1997), *Bacillus agaradhaerens* cellulase Cel5a (Davies, G. et al. 1998), *Streptomyces lividans* CelB2 (Sulzenbacher, G. et al. 1999), *Bacillus circulans* xylanase (Sidhu, G. et al. 1999), *Candida* sp. β-glucosidase (Cutfield, S. et al. 1999), and *B. circulans* cyclodextrin glucanotransferase (Uitdehaag, J. et al. 1999)) have been determined (Figure 4). With the evidence provided from the studies of fluoro-substituted sugars, it now seems likely that most if not all retaining glycosidases utilize a double displacement mechanism involving a covalent intermediate.

![Figure 3](image.png)

**Figure 3** The Mechanism-Based Inactivators 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucoside (compound 1.1) and 5-fluoro-β-L-idosyl fluoride (compound 1.2). These inactivators were used to identify the enzymic nucleophiles of *Agrobacterium* sp. β-glucosidase (and other β-glucosidases), and yeast α-glucosidase respectively.
Figure 4 Structures of the covalent intermediates for family 5 *S. lividans* β-endoglucanase (A), family 11 *B. circulans* xylanase (B), family 13 *B. circulans* cyclodextrin glucanotransferase (C). Images recreated from (Sulzenbacher, G. *et al.* 1999; Sidhu, G. *et al.* 1999; Uitdehaag, J. *et al.* 1999).

Table 2 Glycosidases That Have Had Their Nucleophiles Identified By Mechanism-Based Inactivators or Affinity Labels.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GH Family</th>
<th>α/β</th>
<th>Species</th>
<th>Inactivator</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrosinase</td>
<td>P</td>
<td>β</td>
<td><em>Sinapis alba</em></td>
<td>2deoxy2F glucotropaeolin</td>
<td>(Burmeister, W.P. <em>et al.</em> 1997)</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>1</td>
<td>β</td>
<td><em>Sulfolobus</em></td>
<td>conduritol B epoxide</td>
<td>(Febbraio, F. <em>et al.</em> 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>solfataricus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosidase</td>
<td>1</td>
<td>β</td>
<td><em>Agrobacterium</em></td>
<td>2FDNPβGlu</td>
<td>(Street, I.P. <em>et al.</em> 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>sp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosidase</td>
<td>1</td>
<td>β</td>
<td><em>Sweet Almond</em></td>
<td>2FDNPβGlu</td>
<td>(He, S.M. and Withers, S.G. 1997)</td>
</tr>
<tr>
<td>Glucuronidase</td>
<td>2</td>
<td>β</td>
<td><em>Homo sapien</em></td>
<td>2FβGlucuronyl F</td>
<td>(Wong, A.W. <em>et al.</em> 1998)</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>3</td>
<td>β</td>
<td><em>Vibrio furnisii</em></td>
<td>2NAc5FoL-IdoF</td>
<td>(Vocadlo, D.J. <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>Glucosidase</td>
<td>3</td>
<td>β</td>
<td><em>Aspergillus niger</em></td>
<td>2FβGluF</td>
<td>(Dan, S. <em>et al.</em> 2000)</td>
</tr>
<tr>
<td>Exo-β-(1,3)</td>
<td>5</td>
<td>β</td>
<td><em>Candida albicans</em></td>
<td>2FDNPβGlu</td>
<td>(Mackenzie, L.F. <em>et al.</em> 1997)</td>
</tr>
<tr>
<td>glucanase</td>
<td></td>
<td></td>
<td></td>
<td>2FDNPβCel</td>
<td>(Mackenzie, L.F. et al. 1999)</td>
</tr>
<tr>
<td>Enzyme Type</td>
<td>Residue</td>
<td>Enzyme Family</td>
<td>Species</td>
<td>Code</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------</td>
<td>------------------------</td>
<td>--------------------------</td>
<td>------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Cellulase/</td>
<td>β</td>
<td>Cellulase</td>
<td><em>Cellulomonas fimi</em></td>
<td>2FPNβCel</td>
<td>(White, A. et al. 1996)</td>
</tr>
<tr>
<td>Xylanase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2FPNβX2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Bacillus circulans</em></td>
<td></td>
<td>2FDNPC</td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>β</td>
<td>Endoglucanase</td>
<td><em>Streptomyces lividans</em></td>
<td></td>
<td>4&quot;DαG3F</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Bacillus circulans</em></td>
<td></td>
<td>5FβL-IdoF</td>
</tr>
<tr>
<td>CGTase</td>
<td>α</td>
<td>CGTase</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>5FβG3F</td>
<td>(Braun, C. et al. 1996)</td>
</tr>
<tr>
<td>E257Q mutant Glucosidase</td>
<td>α</td>
<td></td>
<td><em>Rabbit</em></td>
<td>4&quot;DαG3F</td>
<td></td>
</tr>
<tr>
<td>Glucocerebrosidase</td>
<td>β</td>
<td>Glucocerebrosidase</td>
<td><em>Homo sapien</em></td>
<td>2FβGluF</td>
<td>(Miao, S. et al. 1994)</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>α</td>
<td>Galactosidase</td>
<td><em>Phanerochaete chrysosporium</em></td>
<td>22FTNPαGal</td>
<td>(Hart, D.O. et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Xanthomonoas manihotis</em></td>
<td>2FPNβGal</td>
<td>In preparation</td>
</tr>
<tr>
<td>Lysosomal Galactosidase</td>
<td>α</td>
<td></td>
<td><em>Jack bean</em></td>
<td>5FGuIF</td>
<td>(Howard, S. et al. 1998)</td>
</tr>
<tr>
<td>Mannosidase</td>
<td></td>
<td></td>
<td><em>T. saccharolyticum</em></td>
<td>2FDNβX</td>
<td>(Vocadlo, D.J. et al. 1998)</td>
</tr>
</tbody>
</table>
1.2 Digestion of Starch in Humans

1.2.1 Dietary Carbohydrates

Since glucose is a principal source of readily available energy for most organisms, methods had to be evolved for handling large quantities of this potentially harmful substance. Because glucose is a reducing sugar, and therefore contains a reactive aldehyde functionality, large amounts of glucose represent a potential threat to certain biological systems, especially proteins with a low rate of turnover (e.g., proteins in the eye). In addition, high concentrations of glucose will alter carefully regulated osmotic pressures in and around cells, again having detrimental effects. In order to avoid these problems, organisms have developed means of storing large amounts of glucose while decreasing the numbers of reactive “reducing ends”, and controlling the osmotic pressure. Plants store their glucose as the large branched polymer starch, mammals store glucose as glycogen, which is also a highly branched polymer, and some bacteria use another branched polymer known as pullulan. The common features of all of these polymers is that, while they contain many non-reducing ends, they only have one reducing end for thousands of glucose units thus drastically reducing the potential harm of handling such large amounts of glucose. In addition, there is only one molecule of the polymer representing thousands of glucosyl moieties, thus the osmotic pressure is satisfactorily regulated. Some organisms have taken even more extreme measures and store glucose in a form that contains no reducing ends. Insects store glucose as the α-(1-1) disaccharide trehalose, while some bacteria use cyclodextrins, i.e., sugar rings, to store glucose (Figure 5).
Figure 5 Different Storage forms of Glucose Used in Nature. Branched polymers (A) such as starch (amylopectin shown) and glycogen; • = non reducing glucose, Δ = reducing glucose. The shape of amylose (B) from starch (● = carbon, ○ = oxygen), α-cyclodextrin (C), trehalose (D), and cellulose (E) (● = carbon, ○ = oxygen, ——— —= H-bonding). Images (B) and (E) taken from (Voet, D. and Voet, J.G. 1990)
1.2.2 Starch

In humans, starch is the principal source of dietary glucose. This polymer is formed in the plant plastids, primarily the amyloplast but occasionally in other plastids such as chloroplasts, as a result of photosynthesis. Starch is stored primarily, but not exclusively, in seeds, fruits, tubers, and storage roots where it is used as a readily available energy source (Stark, J.R. and Lynn, A. 1992).

Starch is a large glucose polymer that can be fractionated into two different classes, amylose and amylopectin. The amounts of amylose and amylopectin differ significantly in starches from different sources. For example, high-amylose corn starch can have up to 70% amylose while waxy maize starch is composed of about 98% amylopectin and only 2% amylose. Potato starch, another common source of dietary glucose, contains 21% amylose. On average, starch is composed of about 30% amylose and 70% amylopectin. Different α-amylases appear to be able to hydrolyze amylose and amylopectin to different degrees such that an α-amylase will often “prefer” hydrolyzing starch from a particular sources. For example, human salivary amylase hydrolyzes corn starch more effectively than potato starch whereas the human pancreatic enzyme has the opposite preference (Stark, J.R. and Lynn, A. 1992).

Starch contains both crystalline and amorphous regions, the amorphous areas being much more susceptible to enzymatic attack than the crystalline regions. It is thought that crystalline starch is formed primarily from highly branched amylopectin, while the amylose is largely packed into the amorphous regions. One purpose of cooking starchy foods therefore, is to swell the starch and make it more susceptible to enzymatic degradation. However, upon cooling, the starch in many foods begins to crystallize
forming a highly crystalline retrograde starch, which is even more resistant to enzymatic hydrolysis than it was initially.

The two oligosaccharide components of starch, amylose and amylopectin, are large glucose polymers. Structurally, amylose is an “essentially” linear chain of α(1-4) linked glucose units about 1000 residues long. It contains infrequent α(1-6) glucosyl branch points (about 1 per 1000 residues). Amylopectin is a much larger, more complex glucose polymer (Figure 5B). The reader is directed to (Stark, J.R. and Lynn, A. 1992) for detailed descriptions of amylopectin structure. Briefly, amylopectin is composed of three different glucose chains, A, B, and C which differ in their amount of α(1-6) branching and in their degree of attachment to the other amylopectin chains. Only the C chain has a free reducing end, and there is one C chain per amylopectin molecule. It is important to note that both amylose and amylopectin contain only one reducing end per molecule (one molecule can be several thousand glucose units). This is important in decreasing the reactivity of the sugar anomeric center while still allowing storage of very large amounts of sugars as an energy supply.

The 3-dimensional conformation of maltose (the α(1-4) glucose disaccharide) is such that a hydrogen-bond exists between the 3- and 2’- hydroxyl groups. This has the effect of inducing a helical structure into α(1-4) linked glucose polymers (Figure 5B). Since the 6-hydroxyl will be positioned on the outside of the helix, starch can be thought of as a series of helices (α(1-4) regions) covalently associated through α(1-6) linkages. Indeed, x-ray diffraction studies have demonstrated that amylose commonly exists in one of several helical forms (A, B, or V and other rare forms) depending on the relative humidity and the presence of small, included molecules such as iodide or KOH. This 3-
dimensional organization is significantly different from the highly planar, stacked arrays found in \( \beta(1-4) \) glucose polymers such as cellulose (Figure 5E). The differences in substrate secondary structure have implications for the active site geometries of \( \alpha- \) and \( \beta- \) endoglycosidases. \( \beta-(1-4) \) Endoglycosidases tend to have shallow grooves for active sites in order to accommodate the linear substrate, while the active-site of \( \alpha-(1-4) \) endoglucanases have deeper clefts to properly bind the helical substrate.

1.2.3 Location and Action of Digestive Glycosidases

Due to the large, compact nature of starch, the limited time the digestive \( \alpha- \) glycosidases have to act on it, and the desire for gradual release of glucose into the blood stream, a collection of enzymes is needed to properly and fully hydrolyze this polymer (Table 3, Figure 6).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salivary ( \alpha )-amylase</td>
<td>Oral cavity / upper GI tract</td>
<td>Starch</td>
</tr>
<tr>
<td>Pancreatic ( \alpha )-amylase</td>
<td>Lumina of small intestine</td>
<td>Starch</td>
</tr>
<tr>
<td>Lactase</td>
<td>Brush border</td>
<td>Lactose</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>Brush border</td>
<td>Malto-oligosaccharides (G2-G9)</td>
</tr>
<tr>
<td>Sucrase-( \alpha )-dextrinase</td>
<td>Brush border</td>
<td>Sucrose ( \alpha )-Dextins</td>
</tr>
<tr>
<td>Trehalase</td>
<td>Brush border</td>
<td>Trehalose</td>
</tr>
</tbody>
</table>

The first glycosidase to act on ingested starch is the salivary \( \alpha \)-amylase. Highly analogous to its pancreatic counterpart, this enzyme is an endoglycosidase produced by
the salivary glands and secreted in saliva. The salivary amylase is the sole endogenous glycosidase to act on starch in the upper GI tract. By the time the ingested starch has reached the stomach it has been hydrolyzed into smaller oligosaccharides of an average chain length of 8 glucose units (Gray, G.M. 1975). The stomach acids and proteases then largely denature the salivary amylase, although about 10% activity remains.

The pancreatic α-amylase, which is secreted by the pancreas into the intestinal lumina, is the next glycosidase to act on the starch remnants. The crude starch hydrolysate entering the small intestine is composed of both α(1-4) and α(1-6) linked glucose units. Pancreatic α-amylase generates maltose and maltotriose from the α(1-4) linked glucose chains while producing small oligosaccharides of 4-8 glucose units known as limit dextrins from around the α(1-6) branch points, which remain intact.

The disaccharidases produced by the brush border of the intestine then hydrolyze the small oligosaccharides to glucose. Glucoamylase, α-glucosidase and sucrase act on maltose and maltotriose, while α-dextrinase acts to hydrolyze the limit dextrins.

The glucose produced is transported into the brush border cells and eventually to the blood stream by way of a specific glucose transporter, where it is distributed throughout the body. From this point glucose can either enter into glycolytic pathways for conversion to energy or it can be stored as glycogen for future use.
Figure 6 Digestion of Starch in Humans. Digestive glycosidases and their locations are shown. The expanded inset is a micrograph of the intestinal brush border, where glucoamylase, sucrase, and α-dextrinase are located.

There are several disorders related to improper digestion of carbohydrates (discussed in 1.3.7). Many of these, which are related to disaccharidase deficiencies, are
beyond the scope of this thesis. Diseases such as diabetes and obesity can be related to excessive blood glucose levels, which can be regulated by controlling the rate of starch hydrolysis by pancreatic $\alpha$-amylase. Since the oligosaccharides produced from the hydrolysis of starch by salivary $\alpha$-amylase are too large to be efficiently hydrolyzed by the brush border disaccharidases the hydrolysis of these sugars by the pancreatic $\alpha$-amylase is necessary for full digestion of the starch. Regulation of the pancreatic $\alpha$-amylase activity can therefore be useful for controlling the amount of glucose released into the blood stream. In fact, this strategy has been used successfully with the commercially available amylase inhibitor acarbose (Hillerbrand, I. and Berchtold, P. 1980).
1.3 Human Pancreatic α-Amylase

As discussed above, human pancreatic α-amylase (HPA) is crucial for complete and efficient digestion of dietary starch as it is responsible for the hydrolysis of larger malto-oligosaccharides into smaller units that can be hydrolyzed by the brush border disaccharidases. Therefore, an understanding of the detailed catalytic mechanism of this enzyme will be useful in developing methods to control several dietary carbohydrate disorders.

HPA is a member of glycosyl hydrolase family 13, the α-amylase superfamily, and has been grouped into clan GH-H (Table 1) along with enzymes from families 70 (α-amylases) and 77 (α-glucanotransferases) (Coutinho, P.M. and Henrissat, B. 1999). Family 13 enzymes are capable of hydrolyzing α(1-1), α(1-6) or α(1-4) glucosidic bonds. Each of these enzymes possess four regions of high sequence identity (Table 4), while having low overall sequence similarities. Despite this low sequence similarity, a large number of crystal structures of enzymes in this family demonstrate that there is strong conservation of the global folds of the three common domains.
Table 4 Conserved Regions of Selected Members of Glycosyl Hydrolase Family 13
(α-Amylase Family)

<table>
<thead>
<tr>
<th>Enzyme / Species</th>
<th>Region Around Conserved Residue #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96</td>
</tr>
<tr>
<td>MAMMALIAN</td>
<td></td>
</tr>
<tr>
<td>AMYP HUMAN</td>
<td>RIYVDAVINH</td>
</tr>
<tr>
<td>AMYS HUMAN</td>
<td>RIYVDAVINH</td>
</tr>
<tr>
<td>AMYP PIG</td>
<td>RIYVDAVINH</td>
</tr>
<tr>
<td>AMYP MOUSE</td>
<td>RIYVDAVINH</td>
</tr>
<tr>
<td>GLGB HUMAN</td>
<td>IVLLVDVHSH</td>
</tr>
<tr>
<td>INSECT</td>
<td></td>
</tr>
<tr>
<td>AMY DROMA</td>
<td>RIYVDVIFNH</td>
</tr>
<tr>
<td>PLANT</td>
<td></td>
</tr>
<tr>
<td>AMY WHEAT</td>
<td>SCVADIVINH</td>
</tr>
<tr>
<td>AMY HORVU</td>
<td>QAIADIVINH</td>
</tr>
<tr>
<td>SINGLE-CELLED EUKARYOTES</td>
<td></td>
</tr>
<tr>
<td>AMY ORYSA</td>
<td>QVIADIVINH</td>
</tr>
<tr>
<td>GLGB YEAST</td>
<td>LVLLDVV--H</td>
</tr>
<tr>
<td>PROKARYOTES</td>
<td></td>
</tr>
<tr>
<td>AMY ECOLI</td>
<td>AVLLDVVVNH</td>
</tr>
<tr>
<td>AMY BACSU</td>
<td>KVIVDAVINH</td>
</tr>
<tr>
<td>SUCP ECOLI</td>
<td>HLMFDFVCNH</td>
</tr>
<tr>
<td>TREC BACSU</td>
<td>KVVMDLVVNH</td>
</tr>
<tr>
<td>CDGT BACST</td>
<td>EVIIDFAPNH</td>
</tr>
<tr>
<td>GLGX HAEGIN</td>
<td>EVILDVVFNH</td>
</tr>
<tr>
<td>ISOA PSESP</td>
<td>KVYMDVYNH</td>
</tr>
<tr>
<td>PULA KLEPN</td>
<td>NVIMDVYNH</td>
</tr>
</tbody>
</table>

† Enzyme: AMY = α-amylase (P=pancreatic, S=salivary); GLGB = glycogen branching enzyme; GLGX = glycogen debranching enzyme; SUCP = sucrase; TREC = trehalase; ; CDGT = cyclodextrin glucanotransferase; ISOA = isoamylase; PULA = pullulanase

Species: DROMA = Drosophila melanogaster; HORVU = barley; ORYSA = Aspergillus orysae; BACSU = Bacilis Subtilis; BACST = Bacilis stereothermophilus; HAEGIN = Haemophilus influenzae; PSESP = Pseudomonas sp.; KLEPN = Klebsiella pneumoniae
Chapter 1 Introduction

1.3.1 The Structure of $\alpha$-Amylase

The 3-dimensional structures of several $\alpha$-amylases have been determined since 1980 (Table 5). The first $\alpha$-amylase structure determined was the 3 Å structure of *Aspergillus oryzae* $\alpha$-amylase (TAKA) (Matsuura, Y. et al. 1980). In the early 1990's several high-resolution $\alpha$-amylase structures were determined including those of *Aspergillus niger* (Boel, E. et al. 1990), TAKA amylase (Swift, H.J. et al. 1991) and porcine $\alpha$-amylase (Qian, M. et al. 1994). Recently the structures of several more $\alpha$-amylases have been determined both with and without inhibitors bound, including the human pancreatic (Figure 7) (Brayer, G.D. et al. 1995) and salivary $\alpha$-amylases (Ramasubbu, N. et al. 1996) whose structures have been solved to 1.8 Å resolution. In all cases, despite very limited sequence similarities (Table 4), there have been strong similarities in the global folds.

While many members of the glycosyl hydrolase family 13 are composed of up to 5 or 6 domains, the $\alpha$-amylases are composed of three domains, all of which are shared by every member of this family.

*Domain A* is a ($\beta/\alpha$)$_8$ barrel that contains the active site and binds a chloride ion in the mammalian $\alpha$-amylases. All four of the highly conserved sequences used in classifying members of family 13 are contained in loops crucial to the active-site structure. Three completely conserved carboxylic acids (D197, E233, D300) are located at the bottom of the “V” shaped active-site cleft. The bound chloride acts as an allosteric effector, and removal of this
ion results in a 30-fold decrease in activity (Thoma, Spradlin et al.).

Figure 7 The structure of human pancreatic $\alpha$-amylase expressed in *Pichia pastoris* (Rydberg, E.H. et al. 1999). The three domains are labeled, and the metals are displayed in space-filled representation (calcium – orange, chloride – green). In addition, the three conserved active site carboxylic acids are shown in yellow. Structural coordinates from (Brayer, G.D. et al. 2000).

*Domain B* is a small domain composed almost entirely of loops and is responsible for binding a structurally crucial calcium ion. The calcium, bound by residues N100, R158, D157, H201 (Brayer,
G.D. et al. 1995), is believed to be responsible for stabilizing the active-site loop containing D197 (Buisson, G. et al. 1987).

Domain C is largely composed of β-strands in the form of two Greek Key motifs. This domain appears to share similarities with a starch-binding domain in the family 14 glucoamylases, however, only limited research has been performed on the family 13 domain (Brayer, G.D. et al. 1995).

Table 5 Three-Dimensional Structures of α-Amylases Determined, and Their Protein Data Bank Codes

<table>
<thead>
<tr>
<th>Species</th>
<th>Ligands Bound</th>
<th>Protein Data Bank Code</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus oryzae</td>
<td>Acarbose</td>
<td>6TAA</td>
<td>(Swift, H.J. et al. 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Brzozowski, A.M. and Davies, G.J. 1997)</td>
</tr>
<tr>
<td></td>
<td>Aspergillus niger</td>
<td>2AAA</td>
<td>(Boel, E. et al. 1990)</td>
</tr>
<tr>
<td>Sus scrofa</td>
<td>Acarbose</td>
<td>1PPI, 1PIF, 1PIG</td>
<td>(Qian, M. et al. 1994)</td>
</tr>
<tr>
<td>(Pig pancreas)</td>
<td></td>
<td></td>
<td>(Gilles, C. et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>Tendamistat</td>
<td>1BVN</td>
<td>(Wiegand, G. et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>Bean Lectin-Like Inhibitor</td>
<td>1DHK</td>
<td>(Bompard-Gilles, C. et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>Methyl 4,4'-Dithio-α-Maltotrioside</td>
<td>1JFH</td>
<td>(Qian, M. et al. 1997)</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td></td>
<td>1BSI</td>
<td>(Rydberg, E.H. et al. 1999)</td>
</tr>
<tr>
<td>(Pancreatic - cloned into yeast)</td>
<td>Acarbose (D300N)</td>
<td>1CPU</td>
<td>(Brayer, G.D. et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Acarbose (bound to D300N)</td>
<td>2CPU</td>
<td>(Brayer, G.D. et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3CPU</td>
<td>(Brayer, G.D. et al. 2000)</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td></td>
<td>1HNY</td>
<td>(Brayer, G.D. et al. 1995)</td>
</tr>
</tbody>
</table>
Human pancreatic α-amylase is a 56 kDa monomeric protein that binds a single calcium ion and a single chloride ion, both of which are necessary for full catalytic activity. Attempts to replace the calcium ion with rare earth metals, or the chloride ion with other small anions have led to enzymes with greatly reduced activity. Crystallographic evidence suggests that the calcium ion is necessary to stabilize the conformation of an active site loop. However, the role of the chloride ion is a point of contention, and the presence of a negatively charged ion near several crucial negatively
charged residues seems at first, unusual. It has been suggested that the chloride ion is responsible for perturbing the pK\textsubscript{a} values of the active site carboxylic acids in order to allow optimum activity at higher pH values (Feller, G. et al. 1996). In addition to this effect on \(\alpha\)-amylase pH optima, binding of the chloride has also been observed to increase the PPA-Ca\textsuperscript{2+} binding constant by 240-fold (Levitzki, A. and Steer, M.L. 1974).

In the absence of rigorous kinetic analyses aided by site-directed mutagenesis, the putative roles of the active-site residues, such as the three conserved active-site carboxylic acids, can be inferred through detailed analysis of the many crystal structures (Table 5). Product analysis studies by HPLC have indicated that \(\alpha\)-amylase is a retaining endoglycosidase (Braun, C. et al. 1993), while the sequence and structural data in conjunction with studies on other family 13 enzymes, support the theory that \(\alpha\)-amylase utilizes a pair, or perhaps, a trio of conserved carboxylic acids in a double displacement mechanism. It is interesting to note that, for family 13 enzymes, sequence and structural studies had, until recently, out-paced the kinetic analyses and that this situation is quite the opposite to that of many \(\beta\)-glycosidase families (especially families 1, 10 and 11).

### 1.3.2 The Catalytic Nucleophile

In 1989, prior to any high resolution 3-dimensional structures of \(\alpha\)-amylases (< 3 Å resolution), the presence of a covalent glycosyl-enzyme intermediate in the hydrolytic reaction of PPA was revealed in a cryo-NMR study (Tao, B.Y. et al. 1989), thus providing direct support for a double displacement reaction as the mechanism for family 13 \(\alpha\)-amylases. Later, analysis of several mutant \(\alpha\)-amylases where completely conserved active site residues were altered, demonstrated that only D197, E233, and D300 (HPA numbering) were absolutely necessary for the hydrolytic activity of \(\alpha\)-
amylose on starch. Structural studies with the pseudo-oligosaccharide acarbose had been interpreted as suggesting that D197, the most likely candidate for the catalytic nucleophile, was positioned 3.2 Å from the sugar anomeric center and was therefore too far away to perform the nucleophilic attack (Qian, M. et al. 1994). However, this was a rather shortsighted interpretation since a separation of 3.1 Å is essentially the minimum possible, given the van der Waals' radii of carbon and oxygen. Indeed, a recent structure of a glycosyl-enzyme intermediate of a family 13 enzyme, CGTase ((Uitdehaag, J. et al. 1999) see §1.3.5), has shown that this distance is shortened by a 2 Å shift in the glucose at the –1 subsite as compared to the valienamine ring of the inhibitor, thereby demonstrating that D197 is appropriately positioned to perform the role of catalytic nucleophile.

In addition to crystallographic studies, the use of mechanism-based inactivators such as 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-glucoside (22FTNPGlu) or 5-fluoro-α-L-idosyl fluoride (5FIdoF) with yeast α-glucosidase (McCarter, J.D. and Withers, S.G. 1996) have allowed the enzymic nucleophile of this enzyme to be covalently modified as described in section 1.1.4. For enzymes acting on larger substrates (thus requiring more challenging syntheses for the inactivators) such as cyclodextrin glucanotransferase (CGTase), trapping of the covalent intermediate has been achieved using a mutant and 4″-deoxy-maltotriosyl fluoride (described in §1.3.5) (Mosi, R. et al. 1997). In both of these examples the labeled residue corresponded to D197 of HPA, strongly suggesting that this carboxylic acid is also the nucleophilic residue in the hydrolytic reaction catalyzed by HPA.

Mutation of D197 to alanine has been performed in several α-amylases, although not in the human enzyme prior to this study. In all cases the resulting enzyme lost all
activity. However, several other conserved active site carboxyl groups have been mutated (E233 and D300) and these enzymes also, reportedly, showed a complete loss of activity (Svensson, B. and Søgaard, M. 1993; Svensson, B. 1994). Unfortunately, these studies were conducted using single point assays on starch and no rigorous kinetic analyses have been published. There is therefore, very little reliable kinetic evidence to probe the identity of the nucleophile in HPA.

### 1.3.3 General Acid / Base Catalysis

Not surprisingly, glycosidase general acid/base catalysts have proven to be less amenable to labeling by mechanism-based inactivators since their role is that of proton donation and abstraction and not nucleophile attack. Insights into the identity of the residue acting as the general acid/base catalyst have primarily come from three sources.

1) Mechanistic studies: kinetic investigations utilizing substrates that either require, or do not require general acid catalysis have been carried out on several β-glycosidases [Agrobacterium sp. β-glucosidase (Kempton, J. and Withers, S. 1992), E. coli β-galactosidase (Richard, J.P. et al. 1996), B. circulans xylanase (McIntosh, L.P. et al. 1996) and C. fimi xylanase (MacLeod, A.M. et al. 1994)]. These studies, involving substrates with aglycones of differing leaving group ability and mutants of active-site carboxyl groups, have shown a clear mechanistic distinction between the mutant of the acid/base catalyst and mutants of other active-site carboxylic acids. While the rate-determining step for most mutants changed as the leaving group ability of the aglycones improved, the mutant of the acid/base catalyst showed no such trend.
2) Structural studies, which have served as corroborative evidence for the mechanistic studies with family 10 enzymes, and have been the principal source of information for α-glycosidases with few exceptions.

3) Labeling of the acid/base catalyst. This has been successful in several cases. Bromomethyl-keto C-glycosides have been utilized to identify the acid/base catalyst of yeast α-glucosidase (Howard, S. and Withers, S.G. 1998), Abg (Howard, S. and Withers, S.G. 1998), and C. fimi CenA and CenD (Howard, S. and Withers, S.G. 1998), in addition N-bromoacetamido β-D-glycopyranosylamines have been used to label a β-glucosidase (Black, T.S. et al. 1993), a β-endoglucanase (Tull, D. et al. 1996), and a β-xylosidase (Vocadlo, D. 2000).

To date, for family 13 α-glycosidases, few kinetic studies have been performed on enzyme mutants of the putative acid/base catalyst. Therefore, essentially all conclusions regarding the acid/base catalyst are based on structural studies. Many three-dimensional structures exist for family 13 enzymes, several with bound inhibitors, a few Michaelis complexes, and one structure of a covalently bound glycosyl-enzyme intermediate. From these structures it appears that E233 is best situated to provide general acid/base catalysis. However, the picture is complicated in this enzyme family since the active-site contains a cluster of three carboxylic acids. A bromoketone C-glycoside affinity label has been found to covalently bind to the yeast α-glucosidase active site (Howard, S. and Withers, S.G. 1998). The labeled residue corresponded to E233 of HPA, which demonstrated that this residue is properly situated to act as the acid/base catalyst. However, the possibility also exists that E233 and D300, which
together bind a conserved active-site water molecule (Figure 8) that may be the nucleophile for deglycosylation, may each play the role of either general acid or general base catalyst.

![Figure 8 Stereo view of the active site of human pancreatic α-amylase with the enzyme-modified version of the natural inhibitor acarbose bound. Acarbose is shown in a stick representation while the active site residues are represented as solvent-accessible surfaces coloured to indicate electrostatic potential (red is negative, blue is positive). Structural coordinates from (Brayer, G.D. et al. 2000).](image)

**Figure 8** Stereo view of the active site of human pancreatic α-amylase with the enzyme-modified version of the natural inhibitor acarbose bound. Acarbose is shown in a stick representation while the active site residues are represented as solvent-accessible surfaces coloured to indicate electrostatic potential (red is negative, blue is positive). Structural coordinates from (Brayer, G.D. et al. 2000).

### 1.3.4 Non-Covalent Active Site Interactions

Over half a century ago it was first suggested that much of the catalytic power of enzymes is derived from specific, non-covalent enzyme/substrate interactions in the transition state (Pauling, L. 1946). Attempts to quantitate these interactions in glycosidases have naturally focussed upon kinetic analysis of substrates modified at the numerous hydroxyl groups. Analysis of the strength of enzyme-substrate hydrogen
bonds in the transition state has been accomplished utilizing deoxy sugars and
deoxyfluoro sugars for glycogen phosphorylase (Street, I.P. et al. 1989), β-glucosidase
(Namchuk, M.N. and Withers, S.G. 1995), β-galactosidase (McCarter, J.D. et al. 1992),

Interesting differences in the contribution of each hydrogen bond at the transition
state are apparent between the three glycosidases studied. For both of the retaining β-
glycosidases it was found that, although the 3, 4, and 6 hydroxyls on the sugar in the −1
subsite contribute about 8-9 kJ/mol to the transition state, the 2-OH of the same sugar is
responsible for 17-18 kJ/mol of stabilization (McCarter, J.D. et al. 1992; Namchuk, M.N.
and Withers, S.G. 1995). In contrast, for the inverting α-glycosidase glucoamylase, the
energy contributions seem to be the opposite. The 4- and 6-hydroxyls of the sugar bound
at the −1 subsite contribute approximately 18 kJ/mol to the transition state, while the 3-
OH contributes only 9 kJ/mol, and the 2-hydroxyl group apparently provided almost no
stabilization to the transition state (Sierks, M.R. et al. 1992). Unfortunately, no
allowance was made for inductive effects in this study. Although it might be tempting to
suggest that the differences in hydrogen bonding patterns result from inherent differences
between α- and β-glycosidases in general, the many 3-dimensional structures of retaining
α-glycosidases (for example, structures listed in Table 5) suggest that in these enzymes
the 2-OH also plays an important role at the transition state. This therefore suggests that
the differences in hydrogen bonding patterns result from mechanistic differences between
inverting and retaining enzymes.

In addition to the important hydrogen bonding interactions at the transition state
of enzyme catalyzed glycoside hydrolysis, other non-covalent interactions undoubtedly
played significant roles in hydrolysis. Another possible role for non-covalent interactions, suggested by a recent series of crystal structures of CGTase bound to substrates and inhibitors, is in distorting the conformation of the sugar at the \(-1\) subsite to a conformation more favourable for attack by the catalytic nucleophile (Uitdehaag, J. \textit{et al.} 1999).

\textbf{1.3.5 The Nature of the Enzyme—Substrate Complex and the Glycosyl—Enzyme Intermediate in CGTase}

Several structures along the reaction coordinate of the reaction catalyzed by CGTase, a family 13 enzyme, have been determined recently (Uitdehaag, J. \textit{et al.} 1999) and they have given significant insights into the detailed catalytic mechanism of the \(\alpha\)-amylase family. CGTase is predominantly a transglycosidase, forming cyclodextrins from long glucose chains. Its active site binds 9 sugar residues, utilizing subsites in a domain not present in \(\alpha\)-amylase. However, the catalytic machinery is located on loops of domain A, a \((\beta/\alpha)_8\) barrel, and is essentially unchanged from that of \(\alpha\)-amylase, including the four conserved histidines, and the conserved catalytic carboxylic acids. Only the details with respect to the three catalytic acids (using HPA numbering) will be discussed here.

The structure of the enzyme substrate complex was acquired by freeze-trapping a maltononaose substrate in the active site of the D197N/E233Q mutant. The substrate was observed to bind in a competent fashion with the scissile bond intact. In addition, the \(-1\) sugar ring appears flattened from the normal \(4C_1\) chair conformation towards a \(2H_3\) half-chair conformation (Figure 9B) with C2, C1, O1 and C5 lying in a plane. A significant driving force for the ring distortion appears to be the alleviation of potentially severe van
der Waals interactions between D300 and O2, which would occur were the ring not
distorted. D300 appears to hydrogen bond with OH-2, which may decrease the
electronnegativity of OH-2, thus facilitating the development of the partial positive charge
at C1 that forms at the transition state. It has been suggested that it is the loss of this
hydrogen bond between D300 and OH-2 that is the reason for the dramatically lower
activity (by $10^4$ - $10^5$ fold) of D300N mutants relative to wild-type (Uitdehaag, J. et al.
1999). In addition, when compared with the free enzyme, E233 in the complex appears
to be reoriented so that it interacts with the glycosidic oxygen of the scissile bond, thus
suggesting a role as general acid/base catalyst.

The recent structure of the mutant CGTase (E233Q) - 4”-deoxy maltotriosyl
covalent complex (Figure 9C) was the first, and remains the only, structure of a glycosyl-
enzyme intermediate for an $\alpha$-glycosidase/$\alpha$-glycosyl transferase. The use of the 4”-
deoxy substrate was necessary to prevent reactivation through transglycosylation. Since
the substrate employed was unmodified at the reducing end sugar, this structure provides
a view of the reaction intermediate as it would exist naturally, without the possibility of
interference from fluoro substituents. In this structure a short distance (1.47 Å) is
observed between O61 of D197 and C1 of the sugar bound to the $-1$ subsite, clearly
demonstrating the presence of a covalent glycosyl-enzyme intermediate and not an
oxacarbenium ion intermediate. In addition, the $^2$H$_3$ conformation of the $-1$ sugar in the
enzyme-substrate complex has relaxed to a $^4$C$_1$ chair conformation. This suggests that
stereo-electronic assistance is not a significant factor in hydrolysis of the glycosyl-
enzyme intermediate (Uitdehaag, J. et al. 1999), since that would require distortion of the
sugar ring into a $^{2,5}$B conformation (Hosie, L. and Sinnott, M.L. 1985).
Figure 9 Structures of the free enzyme (A), noncovalent enzyme-substrate complex (B) and the glycosyl-enzyme intermediate (C) of cyclodextrin glucanotransferase with a 4''-deoxy-α-maltotriosyl moiety covalently bound to D229 (D197 in HPA). In B, the glucosyl moiety bound at the -1 subsite has been forced into a half-chair conformation by the steric interaction with D328 (D300 in HPA), while E257 (E233 in HPA) has reoriented to interact with the glycosidic oxygen of the scissile bond. In C, 4''-deoxy maltotriose is covalently bound to D229, while E257 is ideally situated to act as a general base (figures from (Uitdehaag, J. et al. 1999)). Images are orthogonal views of the -1 and +1 subsites and surrounding residues.

Thus, these structures clarify the role of D197, as catalytic nucleophile, which has been proposed by several kinetic studies and suggest roles for E233 and D300. As the
roles ascribed to E233 and D300 here cannot be confirmed by direct observation, it remains for kinetic studies to provide the necessary evidence.

### 1.3.6 The Role of HPA in Human Disease

As discussed in section 1.2.3, HPA plays a crucial role in starch metabolism owing to the necessity of processing the intermediate sized oligosaccharides produced by salivary α-amylase for use by the intestinal disaccharidases. This crucial role of HPA provides an attractive target for restricting glucose uptake into the blood stream since inhibition of HPA should reduce the amount of substrate available to the disaccharidases and, therefore, the amount of glucose absorbed by the small intestine. Attempts at utilizing this strategy have been undertaken since the mid-1970’s. Glycoprotein α-amylase inhibitors from beans were first reported in 1943 (Kneen, E. and Sandstedt, R.M. 1943). However, it was not until the large-scale purification of the kidney bean α-amylase inhibitor Phaseolamin in 1974 (Marshall, J.J. and Lauda, C.M. 1975) that the concept of a “starch blocker” became feasible. The initial commercial attempt at reducing glucose uptake by inhibiting intestinal starch hydrolysis resulted in the Starch Blocker tablets. These popular tablets contained 500 mg of Phaseolamin, and reportedly prevented the absorption of 100 g of starch when taken 10 minutes before a meal. However, several independent studies demonstrated that this product was ineffective at reducing the amount of starch absorbed from a meal or for reducing postprandial blood glucose levels (Bo-Linn, G.W. *et al.* 1982; Carlson, G.L. *et al.* 1983; Garrow, J.S. *et al.* 1983; Hollenbeck, C.B. *et al.* 1983). Possible explanations for the failure of this method included degradation of the glycoprotein in the GI tract, or an alternate pathway for intestinal starch hydrolysis, perhaps emphasizing hydrolysis by glucoamylase instead of
α-amylase (Hollenbeck, C.B. et al. 1983). At one point the strategy itself was questioned (no author specified, 1983). However, the discovery of a homologous series of natural α-amylase and α-glucosidase inhibitors from *Actinoplanetes* sp. (the acarbose family of inhibitors isolated in 1977) has since lead to the demonstration that this strategy is effective. Inhibition of glucose uptake in this manner has been demonstrated to be an effective method for controlling both insulin-dependent (type I) and insulin-independent (type II) diabetes mellitus (Hillerbrand, I. and Berchtold, P. 1980). It may also prove useful for controlling caloric intake in obesity treatments.

1.3.6.2 Acarbose: A Natural α-Amylase Inhibitor

In 1977 a homologous series of α-amylase/α-glucosidase inhibitors were isolated from *Actinoplanetes*. The structures of these pseudo-oligosaccharides were based around acarviosine, a 4,6-dihydroxylated-2-amino-cyclohexenyl moiety linked through the amino group to C-4 of 6-deoxyglucose, with a variable number of glucose units bound to the reducing and non-reducing ends (Müller, L. et al. 1980). The most efficacious of these is acarbose (Figure 10A), which is a pseudo-tetrasaccharide composed of a maltosyl unit bound to the reducing end of the acarviosine. Although acarbose is one of the least potent members of this series against α-amylase *in vitro, in vivo* its inhibition of pancreatic α-amylase is essentially the same as for other members of the series. In addition, acarbose is a very potent inhibitor of virtually all intestinal α-glycosidases (with the exception of isomaltase, which exclusively hydrolyzes α-(1-6) glucosidic bonds), even those not in family 13 (Junge, B. et al. 1980).
Figure 10 The structure of acarbose in solution (A) and as observed in the active site of HPA (B). Acarbose is composed of a maltosyl unit linked to the reducing end of an acarviosine moiety (valienamine plus 6-deoxy glucose). Family 13 enzymes, such as HPA, appear to rearrange acarbose, through a series of hydrolytic and transglycosylation reactions, to form an inhibitor that fills all binding subsites, with the valienamine bound at the $-1$ subsite.

Although acarbose has been characterized as a potent inhibitor for many family 13 enzymes there are still questions regarding its mechanism of action. Much of the uncertainty comes from the unexpected forms of this inhibitor that are observed in the crystal structures of the complexes of family 13 enzymes with acarbose (Qian, M. et al. 1994; Gilles, C. et al. 1996; Brzozowski, A.M. and Davies, G.J. 1997; Brayer, G.D. et al. 2000). These crystal structures show extended forms of acarbose bound to the active site, with the valienamine ring always bound in the $-1$ subsite (Figure 10B). It has been suggested that the enzyme rearranges the acarbose molecule, possibly through a series of hydrolytic and transglycosylation reactions, to form the modified structures observed. However, not only is it difficult to imagine an enzyme performing such a rearrangement
particularly in the crystallized form, but also it is difficult to derive a plausible mechanism that would satisfactorily explain most of the observed structures.

In addition to the structural studies with acarbose, several mechanistic investigations have also been reported for the family 13 enzymes CGTase and porcine pancreatic α-amylase with this inhibitor (K_i values 0.03 μM and 1.0 μM respectively). Kinetic analysis of a series of mutants was used to provide evidence for transition-state analogy in the binding of acarbose to CGTase, although significant ground state mimicry was also observed (Mosi, R. et al. 1998). No such studies with α-amylase mutants have been reported. However, detailed kinetic analyses with the wild-type enzyme have been performed. Interestingly, while the mode of inhibition of CGTase is competitive, inhibition of α-amylase appears to occur in a non-competitive fashion (Alkazaz, M. et al. 1997), suggesting that, in addition to binding at the active site, acarbose binds at an inhibitory site removed from the active site. Although potential secondary binding sites have been characterized kinetically and have been noted in a few structures, there have been few conclusions that explain the unusual rearrangement of acarbose observed in the crystal structures, and no conclusive data to suggest the location or mechanistic details of the second inhibitory site.

Therefore, many questions regarding the mechanism of inhibition of family 13 enzymes by acarbose remain to be answered.
1.4 Aims of the Project

The goals of this project were to characterize the roles of the three crucial active-site carboxylates (D197, E233, and D300) in the hydrolytic mechanism of human pancreatic α-amylase. According to the Koshland double displacement mechanism (Koshland, D.E. 1953), one residue is the enzymic nucleophile, and one residue is the general acid/base catalyst. The role of the third residue was also investigated.

The establishment of an over-expression system is crucial to achieving the goals of this thesis. The methylotrophic yeast *Pichia pastoris* has been successfully utilized by the laboratory of Professor R.T.A. McGillivray for the expression of other human enzymes, and their aid and expertise will be instrumental in the successful adaptation of this expression system for use with human pancreatic α-amylase. In addition, collaboration with the laboratory of Professor C.M. Overall will allow the generation of several active site mutants of the three acids. The amide and alanine mutants are of initial interest since they should give rise to mutant enzymes with significant changes in the catalytic machinery while having minimal effect on the global fold.

Once the expression system is operating and the mutants have been generated, a new purification method must be developed to successfully isolate the mutant proteins. The current purification for the wild-type enzyme is based on affinity purification with starch followed by digestion of the starch by the enzyme being purified. The expected reduction in catalytic activity resulting from the active-site mutations will render this purification protocol ineffective.
Kinetic characterization will begin with determination of the Michaelis-Menten parameters for the recombinant enzymes with a series of substrates, both malto-oligosaccharides and glycosyl fluorides. It is expected that $k_{cat}/K_m$ values for hydrolysis of the natural sugars (which require general acid catalysis) by the mutant of the acid/base catalyst will be more drastically reduced relative to wild-type than will $k_{cat}/K_m$ values for the glycosyl fluorides (which do not require general acid catalysis). The variation of rate constants with pH will provide information about the ionization states of these residues during the reaction. Studies of the inhibition offered by the pseudo-tetrasaccharide inhibitor acarbose with the recombinant enzymes should give some much needed information on the mechanism of action of this inhibitor and may provide insights into general mechanisms of inhibition of HPA that can aid future inhibitor design.

Structural characterization will also be undertaken in collaboration with the laboratory of Professor G.D. Brayer. The three dimensional structures of the mutants will be determined alone and with a series of ligands bound. It is anticipated that the structures of the mutants with the pseudo-tetrasaccharide inhibitor acarbose will be especially interesting.

The work from this thesis will provide new insights into the mechanism of human pancreatic α-amylase and retaining α-glycosidases in general. In addition, the work described herein will provide a foundation upon which to support future mechanistic studies of HPA.
CHAPTER 2

Generation
and Isolation
of Recombinant $\alpha$-Amylases
2.1 Introduction

2.1.1 Human α-Amylase Genetics and Expression

Two closely linked human α-amylase gene loci are located on the p21 arm of chromosome 1 (Zabel, B.U. et al. 1983; Muke, M. et al. 1984) and comprise a total of five genes and two truncated pseudogenes (Gumucio, D.L. et al. 1988). One locus of three genes is responsible for expression of salivary α-amylase (1A, 1B, and 1C) and the other locus contains two genes (2A and 2B) for the pancreatic α-amylase expression. All alleles are expressed in a codominant mode.

The α-amylase gene loci appear to have arisen from a single ancestral pancreatic α-amylase (2B). Around 40 million years ago there was an insertion of a γ-actin pseudogene in the 5’ non-translated region of the 2B gene. A series of gene duplication events, about 1 million years ago, followed by insertion of a retroviral-like sequence into the γ-actin pseudogene appear to have been responsible for the creation and tissue-specific expression of the salivary α-amylase gene locus (1A, 1B, 1C). Excision of the retroviral sequence, possibly from a fourth salivary α-amylase gene, leaving only the long terminal repeats, resulted in the generation of the second α-amylase gene with pancreas-specific expression (Gumucio, D.L. et al. 1988; Samuelson, L.C. et al. 1990).

Although many mammalian species express a salivary α-amylase there are some, including species of primates and rodents, that do not. A comparison of the α-amylase genes from mice and humans suggests that the salivary enzyme arose separately in each of these species (Meisler, M.H. and Ting, C.N. 1993). Since species lacking the salivary α-amylase appear to function similarly to their counterparts that possess the enzyme,
roles in addition to carbohydrate hydrolysis have been proposed. The salivary α-amylase may play a role in microbial colonization of the oral cavity (Scannapieco, F.A. et al. 1989; Scannapieco, F.A. et al. 1992), or possibly the sweet taste of monosaccharides liberated from complex carbohydrates by the action of salivary α-amylase could be used as a signal to indicate a source of nutritious food (Meisler, M.H. and Ting, C.N. 1993).

Numerous different phenotypes of human α-amylases are known – 12 salivary and 6 pancreatic, although the degree of α-amylase heterogeneity seems to be different for each race. It stands to reason that if there are only five genes, many of the isoforms arise from a variety of post-translational modifications. Common post-translational modifications of human α-amylases include; pyroglutamate formation from the N-terminal glutamine, glycosylation, deglycosylation, and deamidation. Deglycosylation and deamidation of human α-amylases are known to occur both by enzymatic and non-enzymatic means (reviewed in (Zakowski, J.J. and Bruns, D.E. 1985)).

Many of the post-translational modifications of the α-amylases, especially glycosylation, have been well characterized. The pancreatic α-amylase is known to lack glycosylation, while the salivary α-amylase is expressed in both slightly glycosylated, and highly glycosylated forms (N-linked glycosylation). This differential glycosylation occurs despite the presence of the same two (and only two) N-linked glycosylation consensus sequences in both enzymes (N^{412}GS and N^{461}CT). The oligosaccharide structures of four different forms of the salivary enzyme have been characterized but will not be presented here (reviewed in (Zakowski, J.J. and Bruns, D.E. 1985)).
2.1.2 Cloning and Mutagenesis of α-Amylases

By the mid 1990’s many mutants of α-amylases from a variety of species had been constructed. These studies have been admirably detailed in several reviews (Svensson, B. and Søgaard, M. 1993; Svensson, B. 1994), and therefore only selected mutants will be discussed here.

While the sheer number of different mutant α-amylases that have been generated is almost overwhelming, there are two significant gaps in the information gathered from the study of these mutants. First, very few of the mutants generated were subjected to detailed kinetic characterization. Most studies have only reported relative activities with a single substrate concentration, and often only a qualitative appraisal of activity on starch has been presented. This level of analysis, while giving a general impression of the importance of a residue to the catalytic activity, is virtually useless for meaningful mechanistic analysis. The notable exceptions to this trend are the detailed kinetic studies performed on a series of porcine and human α-amylase active-site histidine mutants (Ishikawa, K. et al. 1992; Ishikawa, K. et al. 1993) which were discussed in Chapter 1.3.5., and a brief study of the barley α-amylase active site carboxylic acids and histidines (Sogaard, M. et al. 1993).

The second gap in the studies of α-amylase mutants, is a noticeable lack of study of the human α-amylases. Undoubtedly this has resulted from the difficulty in large-scale production of many mammalian α-amylases, human among these, from heterologous expression systems (Svensson, B. and Sogaard, M. 1992). The three human α-amylase histidine mutants mentioned above have been characterized in significant detail, but these studies are the exception rather than the rule. This thesis is aimed at
addressing the lack of detailed kinetic investigation into the roles of the active site carboxylic acids of human pancreatic $\alpha$-amylase.

### 2.1.3 Aims of This Part of the Project

Until the start of this project the primary source of $\alpha$-amylase for studies in this laboratory has been pancreas acquired from the University Hospital. However, owing to the difficulty in obtaining human pancreas in the last decade and the desire to utilize site-directed mutagenesis to further our understanding of the enzyme, cloning of the human pancreatic $\alpha$-amylase was undertaken. The cDNA was successfully constructed by Dr. H. Vo and the initial stages of incorporating it into the *Pichia pastoris* expression system were accomplished by Dr. H. Côté (Rydberg, E.H. *et al.* 1999). The aims of this part of the project therefore, were three-fold:

1) The establishment of an over-expression system, followed by the generation of several mutants of the three acids. The amide and alanine mutants were of particular interest since they should give rise to mutant enzymes with significant changes in the catalytic machinery while having minimal effect on the global fold.

2) Once the expression system was developed and the mutants had been generated, a new purification method must be developed to successfully isolate the mutant proteins. The current purification for the wild-type enzyme is based on affinity purification with starch followed by digestion of the starch by the enzyme being purified. The expected reduction in enzyme catalytic activity resulting from the active-site mutations would render this purification protocol ineffective.
3) The recombinant proteins must be shown to be structurally and kinetically viable for use in further studies. The pancreas-derived enzyme has been successfully crystallized and its three-dimensional structure determined (Brayer, G.D. et al. 1995). Ideally, the recombinant enzyme should crystallize under similar conditions and a three-dimensional structure could be determined for comparison with the wild-type protein. Likewise, kinetic parameters for the native and recombinant HPA should be similar if the yeast expression system has induced no significant changes in the enzyme.

Successful accomplishment of the above goals would bring this project to a position where more detailed structural and kinetic studies could be carried out.
2.2 Results

2.2.1 Cloning and Mutagenesis

Human α-amylase and the D197A and D197N mutants were expressed in *Pichia pastoris* using the secretion vector pPIC9 (Invitrogen Corp., Carlsbad, California). In this vector, secretion of recombinant proteins is directed by the yeast α-factor sequence. Details regarding the cloning and mutagenesis of the wild-type α-amylase and the D197A and D197N recombinant proteins by our collaborators Drs. H. Vo and H. Côté have been reported previously (Rydberg, E.H. *et al.* 1999). Briefly, the D197 mutants were generated in the secretion vector pPIC9 using a PCR methodology. Transformation of *Pichia* spheroplasts was highly inefficient, with 1-4% of the resulting clones being successful in expressing the recombinant protein, as determined by a western blot screening protocol.

To successfully utilize the Künkel method of mutagenesis to generate the other mutants, an F1 (M13 phage) origin of replication needed to be inserted into the pPIC9 vector. The HPA gene could have been ligated into the commercially available pHIL-S1 shuttle vector (Invitrogen Corp., Carlsbad, California), as this vector contained an F1 origin and was almost identical to pPIC9. However, pHIL-S1 contained the sub-optimal Phol sequence to drive secretion of the recombinant protein. It was decided, therefore to generate the unique pHICL-AMY vector, which would contain the F1 origin of pHIL-S1 along with the efficient α-factor secretion sequence and HPA gene from pPIC9-AMY. Since the Sac1/Sal1 digested fragments of pPIC9-AMY were almost identical in size (5040 bp and 4900 bp), further digestion of the fragment not desired was necessary in order to allow for gel purification of the appropriate fragment. Successful ligation of the
pPIC9-AMY and pHIL-S1 fragments generated the 10.6 kbp pHICL-AMY vector containing the F1 origin, α-factor secretion sequence, and the α-amylase gene (Scheme 1).

Following the generation of pHICL-AMY, the orientation of the F1 origin had to be determined in order to allow synthesis of mutagenic primers to the correct strand. Non-dUMP containing single-stranded DNA for sequencing was generated in a manner analogous to the early steps in the Künkel method of mutagenesis (Kunkel, T.A. et al. 1987), except the initial transformation was of *E.coli* strain DH5α (DUT + , UNG + ) instead of RZ1032 (DUT −, UNG −). Attempted sequencing of the AMY gene using complementary primers in separate reactions enabled the orientation of the F1 origin to be determined (as only the strand with an F1 origin is packaged by the phage). The successful sequencing reaction generated the sequence of the strand complementary to the single DNA strand isolated from the phage. This experiment showed that the F1 origin was oriented in the opposite direction to the amylase gene.

With this knowledge, mutagenic primers to the anti-sense strand were then generated. It was determined that the primers used to generate the E233Q and D300N mutants, if degenerate at positions 1 of each codon, could also be used to generate the potentially interesting mutants E233K, D300H, and D300Y. After mutagenesis, the Künkel method of strand selection was used. This method proved to be very efficacious as the desired mutation was consistently present in greater than 80% of the colonies selected.
Scheme 1 Generation of pHICL-AMY from pPIC9-AMY and pHIL-S1. The 5560 bp, F1 origin-containing fragment from the SacI/SalI digestion of pHIL-S1 was ligated with the 5040 bp pPIC9-AMY fragment containing the AMY gene, resulting in the pHICL-AMY vector. The shaded regions were digested or discarded leaving the desired regions for ligating.
Figure 11 Growth curves for *P. pastoris* transformed with wild-type HPA (•) and E233A/D300A (V) on Glycerol (A) and Methanol (B) containing media. The growth curves for each strain are similar when in the same media. The doubling time in the logarithmic phase is 2 hours with glycerol as the carbon source and 4 hours with methanol as the carbon source.

Attempted transformation of *Pichia pastoris* by a PEG methodology was unsuccessful; however, electroporation seemed to be a simple and effective alternative (Invitrogen Inc., 1995). Approximately 20% of the clones generated from the transformation were positive for expression of the recombinant protein. Although little difference was observed between colonies grown on dextrose or methanol-containing plates, growth curves in methanol containing media (Figure 11) showed the transformants to have doubling times of 2 hours (BMGY) and 4 hours (BMMY), demonstrating that all transformed clones were of the MUT\(^+\) (Methanol UTilizing) phenotype (Invitrogen, Inc. 1995). This suggests that, in all cases, integration into the *Pichia* genome occurred at the AOX1 gene. This is the expected result when the pHICL-AMY vector is linearized by SacI prior to transformation, since the 3' and 5' ends of the AOX1 gene remain intact and appropriately oriented for such an integration. However, despite the MUT\(^+\) phenotype, greater protein concentrations in the supernatant were achieved using
induction conditions for the MUT\(^S\) (Methanol Utilizing - Slow growing) phenotype (discussed further in §2.2.3).

### 2.2.2 Selection of Successful Transformants

Upon western-blot screening of 2 mL cultures of *Pichia* transformed with wild-type, D197A or D197N (using a spheroblast transformation method), 4 clones out of 100 were found to express wild-type protein, while only one clone of 100 for each of the D197 mutants were found that successfully expressed the desired protein. The clone showing greatest expression for each protein, as judged by SDS-PAGE stained with Coomassie Blue, was chosen for use in large-scale overexpression.

Screening of the *Pichia* colonies electroporated with E233 and D300 mutant DNA was performed by two methods. Western-blot screening of 2 mL induced cultures was initiated, however, growth and induction were unsatisfactory for these clones in the small cultures and 10 mL induced cultures were then used with greater success. Another method attempted for screening was the newly developed “yeastern” (Wung, J.L. and Gascoigne, N.R.J. 1996), which has been designed specifically for screening *Pichia* cultures that secrete foreign proteins. This method, which is essentially non-electrotransferred Western-blotting of secreted proteins from plates of methanol-induced *Pichia* colonies, seemed to be prone to high backgrounds and excessive washing of the membrane during development was necessary to generate a useful blot. The yeastern method, therefore, was used as a crude screen to guide the colony selection process for the more time-consuming western-blot expression screening. Transformation with D300 mutant DNA seemed to be quite successful, as most of the D300 mutants were found to be expressed by a large number of the transformants (about 40%) at levels estimated to
be about 0.1 mg/mL from the Coomassie-stained SDS-PAGE. The E233 mutant transformations were not as successful, and the E233Q transformation was performed twice in order to generate successful transformants. In general, there was about a 5% success rate in the transformation and the protein appeared to be expressed in quite low levels (\( \leq 0.025 \) mg/mL as estimated from Coomassie-stained SDS-PAGE). In all cases, the clones appearing to have the highest expression levels, as judged by SDS-PAGE, were saved for use in large-scale overexpression.

The variability in expression levels observed with the E233 and D300 mutants appears to be a common characteristic of the \textit{Pichia} expression system. Reports in the literature have demonstrated expression levels as high as 2.5 g/L (Paifer, E. \textit{et al.} 1994) and as low as 0.2 mg/L (Lam, L.P. and Berger, S.A. 1997).

\textbf{2.2.3 Expression and Purification}

Expression of the recombinant proteins was performed in shake flasks using BMGY medium for growth and BMMY medium for induction. The recombinant proteins were expressed at between 3 and 20 mg/L and were the only proteins in the media that could be detected by SDS-PAGE with Coomassie Brilliant Blue staining. It was determined by SDS-PAGE (Figure 12) and saccharogenic assays (Bernfeld, P. 1955) of the wild-type enzyme that the BMGY/BMMY media combination gave the highest level of protein expression. Use of the minimal media either BMG or BMM (which do not contain yeast extract or peptone), instead of the more complete media, resulted in a noticeable decrease in the amount of protein expressed.
Figure 12 SDS-PAGE Showing Differential Protein Expression Levels of Recombinant HPA in *Pichia pastoris* with Different Growth and Induction Media. Activity assays of the supernatants, using the DNS assay, are shown superimposed on the gel.

Saccharogenic assays of the crude mutant proteins indicated that they had very low activity (Figure 13 shows specific activities of purified recombinant α-amylases on starch for comparison). Affinity precipitation with glycogen (Schramm, M. and Loyter, A. 1966) could therefore not be used for the purification of these proteins since they would be unable to digest the glycogen after precipitation. Instead, purification using several commercial chromatographic resins was attempted under a range of buffer conditions in an attempt to separate the recombinant protein from a coloured contaminant produced by *Pichia* under conditions of methanol metabolism and to achieve concentration of the 1 L supernatant. Neither anion nor cation exchange resins were initially successful at separating the α-amylase from the media contaminant. The anion exchange resins (Q-Sepharose, Mono-Q) did not bind the recombinant protein satisfactorily even at high pH and rapidly became overloaded with the media contaminant, while the cation exchange resins (CM-Sepharose, SP-Sepharose) did not bind either the recombinant protein or the contaminant. Nickel-chelate chromatography
was also attempted with the thought that some of the colour substance may have a heme component. Heme porphyrins have been demonstrated to bind nickel (Alston, K. et al. 1984), therefore a nickel-chelate column was also attempted in hopes of removing the colour contaminant. This also proved unsuccessful, as neither the amylase nor the coloured contaminant bound to the nickel-chelate column.

Figure 13 Specific Activities for Purified Recombinant Proteins on Starch. One unit is defined as the μmoles of reducing ends, measured as maltose, generated per minute at 30°C.

Since HPA failed to bind significantly to any of the ion exchange resins used, the hydrophobic interaction resin phenyl-sepharose was tested. The recombinant proteins were shown to bind successfully to phenyl-sepharose under ionic strengths equivalent to that in the media, while only binding a relatively small amount of the media contaminant. Elution from the hydrophobic column was achieved using distilled water, as the α-amylase remained bound to the resin even under relatively low ionic strength conditions (as low as 20 mM phosphate, 25 mM NaCl at pH 7.0). The remaining media
contaminant could then be separated from the amylase by passing the buffered and concentrated phenyl-sepharose eluant through a small Q-Sepharose column. The modest amount of remaining contaminant bound tightly to the anionic resin while the majority of the recombinant protein did not bind, and appeared in the flow-through. The recombinant proteins were now better than 99% pure as judged by Coomassie Brilliant Blue stained SDS-PAGE and visual inspection showed the protein solutions to be clear and colourless.

2.2.4 Fermentation Trials
Since only modest levels of expression were being achieved (3-20 mg/L) it was decided to attempt to increase expression levels by expression in a 1 L fermentation reactor. The *Pichia* expression system has been optimized for use with fermentors and an increase in expressed protein levels of up to ten times is not uncommon. Some of the highest expression levels using *Pichia* reported in the literature have been achieved through the use of fermentors (White, C.E. *et al.* 1994; Heimo, H. *et al.* 1997).

Initial attempts at overexpressing HPA in a 1 L fermentor utilized BMGY medium with regular additions of 0.5% methanol after the growth phase was completed. This procedure proved unsuccessful and less protein was expressed in the fermentor than was expressed in the shake flasks. Further attempts included varying the methanol concentration during induction (followed by a methanol probe), and using a basal salts medium. In all cases aliquots of the medium were assayed for amylase (by saccharogenic assay and by SDS-PAGE) at regular intervals throughout the expression period. Unfortunately, none of the conditions appeared to give expression of HPA at concentrations near that achieved in shake flasks.
2.2.5 Glycosylation Site

Despite obtaining the recombinant enzyme in modest, but useful, amounts (3-20 mg/mL), crystallization was unsuccessful under a broad range of conditions, including the conditions used for the native enzyme (Brayer, G.D. et al. 1995). In some cases glycosylation is known to interfere with crystallization (Nersissian, A.M. et al. 1996; Liu, J. et al. 1996; Linnevers, C.J. et al. 1997), and since Pichia pastoris is known to glycosylate foreign proteins heterogenously, it seemed likely that the recombinant α-amylase was glycosylated.

The first direct evidence that the recombinant amylase might be glycosylated was the presence of two bands on Coomassie-stained SDS-PAGE and Western blots that migrated within about 5 kDa of each other (Figure 14). These two bands were present in all preparations of the recombinant enzyme. Schiff staining (Stromqvist, M. and Gruffman, H. 1992) of the protein after transfer to a PVDF membrane confirmed the presence of protein glycosylation. Electrospray mass spectrometry showed that the recombinant amylase was expressed in a series of forms with masses differing by approximately 165 Da – the mass of a sugar residue (Figure 15A). A peak due to the non-glycosylated protein (predicted mass 55887 Da, observed 55894 Da) was also observed. This suggests the presence of ten to thirteen sugar residues on the glycosylated enzyme forms.
Figure 14 SDS-PAGE Showing Evidence for Glycosylation. HPA from the Pichia supernatant (glyHPA) was 2 kDa larger than that treated with Endoglycosidase F.

_Pichia pastoris_ is known to perform both _N_- and _O_-linked glycosylation of foreign proteins (Grinna, L.S. and Tschopp, J.G. 1989; Trimble, R.B. _et al._ 1991; Cregg, J.M. _et al._ 1993; Juge, N. _et al._ 1996). Since _N_-linked glycosylation occurs at a consensus sequence (Asn-X-Ser(Thr), where -X- is any residue except proline) while _O_-linked glycosylation does not, we first looked for evidence of _N_-linked glycosylation. Human pancreatic α-amylase has two potential sites for _N_-glycosylation, Asn412 and Asn461; both of these residues are located on the surface of domain C (Brayer, G.D. _et al._ 1995). Deglycosylation with endoglycosidase F, an enzyme that hydrolyzes the GlcNAc–β(1-4)–GlcNAc glycosidic bond of _N_-linked high mannosyl groups, resulted in an α-amylase with a mass of 56088 Da, (Figure 15B). This corresponds to the native enzyme with a single GlcNAc present therefore indicating a single _N_-glycosylation site and no _O_-linked glycosylation. These data also confirm that the recombinant α-amylase is heterogeneously _N_-glycosylated containing, at most, a single _N_-linked oligosaccharide
chain likely ranging from Man₈GlcNAc₂ to Man₁₁GlcNAc₂ (Grinna, L.S. and Tschopp, J.G. 1989; Cregg, J.M. et al. 1993).

Figure 15 Reconstructed electrospray mass spectra of recombinant human pancreatic α-amylase expressed in the yeast *Pichia pastoris*. The protein is expressed as a heterogeneously glycosylated product (A) with the major species possessing from 10 to 13 sugar residues. After hydrolysis with endoglycosidase F (B) the recombinant α-amylase has a mass indicative of the presence of only one sugar residue (GlcNAc).

Samples of untreated and endoglycosidase F-treated recombinant HPA were then denatured with urea, reduced with dithiothreitol (DTT) and the cysteine residues derivatised with iodoacetamide prior to tryptic digestion. A comparison of tryptic digest maps for the two HPA samples revealed a peptide of m/z 1028.5 amu seen only in the treated sample. By Edman degradation, the peptide was shown to have the apparent sequence EDVISGDKINGDETGIK. The nearest HPA sequence to this was CDVISGDKNCTGIK. The obvious differences between the two sequences (Glu instead of Cys, and Asp₄₆₁ replacing Asn₄₆₁) were apparently due to the acetamidation of the cysteines yielding residues for which the PTH derivative was indistinguishable chromatographically from the Glu PTH derivative. Similarly, the presence of the GlcNAc on Asn₄₆₁ apparently modified the chromatographic behaviour so as to
resemble Asp. This strongly suggested that the glycosylated residue in the recombinant amylase was Asn461.

Upon deglycosylation with endoglycosidase F, the recombinant amylase could be crystallized under the same conditions and in the same space group as the native enzyme (Brayer, G.D. et al. 1995). However, despite this the unit cell dimensions for the recombinant enzyme differed, as did the packing of the molecules in the unit cell (Rydberg, E.H. et al. 1999). These differences appear to be due to the glycosylation of Asn461. As Figure 16 shows, there is well-defined electron density off the amide nitrogen of Asn461, which fits a GlcNAc sugar residue. No other sites of glycosylation were evident from electron density maps.

Although somewhat different crystal packing arrangements were observed, the folds of the recombinant and native HPA's are comparable with an overall average main chain deviation of 0.22 Å. The largest differences occur at residues 304-308, which form a flexible loop near the active site and may be involved in substrate binding. In the structures of both the native and recombinant proteins, these residues have high thermal factors indicating significant polypeptide chain mobility. The next largest differences occur near the glycosylation site at Asn461, which is part of a surface loop region. Both areas of conformational differences are probably the result of mobile loops adapting to a new crystal packing arrangement (Rydberg, E.H. et al. 1999).
Figure 16  A portion of the 2F₀-F₀ difference electron density maps of *Pichia*-HPA in the vicinity of residues N412 (A) and N461 (B). The electron density maps clearly indicate the N-acetylglucosamine (GlcNAc) residue bound to Asn461. This map has been contoured at the 5σ level and the final refined coordinates for both the N412 and N461 are overlaid. Plots are from (Rydberg, E.H. *et al.* 1999).

The conformational similarity between the recombinant and native proteins extends to both the chloride and calcium binding sites, as well as the active site region. The largest active site conformation difference is an approximate 0.3 Å shift in a water molecule bound to Asp197 OD1.

**2.2.6 Characterization by ESI-MS**

To confirm the homogeneity and mass of the purified recombinant proteins, electrospray ionization mass spectrometry (ESI-MS) was used to characterize the deglycosylated recombinant proteins (Table 6).

In order to compare the ESI-MS mass determinations with the theoretical masses, several modifications had to be accounted for. All of the recombinant proteins had been shown to be *N*-glycosylated by SDS-PAGE. Deglycosylation by endoglycosidase F
would therefore result in apparent masses of 203 amu larger than those determined from
the amino acid sequences, due to the mass of a GlcNAc. Also, the N-terminal glutamine
may be deamidated to form the pyroglutamate. This has been widely observed in HPA
crystal structures (Brayer, G.D. et al. 1995), and also accounts for the resistance of HPA
to N-terminal sequencing (Zakowski, J.J. and Bruns, D.E. 1985). The deamidation would
manifest itself as a reduction in apparent mass of 17 amu. It is interesting to note that the
same sample of recombinant protein has been observed to have masses corresponding to
both the glutamine and pyroglutamate forms of the protein as determined by ESI-MS
when measured at different times. Often, as in the case of the wild-type enzyme, an
initial mass determination performed shortly after purification suggested that the protein
was in the pyroglutamate form while a mass determination several weeks later resulted in
a mass corresponding to the glutamine (or glutamate!) form. For example, the mass of
the wild type HPA has been measured initially as 56073 amu, which corresponds
precisely to the theoretical mass with the addition of one GlcNAc and the loss of one
NH₃. The same sample measured several days later gave the mass of 56095 amu, which
is within error limits of the mass of the protein containing the N-terminal glutamine (or
more likely, glutamate). Aging of purified human α-amylases has been extensively
studied by electrophoretic methods, and has been attributed to slow, non-enzymatic
deamidation (Saeki, S. 1978; Lorentz, K. 1979). Although deamidation would not result
in the change in mass observed, slow hydrolysis of the N-terminal pyroglutamate, a
similar reaction, would result in the addition of 18 Da. This appears to be the case for all
but two of the recombinant proteins (E233Q and D300Y). Therefore, when reporting the
masses, as determined by ESI-MS (as in Table 6) the masses have been reported as the aged mass, since this was most likely the form used for the majority of the kinetic studies.

The ESI-MS data in Table 6 suggests that the masses of all recombinant proteins are within experimental error (2 parts in 10000) of their theoretical values for either N-terminal form discussed above. All proteins appear to revert to the non-pyroglutamate form upon storage, with the exceptions of E233Q and D300Y.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approx Yield (mg/L)</th>
<th>Theoretical Mass (Da)</th>
<th>Observed Mass (Da)</th>
<th>∆Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA</td>
<td>15</td>
<td>56091</td>
<td>56095</td>
<td>4</td>
</tr>
<tr>
<td>D197A</td>
<td>8</td>
<td>56047</td>
<td>56050</td>
<td>3</td>
</tr>
<tr>
<td>D197N</td>
<td>6</td>
<td>56089</td>
<td>56082</td>
<td>7</td>
</tr>
<tr>
<td>E233A</td>
<td>4</td>
<td>56033</td>
<td>56038</td>
<td>5</td>
</tr>
<tr>
<td>E233K</td>
<td>4</td>
<td>56089</td>
<td>56085</td>
<td>4</td>
</tr>
<tr>
<td>E233Q</td>
<td>4</td>
<td>56090</td>
<td>56071</td>
<td>19‡</td>
</tr>
<tr>
<td>D300A</td>
<td>15</td>
<td>56047</td>
<td>56042</td>
<td>5</td>
</tr>
<tr>
<td>D300H</td>
<td>20</td>
<td>56113</td>
<td>56112</td>
<td>1</td>
</tr>
<tr>
<td>D300N</td>
<td>15</td>
<td>56090</td>
<td>56085</td>
<td>5</td>
</tr>
<tr>
<td>D300Y</td>
<td>20</td>
<td>56139</td>
<td>56128</td>
<td>11‡</td>
</tr>
<tr>
<td>E233A/ E233K</td>
<td>3</td>
<td>55989</td>
<td>55992</td>
<td>3</td>
</tr>
</tbody>
</table>

‡ = these masses, that vary as much as 17±6 Da from theoretical, may contain different forms of the N-terminal Glu (which could alter the mass by 18 Da)
2.2.7 Characterization by X-Ray Crystallography

Upon deglycosylation with endoglycosidase F, the recombinant proteins were crystallized under conditions similar to pHPA. A comparison of the refined structures revealed that there was very little difference in the overall global folds and that the active sites were virtually identical, with the exception of the mutated residues (Figure 17). The most significant structural differences were rotation of the C-domain about the flexible linker, and deviations in the flexible, substrate binding loop from residues 301-310 (Brayer, G.D. et al. 1995; Rydberg, E.H. et al. 1999; Brayer, G.D. et al. 2000).

Figure 17 Structural comparison of the three conserved active site carboxylic acids of pHPA (red), D197A (cyan), E233A (yellow) and D300N (green). Alignments were performed using the Swiss PDB viewer. Three active site strands were used in the alignment. The structures of both mutant enzymes aligned with the wild-type structure with 0.03 Å RMS deviations. Wild-type HPA coordinates from (Rydberg, E.H. et al. 1999), structural coordinates of the mutant enzymes courtesy of Dr. G.D. Brayer.
2.3 Discussion

Clearly the expression levels of human pancreatic α-amylase discussed in this thesis, typically around 10 mg/L, are considerably lower than some of the immense expression levels reported (eg. 2.5 g/L) (Paifer, E. et al. 1994). However, a perusal of the literature reveals that the most common expression levels achieved are 10-100 mg/L for shake flask cultures (Figure 18). The large variation in reported expression levels suggests that the degree of overexpression in the *Pichia* system is dependent on the specific exogenous protein introduced into *Pichia*, as well as the particular *Pichia* system used. In this light, an expression level of 10 mg/L is not unusual.

![Figure 18](image)

**Figure 18** Expression Levels for Foreign Proteins (black = from all species, shaded = human) in *Pichia Pastoris* as Reported in the Literature from 1991-1999. (Invitrogen 1995)

Although the native human α-amylases (salivary and pancreatic) are known to exist in several forms resulting from differential modification, SDS-PAGE and x-ray crystallographic studies have shown no evidence that the native pancreatic enzyme is glycosylated (Zakowski, J.J. and Bruns, D.E. 1985; Brayer, G.D. *et al.* 1995). Since
*Pichia pastoris* is known to heterogeneously glycosylate secreted proteins, it was not surprising that the recombinant α-amylases were *N*-glycosylated. Both potential *N*-glycosylation sites are located in domain C and were therefore not anticipated to affect the catalytic function of HPA with small substrates.

Upon initial investigation, it was surprising that glycosylation of the recombinant protein occurs at Asn461 (consensus sequence NCT) and not Asn412 (consensus sequence NGS) since both residues occur in surface loops and contain consensus sequences that allow for high core-glycosylation efficiency (Shakin-Eshleman, S.H. *et al.* 1996). Indeed, comparison of the folds of the loops involved would appear to favor glycosylation at Asn412 since this residue is part of an Asx turn, which has been shown to strongly favor *N*-glycosylation (Imperiali, B. 1997). In contrast, the loop containing Asn461 is not part of either an Asx or a β-turn. Furthermore, the cysteine at the -X-position of the Asn461 NCT consensus sequence is involved in a disulfide bond with Cys450. While the presence of a disulfide bond does not exclude glycosylation, it has been suggested that this is the reason why *N*-glycosylation is not commonly seen at consensus sequences with cysteine at the -X- position (Shakin-Eshleman, S.H. *et al.* 1996). A major factor in the lack of glycosylation at Asn412 may be the strong hydrogen bond formed between the side chain amide of this residue and Asp432. In contrast, the glycosylated sidechain of Asn461 is found to be highly solvent accessible in the X-ray structure of native HPA.

Deglycosylation of the recombinant HPA was crucial for successful crystallization of this protein for structural studies. Follow-up analyses indicate packing between molecules in the crystal lattice would lead to prohibited contacts in the presence
of bulky glycosyl groups (10 to 13 sugar residues). The heterogeneous nature of the glycosylation would also be detrimental to crystallization. Endoglycosidase F digestion appears to completely remove all glycosyl residues except the amide-bound GlcNAc, resolving this problem. In addition, the removal of protein heterogeneity also greatly enhanced the intensity of the ESI-MS signal (Figure 15).

Successful cloning and generation of mutant human pancreatic α-amylases in the *Pichia pastoris* expression system provides the foundation for kinetic and structural studies of the catalytic mechanism and, specifically, determination of the roles that the three conserved catalytic carboxylic acids play in this mechanism. In the longer term, detailed kinetic investigations of mutant enzymes generated from this system will aid in inhibitor design for use in attenuating sugar metabolism or for *in vivo* diagnostic uses.
CHAPTER 3

KINETIC
AND
STRUCTURAL
ANALYSES
3.1 Introduction

The saccharogenic activity of α-amylase is one of the oldest described enzymatic activities. As far back as the early 1800’s the digestion of starch by wheat, and later barley, extracts was observed. These extracts were initially termed “diastases” until the early 1900’s when they were designated as “amylases” (Zakowski, J.J. and Bruns, D.E. 1985). Amylase from saliva was studied as early as 1831, while amylases from serum and urine were described in the mid 1800’s. Although a saccharogenic assay (measuring the generation of products, as an increase in reducing units, from the hydrolysis of starch) was first described by Kjeldahl in the 1880’s, it was not until the development of the amylloclastic assay (measuring of the hydrolysis of starch by detecting the decrease in the abundance of starch-iodine complexes) by Wohlgemuth in 1908 that α-amylase determinations became practical as a clinical diagnostic tool (Zakowski, J.J. and Bruns, D.E. 1985). The relative ease of purification from saliva or urine, coupled with the convenient assay methods, led to the study of α-amylase by Henri (Henri, V. 1902), and Michaelis and Pechstein (Michaelis, L. and Pechstein, H. 1914) in the early 1900’s.

In the almost 200 years since its activity was first discovered, several α-amylase assays have been developed, and many modifications have been made to the early saccharogenic and amylloclastic assays, although only a few of these assays are still in use today. The most convenient, and mechanistically meaningful, assay methods have been developed recently and allow the use of small homogeneous substrates. For this thesis, several different assays were utilized in order to study different properties of the hydrolytic activity of human pancreatic α-amylase.
The saccharogenic dinitrosalicylate (DNS) assay was used initially to evaluate successful expression of wild-type HPA and to locate active fractions throughout the purification. This assay measures the increase in sugar reducing ends from starch hydrolysis through their reaction with dinitrosalicylate. After boiling in base in the presence of Rochelle’s salt, the DNS turns a dark red in the presence of sugars with free reducing ends. This procedure is useful for assaying crude enzyme preparations since it is robust and unaffected by a large range of pH, ion concentrations, and other conditions found in culture media (Bernfeld, P. 1955). However, the DNS assay was generally not useful for assaying the crude mutant enzyme preparations, as their catalytic activities were too low to detect in a convenient manner using the natural substrate. A purified preparation of each mutant enzyme was subjected to the DNS assay in order to determine their relative activities on starch, but these assays required large amounts of enzyme (>1 mg/mL) and extended periods of time (>12 hours).

A more informative means of assaying HPA utilizing small, natural oligosaccharides, involves the use of HPLC. This technique is not only useful for deriving kinetic parameters, but also for determining product composition. HPLC allows the study of potentially different mechanisms with various substrates or mutants since, with certain columns (such as the Waters® Dextropak™), specific anomers of the products can be separated and identified thus eliminating the need for end-labeling of sugars to study cleavage patterns (Brayer, G.D. et al. 2000). Although much more information is acquired in this method than with the DNS assay, the HPLC assay of mutants using natural oligosaccharides still requires extended periods of time.
One of the most convenient, and mechanistically useful, HPA assays recently developed utilizes glycosyl fluorides (Brayer, G.D. et al. 2000). These small oligosaccharides, generally α-maltosyl fluoride or α-maltotriosyl fluoride, contain an anomeric fluorine that is released upon hydrolysis by HPA and can be detected using a fluoride selective electrode. The good leaving group ability of the fluoride allows for rapid enzymatic hydrolysis, and often \(k_{\text{cat}}\) values for the wild-type enzyme with such glycosyl fluoride substrates approach those of much larger, natural substrates. One of the most significant advantages of the glycosyl fluoride substrates is that the fluoride is a much better leaving group than the oligosaccharides that are the natural aglycones and, therefore, cleavage of the glycosyl fluorides does not require general acid catalysis. This can result in enhanced catalytic activity for certain, normally low activity mutants, with respect to others, thus allowing small differences in the activities of certain mutant enzymes, such as the mutants of the catalytic triad of carboxyl groups, to be amplified to a degree where they can be detected. In addition, comparison of the kinetic parameters for hydrolysis of natural oligosaccharides and glycosyl fluorides by the various mutant enzymes may allow elucidation of the roles of one or more of the carboxyl groups in the catalytic mechanism of HPA. Finally, the syntheses of the α-glycosyl fluorides is straight-forward (see §4.4).

Despite the lengthy and varied history of α-amylase enzymology, it is only within the last decade that significant advances have been made into understanding the details of its catalytic mechanism. Unlike the array of detailed kinetic investigations that preceded the crystallographic studies of β-glycosidases, the recent advances in understanding the
mechanism of α-amylases were initiated by a flood of structural data, and the kinetic studies have followed at a slower pace.

### 3.1.2 Specific Aims of the Kinetic and Structural Analyses

With only a few notable exceptions (Ishikawa, K. et al. 1992; Matsui, I. et al. 1992; Ishikawa, K. et al. 1993; Sogaard, M. et al. 1993), there have been but few detailed kinetic analyses of α-amylase mutants. The study of β-glycosidases has benefited from a wide range of kinetic (McCarter, J. and Withers, S.G. 1994; MacLeod, A.M. et al. 1994; Namchuk, M.N. and Withers, S.G. 1995; Macleod, A.M. et al. 1996; Birsan, C. et al. 1998) and others) and, more recently, structural studies (of special note are: Davies, G.J. et al. 1998) where mutants have often played a crucial role in understanding the specific details of the catalytic mechanisms. However, our understanding of the mechanistic details of α-amylases, and α-glycosidases in general, has not benefited from such studies with corresponding mutants. The broad aim of this part of the thesis, therefore, is to address this shortcoming through a series of detailed kinetic and structural investigations, with the following specific goals.

1) **Determining which residue is the catalytic nucleophile**

Three types of experiments will be attempted in order to gather evidence for the identity of the catalytic nucleophile.

A) Determination of the kinetic parameters for the hydrolysis of glycosyl fluoride substrates by the mutants should provide the initial evidence for the identity of the nucleophilic residue. Since the enzymic nucleophile is absolutely essential for the catalytic mechanism of α-amylase, replacement of this residue by alanine or the
corresponding amide should result in drastically reduced activity compared to enzymes likewise altered at the other carboxyl groups, when assayed with the activated substrates.

B) Rescue experiments utilizing a small, strong nucleophile such as azide with a glycosyl fluoride substrate should also provide strong evidence for the identity of the enzymic nucleophile. The product from the successful reaction of the nucleophile mutant with azide and an α-glycosyl fluoride would be the β-glycosyl azide, which would be readily detectable by $^1$H NMR.

C) The strongest support for the identity of the enzymic nucleophile would come from successfully labeling it. Although this is commonly achieved using 2-fluoro, or 5-fluoro glycosyl fluorides, the nucleophile of the family 13 α-glycosyltransferase cyclodextrin glucanotransferase (CGTase) was recently identified by covalently modifying it in a mutant CGTase containing a modified acid/base catalyst. In this case the reagent employed was 4''-deoxy-α-maltotriosyl fluoride, which is a substrate for the wild-type enzyme. This strategy will also be utilized in an attempt to covalently modify the catalytic nucleophile of α-amylase.

2) Providing kinetic evidence to identify the residue that acts as general acid/base catalyst

To aid in the identification of the general acid/base catalyst of α-amylase, three types of experiments will be attempted.

A) Determination of the kinetic parameters of the mutants using glycosyl fluoride substrates may provide some evidence for the identity of the general acid/base catalyst. Since the rate of glycosyl fluoride hydrolysis should be independent of general acid catalysis, owing to the good leaving group ability of the fluoride, the mutant of the
general acid/base catalyst should still have a relatively high rate constant for the glycosylation step. However, if deglycosylation is rate-determining, then this analysis is made more difficult. A potentially more diagnostic means of studying the acid/base catalyst would be to compare the relative decrease in $k_{cat}/K_m$ values between natural and glycosyl fluoride substrates for each mutant. Since the $k_{cat}/K_m$ value reflects the first irreversible step, presumably glycosylation, it should allow direct comparison of the general acid-dependent step.

B) Again, rescue experiments can be utilized, this time to provide evidence for the identity of the general acid/base catalyst. In this case, the product from the successful reaction of the acid/base catalyst mutant with azide and an $\alpha$-glycosyl fluoride would be the $\alpha$-glycosyl azide, which would be easily identified by $^1$H NMR. This product should only be produced by the enzyme mutant with the general acid/base catalyst modified to a neutral group smaller than glutamate.

C) The pH dependence of the catalytic rate constants $k_{cat}$ and $k_{cat}/K_m$ can provide valuable information regarding the ionization states of catalytic residues during the reaction, providing that the reaction is highly dependent on the ionization state of these residues. In most glycosidases for which pH profiles have been studied, a bell shaped dependence of the rate constants on pH is observed. This is generally interpreted to mean that the acidic limb represents the $pK_a$ of the nucleophile and the basic limb represents the $pK_a$ of the general acid/base catalyst. In an elegant experiment (McIntosh, L.P. et al. 1996) this has been shown to be true for $B. circulans$ xylanase by directly monitoring the acid/base and nucleophilic residues during the course of the reaction by $^{13}$C NMR. In addition, what is often observed for the enzymes containing mutations at one of these
residues is a pH profile that shows the dependence of the rate constants on only a single pKₐ. The pH profiles for the mutant α-amylases will be determined with the expectation that the basic limb of the pH profile for the enzyme containing a mutation of the acid/base catalyst should be significantly altered.

3) Elucidating mechanistic details regarding the inhibition of α-amylase by acarbose

Lastly, inhibition by the pseudo-tetrasaccharide inhibitor acarbose with the recombinant enzymes will be studied in two ways.

A) The inhibition constant (Kᵢ) for acarbose and the mode of inhibition will be determined for each recombinant enzyme utilizing standard enzyme inhibition assays.

B) Structures of the recombinant enzymes with acarbose soaked into the crystals will be determined. Since acarbose is thought to undergo an α-amylase catalyzed transglycosylation reaction to achieve its active form (Qian, M. et al. 1994), the structure of the nucleophile mutant with acarbose is anticipated to be especially interesting, since no such reaction should occur.

Since no studies involving retaining α-glycosidase mutants and acarbose have been reported to date, the information from this study should give some new insight into the mechanism of action of this inhibitor, and it may provide insights into general aspects of the inhibition of HPA that may aid future inhibitor design.

The studies presented here provide a timely, detailed mechanistic study of α-amylase and α-amylase mutants and provide evidence for the identities of the catalytic nucleophile and general acid/base catalyst.
3.2 Results

3.2.1 Determining Valid Assay Conditions

Although glycosyl fluorides have been used previously for assaying HPA in our laboratory (Braun, C. 1995; Rydberg, E.H. et al. 1999; Brayer, G.D. et al. 2000), the unique properties of the mutant enzymes may necessitate altering the standard assay conditions (30°C, buffered with 20 mM phosphate containing 25 mM NaCl at pH 7.0). Higher \( K_m \) values, requiring greater substrate concentrations, would require higher buffer concentrations, lower \( k_{cat} \) values could require longer assay times, and studies of the pH dependence of the kinetic parameters will require alternative buffers. In addition, fluoride selective electrodes are known to give more stable responses in solutions of higher ionic strengths, so the effects of increased NaCl concentrations on \( \alpha \)-amylase activity will also be determined.

3.2.1.1 Varying Phosphate and NaCl Concentrations

Increased buffer or NaCl concentrations may be required when assaying with high substrate concentrations or for optimized fluoride electrode response. Thus, phosphate and NaCl concentrations were varied between 20 mM and 100 mM, and the activity of wild-type HPA was assayed with \( \alpha \)G3F utilizing a fluoride-selective electrode. No significant difference in HPA activity was observed upon varying either phosphate or NaCl between 20 mM and 100 mM.
3.2.1.2 Stability to Prolonged Incubation at Assay Temperature

As some assays with the mutant enzymes were expected to require prolonged incubation times to assay the low activities, a time course assay was undertaken in order to assess the validity of results acquired after such prolonged incubations.

Figure 19 Time course assay for incubation of wild-type HPA (•), E233Q (□), and D300N (△) at 30°C. The mutant enzymes were stable for more than 2 days, while the wild-type began losing activity after a few hours.

Solutions of wild-type HPA, E233Q and D300N were incubated at 30°C for 4 days and aliquots were removed periodically during this time for assay with αG3F under standard conditions. All mutant enzymes appear to be stable to incubation at 30°C for at least two days (Figure 19), whereas the wild-type enzyme began losing activity after a few hours.

3.2.1.3 Investigating the Effect of Citrate on α-Amylase Activity

For the pH studies planned, a citrate/phosphate buffer was to be used to allow a constant buffer system throughout the entire pH range (4-10). However, rapid loss of activity was observed with this buffer system even at pH 7.0, where no activity loss was
observed with only phosphate. Since citrate is known to chelate calcium, it seemed possible that the observed inhibition was due to chelation of the α-amylase-bound calcium by citrate. To test this hypothesis, 0.5 mM CaCl₂ was added at various times during the assays (Figure 20). Upon addition, CaCl₂ rescued the enzyme activity, while CaCl₂ added prior to the start of the assay protected the enzyme from inactivation. Therefore, it appears that the citrate in the assay was removing the crucial calcium from the α-amylase. All pH studies were therefore performed using citrate/phosphate buffer containing 0.5 mM CaCl₂.

**Figure 20** Overcoming citrate inhibition of pHPrA with 0.5 mM CaCl₂. Addition of 0.5 mM CaCl₂ at the beginning of assays containing citrate (—) was found to protect against rapid enzyme inactivation. Likewise, addition of 0.5 mM CaCl₂ after 5 minutes of reaction (...) or after almost complete inactivation (—) was found to restore full activity. Assays measured the hydrolysis of 2.0 mM αG3F by detection of the fluoride released. For each assay spontaneous hydrolysis of αG3F was followed for 250 seconds before addition of enzyme.
3.2.2 Assaying a Variety of Substrates

Although crude assays of specific activity of the recombinant enzymes were performed using the DNS method, and HPLC was utilized to assay maltopentaose degradation, the predominant method of assaying the recombinant enzymes was through the use of \( \alpha \)-glycosyl fluorides. Kinetic parameters for the recombinant enzymes were determined in a continuous assay by measuring the rate of release of fluoride ion from \( \alpha \)-maltosyl-fluoride (\( \alpha \)G\(_2\)F) and \( \alpha \)-maltotriosyl fluoride (\( \alpha \)G\(_3\)F) using a fluoride selective electrode. This assay method has been successfully utilized previously for measuring wild-type \( \alpha \)-amylase activity (Braun, C. 1995; Rydberg, E.H. et al. 1999; Brayer, G.D. et al. 2000), and is one of the few convenient, continuous, non-coupled assays for \( \alpha \)-amylases.

Early studies of the reactions of \( \alpha \)G\(_2\)F with \( \alpha \)-amylases suggested that the predominant mechanism of fluoride release was through transglycosylation (Okada, G. et al. 1979). These experiments, however, were performed at high \( \alpha \)G\(_2\)F concentrations (typically greater than 20 mM, \( K_m = 3.0 \) mM) with product determination occurring after 20-30 minutes. Consequently, these studies were reporting conditions that may not be typical of the initial reaction (that is, during measurement of \( v_0 \)). For the studies in this thesis, wild-type \( \alpha \)-amylase showed little evidence of transglycosylation using \( \alpha \)G\(_2\)F or \( \alpha \)G\(_3\)F as substrate under standard assay conditions (substrate concentrations less than 7 times the \( K_m \) value, and the reaction measured over five minutes) by either TLC or HPLC (discussed in §3.2.3), although transglycosylation products were observed after periods of extended incubation. However, the mechanism of action of HPA on \( \alpha \)-glucosyl-fluoride (\( \alpha \)GF) has been demonstrated to be different from that on the longer glycosyl fluorides.
(Brayer, G.D. et al. 2000). Thus, the reaction of α-glucosyl fluoride with a variety of recombinant enzymes was also investigated.

3.2.2.1 Starch

The culture supernatant and purified forms of all recombinant proteins were assayed by the DNS (dinitrosalicylic acid) method as a rapid assay of specific activity on the natural substrate. No activity was detected in the supernatants under standard DNS assay conditions for any of the mutant proteins (data not shown), and the specific activity in the purified form (Table 7) could only be determined after extended incubation (typically at 12 to 24 hours). As only approximate specific activities were deemed necessary, and because large amounts of enzyme were required for each assay (except for the wild-type), only a single assay was performed for each enzyme during the extended incubation. Therefore, the specific activities of the mutant enzymes represent only crude estimates.

From Table 7 it appears obvious that all of the mutations generated at D197, E233, and D300 resulted in enzymes with significantly reduced ability to hydrolyze starch, as their specific activities are at least $10^4$ fold less than that of the wild-type enzyme. Furthermore, with the exception of D197A, D197N, and E233A, virtually all of the mutant enzymes have similar activities, as measured by the DNS-starch assay. These results are essentially the same as previously reported starch assays for enzymes mutated at any one of the three active site carboxylic acids (reviewed in (Svensson, B. and Søgaard, M. 1993; Svensson, B. 1994)).
Table 7 Specific Activities of Recombinant α-Amylases on Soluble Potato Starch.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific Activity on Soluble Starch (‡U/mg protein)</th>
<th>Protein</th>
<th>Specific Activity on Soluble Starch (‡U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA-gly</td>
<td>N/A</td>
<td>E233Q</td>
<td>0.020</td>
</tr>
<tr>
<td>HPA-degly</td>
<td>5.5×10³</td>
<td>D300A</td>
<td>0.010</td>
</tr>
<tr>
<td>D197A</td>
<td>None Detected</td>
<td>D300H</td>
<td>0.080</td>
</tr>
<tr>
<td>D197N</td>
<td>None Detected</td>
<td>D300N</td>
<td>0.020</td>
</tr>
<tr>
<td>E233A</td>
<td>0.280</td>
<td>D300Y</td>
<td>0.020</td>
</tr>
<tr>
<td>E233K</td>
<td>0.060</td>
<td>E233A/D300A</td>
<td>0.050</td>
</tr>
</tbody>
</table>

‡ = a unit is defined as the number of reducing ends (calculated as μmol of maltose) generated per minute

HPA-gly = heterogeneously glycosylated HPA expressed from Pichia pastoris
HPA-degly = endoglycosidase F treated HPA
N/A = not acquired

3.2.2.2 α-Glycosyl Fluorides

α-Maltosyl- and α-Maltotriosyl Fluorides

Upon successful purification of recombinant HPA from Pichia (pHPA), initial kinetic characterization necessarily focussed on ensuring that there were no gross differences between the recombinant wild-type and the native (pancreas-derived) enzymes. Kinetic parameters (Table 8) for pHPA (αG3F: $k_{cat} = 280 \text{ s}^{-1}$, $K_m = 0.3 \text{ mM}$) compared favourably with those of the pancreas-derived enzyme (αG3F: $k_{cat} = 250 \text{ s}^{-1}$, $K_m = 0.5 \text{ mM}$ (Rydberg, E.H. et al. 1999; Brayer, G.D. et al. 2000)) suggesting that the cloning and expression had been successful. In addition, the differential glycosylation of pHPA and the native enzyme did not seem to influence the kinetic parameters since they did not change significantly for pHPA upon deglycosylation with endoglycosidase...
F. This demonstrated that glycosylation on the C-domain, even with a large, 13-residue glycosyl group, did not influence the catalytic properties of the distant active site with either small or large substrates (Table 7 and Table 8).

Following the confirmation that pHPA had similar kinetic parameters to the native HPA, the ten mutant α-amylases were then kinetically characterized. Although no significant activity had been observed for enzymes in which any of the three active-site carboxylic acids (D197, E233, D300) were replaced when assayed with starch (reviewed in (Svensson, B. and Søgaard, M. 1993; Svensson, B. 1994), and Table 7), the kinetic parameters in Table 8 clearly demonstrate that differences in activity between the various mutants can be observed using α-glycosyl fluoride substrates. It is also obvious that most of the mutants show dramatic decreases in their specificity constants ($k_{cat}/K_m$) when compared with the wild-type enzyme. Although the $K_m$ values for the mutants only differ from wild-type by at most an order of magnitude, the $k_{cat}$ values vary over 6 orders of magnitude. Consequently, the specificity constants for the recombinant enzymes vary by seven orders of magnitude.
Table 8 Kinetic parameters for recombinant proteins with $\alpha$-maltotriosyl fluoride and $\alpha$-maltosyl fluoride. All assays were performed at pH 7.0

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>Fold decrease in $k_{cat}/K_m$</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>Fold decrease in $k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA</td>
<td>(283)</td>
<td>(0.51)</td>
<td>(555)</td>
<td></td>
<td>443</td>
<td>4.5</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>$p$HPA-gly</td>
<td>280</td>
<td>0.34</td>
<td>830</td>
<td>1.0</td>
<td>150</td>
<td>2.1</td>
<td>73</td>
<td>1.0</td>
</tr>
<tr>
<td>$p$HPA-degly</td>
<td>215</td>
<td>0.26</td>
<td>850</td>
<td>0.98</td>
<td>130</td>
<td>1.8</td>
<td>75</td>
<td>1.0</td>
</tr>
<tr>
<td>D197A</td>
<td>$1.0 \times 10^{-4}$</td>
<td>3.8</td>
<td>$2.6 \times 10^{-4}$</td>
<td>$3.1 \times 10^{7}$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>D197N</td>
<td>$7.3 \times 10^{-4}$</td>
<td>1.1</td>
<td>$6.7 \times 10^{-4}$</td>
<td>$8.1 \times 10^{7}$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>E233A</td>
<td>0.82</td>
<td>1.1</td>
<td>0.75</td>
<td>$1.1 \times 10^{3}$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>E233K</td>
<td>0.0036</td>
<td>0.45</td>
<td>0.008</td>
<td>$1.0 \times 10^{5}$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>E233Q</td>
<td>0.51</td>
<td>0.033</td>
<td>15</td>
<td>$8.3 \times 10^{2}$</td>
<td>0.10</td>
<td>0.10</td>
<td>0.99</td>
<td>74</td>
</tr>
<tr>
<td>D300A</td>
<td>0.010</td>
<td>0.47</td>
<td>0.021</td>
<td>$4.0 \times 10^{4}$</td>
<td>0.016</td>
<td>6.2</td>
<td>0.0026</td>
<td>$2.8 \times 10^{4}$</td>
</tr>
<tr>
<td>D300H</td>
<td>0.11</td>
<td>2.7</td>
<td>0.044</td>
<td>$1.9 \times 10^{4}$</td>
<td>0.10</td>
<td>6.8</td>
<td>0.014</td>
<td>$5.2 \times 10^{3}$</td>
</tr>
<tr>
<td>D300N</td>
<td>0.15</td>
<td>0.89</td>
<td>0.17</td>
<td>$4.9 \times 10^{3}$</td>
<td>0.18</td>
<td>4.8</td>
<td>0.038</td>
<td>$1.9 \times 10^{3}$</td>
</tr>
<tr>
<td>D300Y</td>
<td>0.0029</td>
<td>2.1</td>
<td>0.0014</td>
<td>$5.9 \times 10^{5}$</td>
<td>0.0020</td>
<td>7.8</td>
<td>0.00026</td>
<td>$2.8 \times 10^{5}$</td>
</tr>
<tr>
<td>E233A/D300A</td>
<td>0.0027</td>
<td>0.79</td>
<td>0.0034</td>
<td>$2.4 \times 10^{5}$</td>
<td>0.021</td>
<td>29.0</td>
<td>0.00074</td>
<td>$9.9 \times 10^{4}$</td>
</tr>
</tbody>
</table>

All kinetic parameters in this table were determined at pH 7.0
HPAgly = heterogenously glycosylated HPA expressed from Pichia pastoris
HPAdegly = endoglycosidase F treated HPA
N/A = Data not acquired
Graphical representation of data is shown in Appendix B, Figure B-1.
Average errors in kinetic parameters: $K_m$ (± 7-10%) and $k_{cat}$ (± 5-7%)
\(\alpha\)-Glucosyl Fluoride

Native human pancreatic \(\alpha\)-amylase has been shown to cleave \(\alpha\)-glucosyl fluoride via transglycosylation rather than hydrolysis (Braun, C. 1995; Brayer, G.D. et al. 2000). Evidence for this mechanism was gathered from kinetic and HPLC analyses. The recombinant wild-type HPA was shown to act in an identical fashion to the native enzyme with \(\alpha\)GF (Figure 21). These data were fit to a model that assumed \(\alpha\)GF was acting as both acceptor and donor, with different affinities, in a transglycosylation reaction where the product was also a substrate (Lougheed, B. et al. 1999). To test whether the mutant enzymes would also act on \(\alpha\)GF in a similar fashion the carboxylic acid amide mutants (D197N, E233Q, and D300N) were assayed with \(\alpha\)GF, as were D197A, and D300H. The only mutant with any detectable activity on \(\alpha\)GF was D300H, which appeared to act via a similar mechanism to that of the wild-type enzyme (Figure 22).

**Figure 21** Reaction of \(\alpha\)GF with native HPA (A – reprinted from (Brayer, G.D. et al. 2000)) and Recombinant HPA (B). Data (B) was fit with a model that suggests HPA acts on \(\alpha\)GF via a transglycosylation mechanism, with \(\alpha\)GF acting as both acceptor and donor with different affinities for each site.
Figure 22 Reaction of αGF with D300H. Data was fit with a model that suggests D300H acts on αGF via a transglycosylation mechanism, with αGF acting as both acceptor and donor with different affinities for each site.

3.2.2.3 Maltopentaose

Maltopentaose (G5) was chosen as a substrate for assays of pHPA and the carboxylic acid amide mutants for three reasons. First, maltopentaose is the smallest natural substrate that completely fills the five binding subsites of the active site. Unlike other small oligosaccharides (G3, G4 and G6), maltopentaose has been shown to bind in only one productive binding mode to produce G2 from the reducing end and G3 from the non-reducing end, thus analysis of its hydrolysis by HPA is quite simple (Brayer, G.D. et al. 2000). Second, because in the reaction catalyzed by HPA the aglycone of G5, namely maltose, is a poor leaving group, this substrate requires general acid/base catalysis – unlike αG2F and αG3F. Thus, mechanistic differences in the utilization of substrates with different requirements for general acid catalysis can be studied. Third, in the hydrolysis of both αG3F and G5, G3 is the glycone, therefore the deglycosylation step will be identical in the two reactions. Any difference in the kinetic parameters for reactions with αG3F compared to G5, with the same enzyme, must be due to changes in the glycosylation step. Comparison of the $k_{cat}/K_m$ values (the 2$^{nd}$ order rate constant) for
mutant enzymes acting on αG3F and G5 with the values for the reactions with pHPA can therefore be used to elucidate information regarding the general acid catalyst in the catalytic mechanism of HPA. A greater decrease in $k_{cat}/K_m$ value for the mutant of the general acid catalyst, with respect to pHPA, should be observed for G5 (where glycosylation requires general acid catalysis) than for αG3F (where glycosylation does not require the assistance of a general acid catalyst).

Table 9 Kinetic parameters for the reactions of pHPA, D197N, E233Q, and D300N with αG3F, and G5, and specific activities on soluble starch. Similar decreases in $k_{cat}/K_m$ values for αG3F compared with G5 were observed for both D197N and D300N with respect to pHPA. However, E233Q has a much greater $k_{cat}/K_m$ value for αG3F than for G5, suggesting that it acts as the general acid catalyst.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
<th>$U$ (mmol$^{-1}$ min$^{-1}$ mg$^{-1}$)</th>
<th>Fold Decrease in $k_{cat}/K_m$ or $U$ compared to wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αG3F</td>
<td>215</td>
<td>0.26</td>
<td>850</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>95</td>
<td>0.70</td>
<td>136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td></td>
<td></td>
<td>5.5 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>D197N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αG3F</td>
<td>7.3 x 10$^{-4}$</td>
<td>1.1</td>
<td>6.7 x 10$^{-4}$</td>
<td>1.3 x 10$^6$</td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>No Reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td></td>
<td></td>
<td>6.7 x 10$^{-4}$</td>
<td></td>
</tr>
<tr>
<td>E233Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αG3F</td>
<td>0.51</td>
<td>0.033</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>0.011</td>
<td>0.3</td>
<td>0.0030</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td></td>
<td>0.020</td>
<td>5.7 x 10$^1$</td>
<td></td>
</tr>
<tr>
<td>D300N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αG3F</td>
<td>0.15</td>
<td>0.89</td>
<td>0.17</td>
<td>5.0 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>0.083</td>
<td>2.3</td>
<td>0.037</td>
<td>1.8 x 10$^3$</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td></td>
<td>0.020</td>
<td>2.8 x 10$^5$</td>
<td></td>
</tr>
</tbody>
</table>

Graphical representation of data is shown in Appendix B, figure B-1.
Average errors in kinetic parameters: $K_m$ (+/- 7-10%) and $k_{cat}$ (+/- 5-7%)%

The results from Table 9 show that the three mutant enzymes (D197N, E233Q, D300N) clearly differ in their preference for natural as compared to activated substrates.
D197N did not measurably catalyze the hydrolysis of G5 or starch, and only very low hydrolysis rates were observed with αG3F. While the specific activity was similar for both E233Q and D300N on starch, the $k_{cat}/K_m$ values differed dramatically when assayed with either αG3F or G5. A similar reduction in specificity constant for either substrate was observed with D300N when compared to wild-type values. However, the decrease in specificity constant of E233Q for G5 is much greater, when compared to wild-type values, than is the corresponding value with αG3F.

### 3.2.3 Product Distribution Studies

Determination of the products of the reactions, particularly with the mutant enzymes, is necessary to fully understand the mechanism of HPA. It is important to determine whether kinetic assays are measuring hydrolysis or transglycosylation, and whether mutation of an active site residue has resulted in a change of this mechanism.

Product distributions were determined by HPLC for the reactions of wild-type HPA, E233Q, and D300N with both αG3F and G5. The substrates were assayed at concentrations of 5 mM since this was a standard assay concentration that gave maximal rates for all enzymes while still resulting in a strong response from the refractive index detector. Higher substrate concentrations were not necessarily representative of the assay conditions in previous studies, while lower concentrations were difficult to detect. The reactions were followed until the substrate was depleted or for 20 hours, whichever came first.
3.2.3.1 α-Maltotriosyl Fluoride

The reactions of the three recombinant enzymes with α-maltotriosyl fluoride resulted in similar initial products, which then varied dramatically as the reaction progressed. All enzymes gave evidence for hydrolysis as the initial means of utilizing αG3F (for the first 5 minutes) with small amounts of G4 and G6F forming, presumably by transglycosylation.

The reaction catalyzed by wild-type HPA was complete after the first hour and the principal products were G3 and G2 (Figure 23). In addition, the two anomers of G4 can be observed (retention times = 5.3 and 6.3 minutes) after 5 minutes of the reaction, as is a small peak (retention time = 13.1 minutes) corresponding to G6F. Maltotriose (retention time = 4.4 minutes) appears to be produced from the hydrolysis of αG3F and possibly from hydrolysis of G6F while, due to the absence of a peak corresponding to glucose (retention time = 3.4 minutes), all of the G2 (retention time = 3.9 minutes) must arise ultimately from hydrolysis of G6F by way of G4.

An interesting observation regarding the time-course of hydrolysis of αG3F by E233Q, which will be discussed further in § 3.2.6, is the apparent inhibition of E233Q early in the reaction. From the progress curves (section 3.2.6, Figure 32) it is clear that the E233Q catalyzed hydrolysis of αG3F proceeds very rapidly for the first 5 minutes after which the hydrolysis reaction appears to virtually cease. Since early studies demonstrated that E233Q was stable at 30°C for several days, and there still appears to be a significant amount of substrate remaining, this effect would appear to be due to the inhibition of the enzyme, perhaps by a transglycosylation product. The observation, by
both TLC and HPLC, of small amounts of a longer oligosaccharide produced in the reaction (Figure 24 and Figure 25) may support this.

![Graph](image)

**Figure 23** Product analysis of the reactions of wild-type HPA with αG3F. The reaction initially proceeds by hydrolysis with small amounts of transglycosylation products being observed after 5-10 minutes. The final products are G3 and G2 (the latter is presumably produced from hydrolysis of transglycosylation products).

The products of the reaction between D300N and αG3F (Figure 24B) are the most numerous and varied of any of the recombinant enzymes tested. The initial reaction appears to proceed almost equally via hydrolysis and transglycosylation, as products from both reactions (G3 from hydrolysis, and G6F from transglycosylation) are detected after 5 minutes. Although G3 could be produced from either the hydrolysis of αG3F or from the hydrolysis of G6F, the kinetic parameters listed in Table 9 suggest that the former proceeds at a much greater rate. Perhaps the most interesting feature of the reaction of D300N with αG3F is the wide variety of transglycosylation products generated and,
specifically, the large amount of G6F produced in the first hour. Apparently, the D300N mutation disrupts the deglycosylation step of the mechanism in such a way that oligosaccharides are favoured over water as acceptors.

**Figure 24** Product analysis of the reactions of E233Q (A), and D300N (B) with \(\alpha\)G3F. Although the reaction of E233Q proceeds predominantly by hydrolysis, note that it appears to be inhibited after about 5 minutes. This is possibly due to a small amount of transglycosylation product (retention time = 15 min.) that functions as a good inhibitor. In contrast, the reaction of D300N with \(\alpha\)G3F seems to proceed by both hydrolysis and transglycosylation, with transglycosylation becoming dominant after about 20 minutes.
Figure 25  TLC product analysis of the overnight reactions of αG3F with all the recombinant α-amylases.

In addition to the HPLC analyses described above, the products from an overnight reaction of each of the 11 recombinant enzymes with 20 mM αG3F were analyzed by TLC (Figure 25). Although this experiment does not provide any information regarding initial reaction conditions, these TLC results seem to corroborate the HPLC analyses quite well, especially regarding the formation of transglycosylation products. Large
oligosaccharide products were observed in the reactions of both E233Q and D300N and the G2 and G3 products observed in the overnight reaction of wild-type HPA with αG3F were also observed in the TLC overnight analyses. It is interesting to note that three of the alterations at D300 (D300A, D300N and D300H) resulted in enhanced transglycosylation.

3.2.3.2 Maltopentaose

Product analysis of the time-course reactions of wild-type HPA, E233Q, and D300N (Figure 26 and Figure 27) suggest that all three of these enzymes hydrolyze G5 in a similar manner. In the reactions with wild-type HPA and E233Q, the only products appear to be G2 and G3. For the native HPA, these products have previously been shown to arise from a single productive G5 binding mode resulting in G2 as the aglycone (Brayer, G.D. et al. 2000). The reaction of D300N with G5 also produces predominantly G2 and G3, however, G4 is also observed as a product (Figure 27). Since no glucose is apparently produced, it is difficult to hypothesize how the G4 was generated. One possibility might be that D300N hydrolyzes G5 in two productive binding modes wherein either G2 or G3 is the aglycone. If deglycosylation of the maltosyl-enzyme complex was slower with water than with an oligosaccharide acceptor, then either G4 (G2 donor, G2 acceptor) or the substrate G5 (G2 donor, G3 acceptor) would be produced. Due to the relatively small amount of G4 produced, it is likely that the most favourable productive binding mode is with G2 as the aglycone, as in the wild-type enzyme. If this were not the case, then either the proportion of G4 produced should be much greater, or the enzyme would appear inactive as it would continuously cycle product back into substrate.
Figure 26 Product analysis of the reactions of wild-type HPA (A) and E233Q (B) with maltopentaose. Both enzymes appear to produce the hydrolysis products G2 and G3.

Figure 27 Product analysis of the reaction of D300N with maltopentaose. Although G2 and G3 are the major products, small amounts of G4 are detected.
Scheme 2 Schematic representations of the "standard" hydrolysis of G5 (A) and the possible transglycosylation pathway utilized by D300N to generate G4 (B). To generate G4 from G5 without producing glucose, D300N could utilize the G2 product from (A) as the acceptor in the transglycosylation reaction in (B).

3.2.4 pH Studies

As with any active site mutations, especially those of catalytically crucial, charged residues, it is possible that the pH dependence of the kinetic parameters for the mutant enzymes will differ from those of the wild-type. In addition to altering the optimal assay conditions, a change in pH profile can give insights into the mechanistic details of catalysis. For example, the bell-shaped pH profile for glycosidases is thought to represent the ionization of the nucleophile (acidic limb), and acid/base catalyst (basic limb). These assignments have been confirmed in the case of Bcx, a *B. circulans* xylanase (McIntosh, L.P. *et al.* 1996), where $^{13}$C NMR of an isotopically labeled enzyme was used to directly show that the pK$_a$ of the acidic limb of the pH dependence of $k_{cat}/K_m$ corresponded to that of E78, the catalytic nucleophile. Perhaps more significantly, these authors also demonstrated that the pK$_a$ for the basic limb of the pH profile was due to the
ionization of E172, the general acid/base catalyst, and that the pK_a of this residue cycled between 6.7 and 4.2 during the glycosylation and deglycosylation steps respectively. Thus these authors conclusively demonstrated that a single carboxylic acid could act as both the general acid and general base catalysts.

Few detailed pH studies have been performed on α-amylases, however, Ishikawa et al. performed a notable series of studies on the mutants of several highly conserved active site histidine residues in porcine and human pancreatic α-amylases (Ishikawa, K. et al. 1992; Ishikawa, K. et al. 1993). These experiments were designed to probe the pH dependence of the “amylase” (hydrolysis of larger substrates that completely fill the active site) and “maltase” (hydrolysis of substrates that bind to only a few active site sub-sites) activities and the influence of the four active site histidines on those activities. The surprising result of these experiments was that the +2 substrate-binding sub-site influences the pH dependence of the α-amylase activity. When this sub-site is filled, a bell shaped pH dependence is observed, where the activity depends on the ionization of two active site residues. However, for smaller substrates where the +2 binding site is empty, the activity appears to depend on the ionization of three active site residues (the authors suggest these are, from acidic to basic pK_a values, D197, H101, and E233). These data seem to suggest that the residue responsible for the basic pK_a with the amylase activity loses “effectiveness” when no substrate is bound at the +2 sub-site, as with the maltase activity. Perhaps a hydrogen bonding network, induced by the substrate, is needed to properly orient the residue for full catalytic activity. Alternatively, there could be a change in mechanism with pH for the small substrates.
As the two examples above demonstrate, pH studies can be powerful tools for elucidating mechanistic details, although the results of such studies can also be difficult to interpret.

### 3.2.4.1 Stability of Recombinant Enzymes Towards Different pH Values

In order to properly interpret the results of any studies on the pH dependence of the kinetic parameters, the pH stability of the recombinant enzymes must first be determined. To this end, the enzymes were incubated for 1 hour at the desired pH to ensure sufficient time for equilibration. They were then assayed at either pH 7.0 for wild-type pHPA or pH 6.0 for the mutant enzymes. All recombinant enzymes tested demonstrated high stability to pH values between 4.0 to 10.0 with the exception of E233Q, which began to denature below pH 5.0 (Figure 28). Therefore, for most of the enzymes, the pH range 4.0 to 10.0 was an acceptable range over which to study the pH dependence of $k_{cat}$ and $k_{cat}/K_m$ values.

![Figure 28](image.png)

**Figure 28** pH stability curves for wild-type HPA (▽), E233A (○), E233Q (●), D300A (□), and D300N (△) assayed with αG3F. Enzymes were incubated for 1 hour at the desired pH before assaying at optimal pH. All recombinant enzymes are stable over the pH range of 4.0 – 10.0 with the exception of E233Q, which denatures below pH 5.0.
Since these and the following pH studies utilized αG3F as substrate, it was impractical to assay below pH 4.0 as the fluoride released would form HF (pK_a = 3.17) and would not be detected by the fluoride selective electrode. Likewise, at pH values too far in the basic range interference by hydroxide becomes significant (the fluoride selective electrode is 1000 fold more sensitive to OH^- than to F^-).

3.2.4.2 Dependence of k_cat and k_cat/K_m on pH

The pH dependence of the first order (k_cat) and second order (k_cat/K_m) rate constants for the enzymatic reactions were investigated for several recombinant enzymes. The k_cat/K_m value for cleavage of αG3F was determined by the substrate depletion method, where k_cat/K_m is the pseudo first-order rate constant for the reaction at substrate concentrations lower than 20% of the K_m value. The k_cat values were studied by assaying with substrate concentrations at which the enzyme catalyzed hydrolysis was at V_max. Full Michaelis-Menten curves were generated at the low and higher pH values to ensure that the K_m value did not change appreciably with pH.

Wild-type HPA demonstrated a pH dependence of its k_cat value for hydrolysis of αG3F which was similar to that reported in the literature (Ishikawa, K. et al. 1992) with pK_a1 = 6.5 and pK_a2 = 9.0 and a pH optimum from 7.0 to 8.5. However, the pH profiles for both D300N and, to a lesser extent, D300A showed evidence that their k_cat values were dependent on the ionization states of three residues (Figure 29) with pK_a values of pK_a1= 5.5, pK_a2 = 6.3, pK_a3 = 9.0. This was also observed in the pH dependence of the k_cat/K_m values for these mutants (Figure 30). Unfortunately, the very low substrate concentrations required in the assays for the pH dependence of k_cat/K_m values of E233Q.
combined with its poor pH stability below pH 6.0, meant that the pH profiles for this mutant could not be reliably interpreted.

![Graph](image1)

**Figure 29** pH Dependence of $k_{cat}$ for hydrolysis of $\alpha$G3F by wild-type HPA (▼), D300A (□), and D300N (◊) (A). Data for HPA-wt shown expanded (B). pH optima for all mutants are shifted 1.2 pH units more acidic than for pHPA. The acidic pK$_a$ values for the mutants were the same, shifted 1 pH unit lower than wild-type HPA, while pK$_a$ values for the basic limbs decreased by up to 2 pH units compared to the values of the wild-type enzyme.

![Graph](image2)

**Figure 30** pH Dependence of $k_{cat}/K_m$ for hydrolysis of $\alpha$G3F by wild-type HPA (▼), E233Q (●), D300A (□), and D300N (◊). pH optima for all mutants are shifted 0.5 to 1.2 pH units more acidic than for pHPA. The acidic pK$_a$ values for the mutants were the same, shifted 1 pH unit lower than wild-type HPA, while pK$_a$ values of the basic limbs decreased by up to 2 pH units from the wild-type enzyme.
3.2.5 Rescue Experiments

One direct method of gathering mechanistic evidence is by the use of rescue experiments. Retaining glycosidases that have had a catalytic carboxylic acid mutated to a small, non-ionic residue (such as alanine or serine) have been shown to experience enhanced activity in the presence of small nucleophiles such as azide, formate, and acetate (MacLeod, A.M. et al. 1994; Macleod, A.M. et al. 1996). Under these conditions it has been demonstrated that mutants of the nucleophile will react through an inverting mechanism while the mutants of the acid/base catalyst will still catalyze the reaction via a double displacement mechanism (Macleod, A.M. et al. 1996).

3.2.5.1 Kinetic Studies

The alanine mutants of all three active site carboxyl groups were assayed in the presence of sodium azide at concentrations from 100 mM to 5.0 M with αG3F as substrate. Enhanced activity was observed for all three mutants under these conditions (Figure 31), although to different degrees. The D197A mutant enzyme experienced a 10-fold increase in activity after addition of 5 M azide, while the rate increases for D300A and E233A were 8-fold and 2-fold respectively. Interestingly, both D300A and E233A were inhibited at azide concentrations greater than 1 M, while D197A did not appear to be inhibited even at 5 M azide.
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Figure 31  Azide rescue of mutant enzyme activities using αG3F as substrate. D197A (A) experiences a 10-fold increase in activity in the presence of >4 M NaN₃, while 1 M azide results in 2-fold and 8-fold increases in the activities of E233A (B) and D300A (C) respectively. E233A and D300A are inhibited by azide concentrations above 1 M.

3.2.5.2 Product Analyses

The products of azide rescue experiments were analyzed by ¹H-NMR for the presence of α- or β-glycosyl azides. No azide products were observed from the enzymatic reactions incubated for 2 days with 10 mM αG3F and 1 M (E233A and D300A) or 4 M (D197A) sodium azide.
3.2.6 Attempts at Labeling the Catalytic Nucleophile

Since the most convincing evidence for the identity of the enzymic catalytic nucleophile is acquired through trapping the covalent enzyme intermediate, several attempts were made to trap the glycosyl-enzyme intermediate of HPA through use of the mutant enzymes. Since the nucleophile of family 13 CGTase had been successfully labeled using the mutant of the acid/base catalyst, several similar strategies were attempted for HPA.

The enzyme mutated at the general acid/base catalyst is a good target for attempting to label the nucleophile since this enzyme has a naturally reduced rate of deglycosylation, while the use of glycosyl fluorides allows a relatively high glycosylation rate to be achieved. In addition, the amide mutant of the acid/base catalyst generally has much lower $K_m$ values on substrates with activated leaving groups than does the wild-type enzyme. This further suggests a prolonged lifetime of the covalent intermediate, since the $K_m$ value reflects the population of all the intermediates in the reaction pathway. As family 13 enzymes are known to possess significant transglycosylation activity that can result in rapid reactivation of the covalent glycosyl-enzyme intermediate, it is sometimes necessary to use substrates lacking an acceptor hydroxyl to detect the labeled enzyme. Therefore, several $\alpha$-glycosyl fluorides were utilized in attempts to label the nucleophile of E233Q, which has the lowest $K_m$ value of all recombinant enzymes on $\alpha$G3F (section 3.2.2).

Since “burst-like” behaviour was observed in the progress curves of the reaction with E233Q and $\alpha$G3F (Figure 32), which suggested the accumulation of an intermediate, attempts were made to observe a covalent maltotriosyl-E233Q species by
Several conditions of substrate and enzyme concentrations were used with several incubation times (5 minutes to 3 hours), however, no evidence for a covalent glycosyl-enzyme complex was observed.

It was possible that a covalent maltotriosyl-E233Q intermediate was formed in the reaction of E233Q with αG3F but that reactivation of the intermediate was occurring via a transglycosylation mechanism. Therefore similar studies to those just described were also undertaken using 4''-deoxy maltotriosyl fluoride as substrate. This substrate was also shown to produce a "burst-like" progress curve with E233Q, only with much less fluoride released (Figure 32). However, again no covalent adduct was observed by ESI-MS under several conditions.

![Figure 32](image)

**Figure 32** Progress curves for E233Q (270 nM) with 2 mM αG3F (solid line) and 2 mM 4DoG3F (dashed line). Both curves show evidence of enzyme inhibition or inactivation.
3.2.7 Inhibition of Recombinant Enzymes

3.2.7.1 Acarbose

The potent, natural α-glycosidase inhibitor acarbose has been widely studied for its role in controlling glucose absorption via inhibition of intestinal α-glycosidases. Acarbose is a pseudo tetrasaccharide composed of maltose bound to the reducing end of an acarviosine moiety (Figure 35A), itself made of valienamine and a 4,6-dideoxy glucose. It was isolated from *Actinoplacetes* sp. as the most potent α-glucosidase inhibitor of a series of pseudo oligosaccharides, although it was one of the least potent *in vitro* α-amylase inhibitors of this series (Müller, L. et al. 1980). Acarbose is now marketed by Bayer Inc. as a drug for control of diabetes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Acarbose $K_I$ (μM)</th>
<th>Mode of Inhibition</th>
<th>Protein</th>
<th>Acarbose $K_I$ (μM)</th>
<th>Mode of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA-degly</td>
<td>0.020</td>
<td>noncompetitive</td>
<td>D300A</td>
<td>170</td>
<td>competitive</td>
</tr>
<tr>
<td>D197A</td>
<td>N/A</td>
<td>N/A</td>
<td>D300H</td>
<td>540</td>
<td>(rf-$K_I$)</td>
</tr>
<tr>
<td>D197N</td>
<td>N/A</td>
<td>N/A</td>
<td>D300N</td>
<td>200</td>
<td>competitive</td>
</tr>
<tr>
<td>E233A</td>
<td>150</td>
<td>competitive</td>
<td>D300Y</td>
<td>1700</td>
<td>(rf-$K_I$)</td>
</tr>
<tr>
<td>E233K</td>
<td>N/A</td>
<td>N/A</td>
<td>E233A/ D300A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>E233Q</td>
<td>110</td>
<td>competitive</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HPAdegly = endoglycosidase F treated HPA
N/A = Data not acquired
rf-$K_I$ = "range-finder" $K_I$ study. Crude estimate of $K_I$ value derived from single data set (one αG3F concentration at various acarbose concentrations). The mode of inhibition can not be determined by this method.
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Kinetic studies of inhibition of \( p\text{HPA} \) and its mutants by acarbose were undertaken to determine the \( K_i \) values and the mode of inhibition for the recombinant enzymes. Initially, "range-finder" \( K_i \) values with respect to \( \alpha G3F \) were determined for each enzyme assuming a non-competitive mode of inhibition (since this mode has been previously reported for wild-type HPA). Following this, 2-line Dixon plots (1/v vs [acarbose]) were used to determine the mode of inhibition and acquire a more accurate determination of the \( K_i \) values. To ensure that the 2-line Dixon plots were valid, a full \( K_i \) determination (5 concentrations of \( \alpha G3F \) at each of 5 acarbose concentrations) was performed for the wild-type enzyme and D300N, a representative mutant (Figure 33). The \( K_i \) values for the 2-line plots were within 3-fold of the \( K_i \) values derived from the full \( K_i \) determination (\( p\text{HPA} \): 60 \( \mu \text{M} \) and 20 \( \mu \text{M} \) respectively; D300N: 0.16 mM and 0.37 mM respectively). Prior to each assay, acarbose was allowed to incubate with the enzyme for 15 minutes to allow for the possibility of time dependent inhibition.

Interestingly, acarbose appears to inhibit the mutant enzymes in a significantly different manner from that by which it inhibits wild-type HPA. Not only were the \( K_i \) values of the mutant enzymes much higher than that of the wild-type enzyme (e.g. 200 \( \mu \text{M} \) for D300N compared to 0.02 \( \mu \text{M} \) for \( p\text{HPA} \)), but acarbose was also found to inhibit the mutant \( \alpha \)-amylases by a different mode. All mutant enzymes studied showed a competitive inhibition mode against acarbose, while the wild-type HPA was inhibited in a non-competitive manner (Figure 33 and Table 10).
Figure 33 Representative plots for the inhibition of the recombinant enzymes by acarbose. 2-Line Dixon plots (shown here for pHPA (A) and D300N (B)) compared well with the full (4 × 5) Kᵢ determinations performed (pHPA (C – Lineweaver Burk plot) and D300N (D – Dixon plot)). The wild-type enzyme appeared to be inhibited in a non-competitive mode (shown as Lineweaver Burk plot for easier analysis of the inhibition mode), while the mutant enzymes were inhibited competitively.

3.2.7.2 Malto-oligosaccharides

As mentioned in sections 3.2.3 and 3.2.6, E233Q appears to lose activity early in its reaction with αG3F. The “burst-like” effect seen on the progress curve for E233Q with αG3F is too large to, in fact, be a burst, and therefore appears to represent inactivation of the enzyme. This is further confirmed by repeated additions of substrate
or enzyme to the assayed sample as only the addition of new enzyme would restore the activity, and then only temporarily (data not shown). Since the enzyme does not appear to suffer from rapid thermal denaturation (Figure 19) or buffer dependent effects, and no covalent modification of the enzyme has been detected, the inactivation appears to be the result of inhibition by a product of the reaction. To determine whether a large oligosaccharide such as that which might be produced in a transglycosylation reaction could result in inhibition, E233Q was assayed with G5, and G6 as inhibitors using αG3F as substrate. In addition, maltotriose was also tested for inhibition since it has been shown to be the product present in the highest concentration. As a control, D300N was also assayed for inhibition by G5. The D300N mutant has been shown to generate large amounts of transglycosylation products (section 3.2.3), but does not appear to be inhibited by these compounds.

Maltopentaose and maltohexaose were found to inhibit E233Q with $K_i$ values of 3 μM and 10 μM respectively, while maltotriose did not appear to inhibit the enzyme even at concentrations of 1 mM. In contrast, G5 was a much worse inhibitor of D300N with a $K_i$ value of 0.5 mM. The low $K_i$ values for G5 and G6 with E233Q suggest that small amounts of transglycosylation products from the reaction of this enzyme with αG3F could be responsible for the observed "inactivation". Larger oligosaccharides have been observed in the reactions of both E233Q and D300N by HPLC (Figure 24) and ESI-MS (data not shown).
3.3 Discussion

3.3.1 Examining the Assay Conditions

To ensure viable enzyme was available for the duration of each assay, the effects of several assay conditions, including phosphate, NaCl and citrate concentration, and the thermostability of the enzymes were studied. Although no significant variation in enzyme activity was observed for phosphate or NaCl concentrations ranging from 20 mM to 100 mM, significant inactivation of α-amylase was observed upon addition of sodium citrate (independent of citrate concentration above 30 mM). This inactivation could be alleviated by the addition of 0.5 mM calcium chloride (Figure 20).

Interestingly, Lecker and Khan (Lecker, D.N. and Khan, A. 1998) have recently studied the effect of citrate on *Bacillus* sp. α-amylase stability and have found that it stabilized the enzyme to thermal denaturation, although not as effectively as NaCl. Further, they demonstrated that the rate of thermal inactivation depended strongly on the cation present, with ammonium citrate exhibiting an up to 6-fold greater rate of inactivation than sodium citrate. This effect was similar for the chloride salts also. Finally, the authors observed saturation of binding of the salts to α-amylase at salt concentrations of 2 mM. Inactivation of α-amylases under such conditions has been shown to be reversible by the addition of calcium ions, if added quickly enough (Lecker, D.N. and Khan, A. 1996), and therefore loss of the enzyme-bound calcium appears to be central to the inactivation. Based on their results, Lecker and Khan proposed a three-stage model for the observed inactivation involving a reversible binding of the anion to the enzyme to stabilize the structure, followed by reversible inactivation upon loss of
calcium, and finally a slow, irreversible inactivation if calcium is not added back. Unfortunately, this model does not provide any role for the cations in the inactivation.

For the studies presented in this thesis additional calcium has not generally been required since no inactivation has been observed for assays performed in a phosphate buffer. In addition, calcium is known to interfere with the fluoride release assays through formation of CaF₂ complexes (Orion 1991) at concentrations as low as 2 mM. This prevents the detection of fluoride by the fluoride selective electrode, which is only sensitive to the soluble fluoride anion. However, the modest amount of calcium added for the pH studies here (0.5 mM) did not present any problems with detection of released fluoride.

The thermostability of all recombinant enzymes tested was sufficient for the duration of their assays. The mutant enzymes appeared to be stable at 30°C for up to 2 days, while the wild-type enzyme began losing activity after several hours under assay conditions. Previous reports have suggested that α-amylases are stable for up to a week at elevated temperatures. The reason for the relatively low thermostability of wild-type HPA in these experiments is probably more related to the low enzyme concentration used than to any inherent instability of the enzyme. The use of a standard protein stabilizing agent, such as BSA, was not desirable, however, since a commercial preparation of BSA has been shown to be contaminated with an α-glucosidase (data not shown). However, despite the lower than expected thermostability of the wild-type enzyme, all of the recombinant enzymes appeared to be sufficiently stable to be used in the desired assays.
3.3.2 Determining the Validity of the Low Enzyme Rates

One question that plagues any detailed analyses of low-activity enzyme mutants is whether the observed activity is due to the mutant itself or whether it results from contaminating wild-type enzyme. This is troublesome for any such mutants, and especially amide mutants of carboxyl groups, as these could be deamidated during expression, purification, or storage thereby restoring some wild-type enzyme (Zakowski, J.J. and Bruns, D.E. 1985).

The results of several experiments can be used to suggest that there is no contamination by wild-type HPA in the low-activity (specifically D197A, and D197N) enzyme preparations. No activity was observed with D197A and D197N with the αGF substrate, even when assayed at protein concentrations of 2 mg/mL (36 μM) and 1 mg/mL (18 μM) respectively. Only 0.5% contamination with wild-type HPA, in this assay, would be required to restore the pHPA activity to the levels observed in Figure 21. In addition, the D197 mutants (20 μM) were incubated for more than 20 hours with starch while similar assays with the wild-type enzyme (0.4 μM) required only three minutes to observe activity, only 10 fM pHPA would be required for detectable activity if assayed for 20 hours! Thus, contaminating wild-type enzyme, if present, must constitute less than 0.00000005 % of the total protein. However, the $K_m$ values of D197A and D197N with the αG3F substrate were 10-fold and 3-fold, respectively, greater than the $K_m$ value of pHPA. As the $K_m$ value for a substrate is a property intrinsic to a given enzyme, this further suggests a lack of contaminating wild-type HPA. Thus these data suggest that the low activity detected for enzymes mutated at D197 on αG3F (section 3.2.2.2) is due to hydrolysis by the mutants themselves.
3.3.2 Evidence for D197 Acting as the Catalytic Nucleophile

The catalytic nucleophile is arguably the single most important amino acid residue in the catalytic mechanism of HPA. Due to its unique nature in the enzyme, this nucleophilic residue should possess several specific properties that would be highlighted by the series of experiments described earlier in this chapter. For example, replacement of the enzymic nucleophile by a more chemically inert residue such as alanine or asparagine should virtually abolish the enzymatic activity (§3.2.2). Also, these enzymes should have a "hole" in their active sites that could be filled by small nucleophiles added to the assay mixture, thus providing a chemical rescue of enzyme activity and a product of analytically useful anomeric stereochemistry. For example, such a mutant should generate a stable β-glycosyl azide upon rescue of the reaction of αG3F with sodium azide (§3.2.5). Ideally, the most conclusive evidence for the identity of the enzymic nucleophile would come from trapping the covalent glycosyl-enzyme intermediate.

Mutants of the putative nucleophile D197 had the lowest $k_{cat}/K_m$ values of all the recombinant enzymes (Table 8), the second order rate constant being six orders of magnitude lower than that of wild-type enzyme. Since the $K_m$ values are increased by only 3 and 10 times (D197N and D197A respectively), and no significant structural changes to the enzyme have been observed (Chapter 2, Figure 17), the drastic reduction in specificity constant must result from severe impairment of the chemical steps ($k_{cat}$). This is precisely what would be expected for enzymes lacking the catalytic nucleophile. In contrast, similar mutations to E233 and D300 result in rate constants that are still several orders of magnitude greater than those of the D197 mutants. It may also be noted that the D197 mutants have a lower $k_{cat}$ than even the double alanine mutant.
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E233A/D300A. This further supports the assignment of D197 as the catalytic nucleophile, since D197 appears to be more important for the activity of the enzyme than the other two carboxylic acids combined.

The rate enhancement for D197A with sodium azide (Figure 31A) was the largest observed for any of the mutants tested (10-fold, compared with 2-fold for E233A and 8-fold for D300A). This effect is consistent with D197 being the nucleophile in a double displacement reaction. Unfortunately, no glycosyl azide product was observed by $^1$H-NMR although several different reaction conditions were attempted. It is possible that the accelerated reaction is still too slow to generate sufficient products to be observed by NMR, or that the enzyme may undergo slow, time-dependent inactivation by the high azide concentrations. Alternatively, a β-glycosyl azide product (which would be expected in the reaction with D197A) itself may act as a substrate since hydrolysis of this compound would only require the deglycosylation machinery of α-amylase, which is largely intact in D197A.

The enzymic nucleophile of HPA will, for now, remain identified only through indirect methods, as several attempts to label this residue have been unsuccessful. However, the observation of a covalent intermediate on PPA by low temperature $^{13}$C-NMR (Tao, B.Y. et al. 1989), and the sequence alignments with labeled enzymic nucleophiles from other family 13 enzymes (Henrissat, B. 1991; Braun, C. et al. 1996; McCarter, J.D. and Withers, S.G. 1996; Mosi, R. et al. 1997), together with the kinetic analyses presented here, build a strong case for D197 acting as the enzymic nucleophile of HPA.
3.3.3 Evidence for E233 Acting as the General Acid Catalyst

The general acid catalyst acts during the glycosylation step of the double displacement reaction to protonate the leaving group of natural substrates such as maltopentaose. In contrast, departure of the leaving group from “activated” substrates such as α-glycosyl fluorides does not require the assistance of a general acid catalyst. Thus, for enzymes in which the general acid catalyst has been mutated, a much larger decrease in $k_{cat}/K_m$ value (which reflects the glycosylation step) should be observed with maltopentaose as compared to the αG3F. In addition, the basic limb of the $pHPA$ pH profile is thought to reflect the ionization of the general acid catalyst. Hence, the basic limb of the pH profile should be drastically altered for an enzyme mutated at this residue. Finally, mutation of the general acid catalyst to alanine should generate a “hole” in the catalytic site that could be filled by the addition of a small nucleophile such as azide. The product of such a reaction should be the α-glycosyl azide.

From Figure 34 it is apparent that mutation of E233 results in the most disproportionate change in $k_{cat}/K_m$ values for hydrolysis of αG3F and G5 compared to the wild-type enzyme (57-fold decrease and $2.2 \times 10^4$-fold decrease, respectively - Table 9). This suggests that E233Q contains enough catalytic machinery to hydrolyze αG3F relatively efficiently, but is missing a crucial component for hydrolysis of G5, the general acid catalyst. Therefore, analysis of second order rate constants for hydrolysis of an “activated” and a natural substrate strongly favour the conclusion that E233 is the general acid catalyst.
The small rate enhancement (2.2-fold) for E233A with sodium azide (Figure 31B) when assayed with α-glycosyl fluoride substrates is far less than that generally observed with β-glycosidases (10^2-10^4-fold) (Wang, Q. et al. 1995; Macleod, A.M. et al. 1996), but is completely consistent with the rate enhancement observed for the CGTase acid/base catalyst mutant E257A (1.8-fold) with sodium azide (Mosi, R.M. 1998). Unfortunately, no glycosyl azide product was observed by ^1H-NMR although several different reaction conditions were attempted. It is possible that the accelerated reaction is still too slow to generate sufficient products to be observed by NMR.
3.3.4 Speculation as to the Role of D300

Since D197 and E233 appear to be the nucleophile and general acid catalyst respectively, the question remains as to the role of D300, the third, highly conserved active site carboxyl group. The kinetic parameters for D300A and D300N demonstrate that D300 is a crucial residue in the catalytic mechanism. As with the other mutants, most of the reduction in the $k_{cat}/K_m$ values ($10^5$ fold) is due to the reduction in $k_{cat}$ values, since the $K_m$ values are less than 3 fold higher than the values for the wild-type enzyme. The recent X-ray crystal structure of the family 13 enzyme CGTase (Uitdehaag, J. et al. 1999) has suggested that D300 aids in the deformation of the sugar bound to the $-1$ subsite from the ground state chair conformation towards the transition state half-chair conformation. In addition, D300 appears to play a role in enhancing the electrophilicity of the sugar anomeric carbon by donating electron density through its hydrogen-bonding interaction with the hydroxyl group at C2 (Uitdehaag, J. et al. 1999). The reduction in $k_{cat}$ with the D300N mutant has, therefore, been postulated to arise from altered hydrogen bonding in the $-1$ subsite resulting in the reduced ability of the amide to aid the nucleophilic attack during glycosylation. These functions however, would be difficult to glean from simple kinetic studies.

Another possible function of D300 could be as the general base catalyst. In all retaining glycosidases where the role of the acid and base catalysts have been elucidated, either through detailed kinetic analyses or NMR experiments, a single active-site residue has been found to perform both functions (for example: (MacLeod, A.M. et al. 1994; Macleod, A.M. et al. 1996; McIntosh, L.P. et al. 1996)). However, to date no such analyses have been performed on retaining $\alpha$-glycosidases, largely due to the technical
challenges inherent in such a study (difficulties in substrate synthesis hamper kinetic studies, while the large size of most of the enzymes prevents NMR investigations). In the early 1990’s suggestions of α-amylases possessing separate general acid and general base catalytic residues were presented (Ishikawa, K. et al. 1990), although without any serious studies into the feasibility of this suggestion. In addition, the presence of a water molecule bound between E233 and D300 (Chapter 1: Figure 8) has led to speculation that each of these residues may play a role in acid/base catalysis (Brayer, G.D. et al. 2000). Possible support for D300 playing a role in general base catalysis may come from the product distribution studies described in Section 3.2.3. Both D300N and D300A (although only D300N data was shown) generate significant amounts of transglycosylation products in the reaction with αG3F and, even with G5 a difference in products is observed as compared to the wild-type enzyme. This suggests that either 1) the ability of the water molecule to act as an acceptor in the deglycosylation step is hampered – due to either the inability of the enzyme to adequately bind the nucleophilic water, or from alteration of the general base catalyst, 2) these mutants selectively favour an oligosaccharide acceptor through an increase in favourable binding interactions, or 3) a general crippling of the reaction has occurred (eg. through alteration of crucial hydrogen bonds) and only the carbohydrate acceptors, which are inherently better acceptors than water, react at an appreciable rate. Given that the apparent binding affinity of G5, which should bind with residues in the +1 and +2 sites, does not appear to differ significantly between wild-type enzyme ($K_m = 0.7$ mM) and D300N ($K_m = 2.3$ mM), it seems unlikely that the mutants possess increased favourable interactions for oligosaccharide acceptors. Although the second possibility seems unlikely, there is little
evidence to favour either the first or third at this time. Therefore, although it may be tempting to suggest that the D300 is the general base catalyst, the increased partitioning between oligosaccharide and water acceptors could result from any or all of the possibilities described in 1) and 3).

3.3.5 General Observations and Trends

pH Profiles for the Recombinant Enzymes

The unusual pH profiles observed for the enzymes mutated at D300 (Figure 29 and Figure 30) have been witnessed previously with small substrates for both porcine and human pancreatic α-amylases during a study of the pH dependence of $k_{\text{cat}}$ for active site histidine mutants (Ishikawa, K. et al. 1992; Ishikawa, K. et al. 1993). The authors suggested a reaction scheme in which the protonation states of three active site residues are responsible for the pH profiles observed. One residue, presumably the nucleophile, must be deprotonated while the other two residues must be protonated for full activity of the enzyme. However, the authors found that binding of a six-membered ring (a sugar or a phenyl ring) at the +2 subsite influenced the pH profile. When the 5 subsites were fully occupied the enzyme activities were only dependent on the ionization states of 2 residues. However, when only the −3 to +1 subsites were occupied the activities were dependent on the ionization of three residues. Furthermore, Ishikawa et al. noted that the H201N mutant demonstrated a dependence on three ionization states regardless of the substrate, while H101N and H299N had bell shaped pH profiles for both amylase and maltase activities. Ishikawa et al. made several suggestions to explain these results. First, they proposed that the 3 pK$_a$ values observed are from two catalytic carboxylic acids (D197 - pK$_{a1}$ and D300 - pK$_{a2}$) and a histidine (H101 - pK$_{a3}$). In addition, they suggested that
Chapter 3  Kinetic and Structural Analyses

H101 is a general acid catalyst and D300 may be a general acid/base catalyst. Further, they proposed that the interaction of H201 with a sugar in the +2 subsite might subtly alter the orientation of D197 (since these two residues are on the same loop) in order to "activate" H101. Finally, a mathematical model was proposed that suggested that if the singly protonated form became inactive, the enzyme should then possess a classic pH dependence involving only 2 ionization values. This last statement could, of course, also be interpreted to mean that mutation of the residue responsible for $pK_a$ should reduce the pH profile to a dependence on only 2 ionization states.

The pH profiles for $k_{cat}$ and $k_{cat}/K_m$ values have been determined for wild-type HPA, E233Q, D300A, and D300N (Figure 29 and Figure 30). The same three-$pK_a$ profiles witnessed by Ishikawa et al. appear to be observed in the dependence of $k_{cat}$ for D300A and D300N ($pK_{a1} = 5.5$, $pK_{a2} = 6.3$, $pK_{a3} = 9.0$), but not for wild-type HPA ($pK_{a1} = 6.5$, $pK_{a2} = 9.0$). In addition, the pH profiles for $k_{cat}/K_m$ values show a similar dependence on three $pK_a$ values in D300A, D300N and wild-type enzyme ($pK_{a1} = 6.5$, $pK_{a2} = 7.5$, $pK_{a3} = 9.0$). Unfortunately, the very low substrate concentrations required in the assays for the pH dependence of $k_{cat}/K_m$ values of E233Q, combined with its poor pH stability below pH 6.0, meant that the pH profiles for this mutant could not be reliably interpreted.

Substrate Binding with the E233 Mutants

It is interesting to note that the $K_m$ values for the E233 mutants are quite different. For cleavage of $\alpha$G3F by E233A and E233Q, $K_m$ values 3 fold higher and 10 fold lower than that of the wild-type enzyme, respectively, are observed, while the $K_m$ value for E233K is essentially unchanged from that of wild type HPA. A low $K_m$ value is
commonly observed in glycosidases mutated at the acid/base catalyst when assayed with glycosyl fluoride substrates, and is often due to trapping of the covalent intermediate. While this is commonly the case, it does not explain why the alanine mutant would have a higher \( K_m \) value than the glutamine mutant. Considerably different \( K_m \) values between the alanine and glutamine mutants at this position have also been documented for CGTase, another family 13 enzyme (Mosi, R.M. 1998). In addition, \( K_m \) values for the acid/base catalyst glutamine mutants of \( \textit{B. subtilis} \) \( \alpha \)-amylase with maltopentaose have been determined to be 100 fold lower than for the wild-type enzyme (Takase, K. \textit{et al.} 1992). Since maltopentaose does not contain an activated leaving group, accumulation of the covalent intermediate would not be expected. In a recent crystal structure of the \( \textit{B. subtilis} \) \( \alpha \)-amylase E208Q mutant (equivalent to HPA E233Q) with maltopentaose bound in the active site, the authors suggested that a hydrogen bond between the Ne2 of Q208 and the glycosidic oxygen of the sugar ring in the -1 subsite may be responsible for the greater affinity of these mutants for G5 (Fujimoto, Z. \textit{et al.} 1998). Since one hydrogen bond from E233Q can result in a reduction of \( K_m \) value of 10-100 fold, it is not unlikely that the removal of all hydrogen bonds from the side-chain, such as with E233A, may result in a drastically decreased affinity of the enzyme for the substrate and therefore an increase in \( K_m \) value. Thus, it is interesting that two conservative mutations (E233A and E233Q) apparently result in significant, and opposite, changes in \( K_m \) values, while a non-conservative mutation (E233K) results in virtually no change in the \( K_m \) value.
Hydrolysis of α-Glucosyl Fluoride

Recombinant and native α-amylases have been observed to cleave α-glucosyl fluoride predominantly by a transglycosylation mechanism (Figure 21). Only one of the mutant enzymes, D300H, cleaved αGF at a significant rate, and it also appeared to utilize transglycosylation as the predominant mechanism (Figure 22). Why only D300H, of all the mutants tested, should cleave αGF is unknown. Although only 0.015% contamination by pHPA would be required to generate the observed D300H activity, it is unlikely that this activity is a result of contamination from wild-type enzyme as the $K_m$ value for D300H assayed with αG3F (Table 8) was 10 fold higher than that of the wild-type enzyme. Since the $K_m$ value is a characteristic of the enzyme that is independent of enzyme concentration, the higher $K_m$ value indicates that the activity measured is not due to wild-type contamination.

The fact that α-amylases cleave αGF predominantly through a transglycosylation mechanism is not unexpected given the very high $K_m$ values estimated for αGF and glucose (30-100 mM (Al Kazaz, M. et al. 1998)). It seems likely that, for significant activity, the enzymes require the additional transition-state stabilization energy realized upon filling multiple binding subsites. Thus the combination of high substrate concentrations (required due to very poor $K_m$ values) and the filling of multiple binding sites would act to favour the transglycosylation reaction.

3.3.6 Inhibition of Recombinant α-Amylases by Acarbose

The natural product acarbose, which is used clinically to control type I and type II diabetes mellitus, is presently the most widely studied α-amylase inhibitor. This pseudo-
tetrasaccharide is an interesting inhibitor, both structurally and kinetically, for several reasons. The first 3-dimensional structure of acarbose bound to an α-amylase was that of its complex with porcine pancreatic α-amylase isozyme I (PPA I) (Qian, M. et al. 1994). Interestingly, instead of observing the pseudo-tetrasaccharide bound to the active site of PPA, a pseudo-pentasaccharide was observed. Since the first observation of this unusual complex, similar structures have been observed for α-amylases from many species, including human pancreatic α-amylase (Brayer, G.D. et al. 2000; Nahoum, V. et al. 2000). In addition, similar observations have been made (Figure 35) for other members of amylase families (for example, family 13: CGTase (Strokopytov, B. et al. 1996)). In all but three of the structures determined (a high-resolution structure of TAKA α-amylase (Bompard-Gilles, C. et al. 1996), a high resolution structure of the PPA isozyme II at low temperature (Gilles, C. et al. 1996), and a low resolution structure of human pancreatic α-amylase (Nahoum, V. et al. 2000)) a complex rearrangement of the acarbose structure appears to occur, resulting in the acarviosine moiety being bound across the −1 and +1 subsites with enough glucose residues bound to its reducing and non-reducing ends to completely fill all subsites in the active site. In the three exceptions, the α-amylase appears to perform a transglycosylation reaction with two acarbose molecules to form the observed pseudo-oligosaccharide. One question that arises from the observed acarbose complexes is whether this is an adaptive inhibitor that is rearranged by its target α-glucosidase into the optimal form?
Figure 35 Acarbose (A) and stereo views of several of the pseudo oligosaccharides that have been observed bound to the active sites of enzymes from the α-amylase family (human pancreatic (B) (Brayer, G.D. et al. 2000), porcine pancreatic, isozyme I (C) (Qian, M. et al. 1994), TAKA (similar to PPA isozyme II) (D) (Brzozowski, A.M. and Davies, G.J. 1997), CGTase (E) (Strokopytov, B. et al. 1995)). The number of active site binding subsites for each enzyme is also shown.
In terms of wt-HPA, the pseudo-pentasaccharide ligand formed could result from an enzyme catalyzed rearrangement of acarbose involving hydrolysis of the reducing end glucose residues (either glucose or maltose) followed by transglycosylation of these sugars onto the non-reducing end of the inhibitor (Scheme 3A). However, this rearrangement mechanism is energetically unfavourable for two reasons; the binding affinity of HPA for glucose is very poor ($K_m > 20$ mM), and the leaving group for the nucleophilic attack on glucose would be water, a very poor leaving group. Another possibility, although not very likely, is that the donor in the transglycosylation is an oligosaccharide contaminant carried through from the purification. Wild-type $\alpha$-amylase has been reported to stay bound to dextrins throughout standard purification methods, and such contamination has been problematic with other polysaccharide-binding enzymes such a glycogen phosphorylase (Withers, S.G. 2000). The transglycosylation shown in Scheme 3B would generate an elongated pseudo-oligosaccharide product. However, neither wt-HPA nor the majority of the other observed inhibitor complexes show this result. Another possibility is presented in Scheme 3C. Here enzymatic modification of the acarbose molecule is not required as two possible binding modes for acarbose in the $\alpha$-amylase active site are proposed. However, once again, this proposal does not match any of the complex structures determined. Therefore, at present, there would appear to be no satisfactory explanation for the observed products of the reactions of $\alpha$-amylases with acarbose.
Scheme 3 Three potential acarbose rearrangements or modified binding schemes that could result in elongated pseudo-oligosaccharides binding in the active sites of α-amylases. In scheme A the reducing end sugars are hydrolyzed from acarbose and added on to the non-reducing end. Scheme B is a transglycosylation reaction between two acarbose molecules. In Scheme C it is proposed that no enzymatic cleavages would occur and additionally, there are two inhibitor binding modes. \( \bigtriangledown \bigtriangledown \bigtriangledown \) = substrate binding sites, \( \bigtriangledown \) = covalent intermediate.

In addition to the structural studies, a number of kinetic studies have been performed with acarbose and family 13 enzymes, specifically CGTase and α-amylase. Acarbose inhibits CGTase in a competitive fashion with a \( K_i \) value of 0.2 µM. In
addition, acarbose has also been shown to inhibit several active site mutants of CGTase with a wide range of $K_i$ values (0.36 $\mu$M – 0.34 mM). Since it is thought that the valienamine ring mimics the transition state conformation of the sugar ring, acarbose has been suggested to act as a transition state analog. This has been supported by recent experiments with CGTase, which show a strong linear correlation between $\log(K_m/k_{cat})$ for cleavage of $\alpha$GluF and $\log(K_i)$ for acarbose inhibition with a series of mutants ($\rho = 0.98$), although evidence of ground state mimicry was also observed ($\rho = 0.9$ for $\log(K_m)$ vs $\log(K_i)$) (Mosi, R. et al. 1998).

In contrast to what is seen with CGTase, inhibition of $\alpha$-amylase by acarbose has been shown to occur by a non-competitive mode (for bovine pancreatic (Wilcox, E.R. and Whitaker, J.R. 1984), barley (Sogaard, M. et al. 1993), porcine pancreatic (Al Kazaz, M. et al. 1998) and human pancreatic $\alpha$-amylases (this thesis)). Secondary acarbose binding sites have been observed by differential UV spectroscopy (for bovine pancreatic, barley and porcine pancreatic $\alpha$-amylases) and X-ray crystallography (for barley and porcine pancreatic $\alpha$-amylases) (Sogaard, M. et al. 1993; Alkazaz, M. et al. 1996), and the binding affinities of these sites are between 2 mM and 13 mM. Since the $K_i$ values for acarbose inhibition of $\alpha$-amylases are generally between 0.05 – 1.0 $\mu$M, depending on the source of the $\alpha$-amylase, it is unlikely that the secondary sites are related to the inhibitory activity of acarbose.

The kinetic analyses of the inhibition of the recombinant enzymes by acarbose (section 2.8) generated unexpected results. Although the data for the wild-type enzyme are similar to those of previously reported studies (noncompetitive inhibition mode, $K_i = 20$ $\mu$M), in all cases inhibition of the mutant enzymes is by a competitive mode with $K_i$
values from 0.2-1.7 mM. Although the decrease in binding affinity of the mutant enzymes for acarbose was not unexpected, the altered mode of inhibition was. The noncompetitive inhibition mode for the wild-type enzyme implies the presence of an inhibitor binding site separate from the active site. Therefore, if active site binding of acarbose was reduced, or eliminated, in a mutant enzyme, an uncompetitive mode of inhibition would be expected. It is therefore difficult to envisage how mutations at the catalytic carboxyl groups could alter the inhibitor binding affinity at a distant site to result in the competitive inhibition mode observed.

As a test of transition state analogy for acarbose, a logarithmic plot of the $K_i$ value for acarbose with each of a series of mutant enzymes against $K_m/k_{cat}$ values for hydrolysis of $\alpha$-G3F by each mutant was plotted (Figure 36). While there seems to be a good correlation in the data from the mutant enzymes, the wild-type data is clearly an outlier. This is not surprising given the difference in inhibition mode between wild-type HPA and the mutant enzymes. This data would also be consistent with the presence of a different (modified) inhibitor binding to the wild-type enzyme. A linear fit of the data from the mutants results in a slope of $\rho = 0.27$, indicating partial transition state analogy.

The X-ray structural data acquired from crystals of the mutant enzymes soaked with acarbose (Figure 37B) do little to clarify the kinetic data. It was expected that the mutant enzymes may bind differently or to a different form of the inhibitor than does the wild-type enzyme. Of particular interest is the structure of acarbose bound to enzymes mutated at D197. These enzymes are essentially inactive and no rearrangement of acarbose structure should occur.
Figure 36 Transition state analogy plot for acarbose with the recombinant enzymes. Log $K_i$ for acarbose inhibition is plotted against log $K_m/k_{cat}$ for $\alpha$G3F hydrolysis by each of a series of mutants. A linear fit was performed to the data for the mutant enzymes. The value for wild-type HPA (●) was not included in the fit.

The structures of acarbose complexes with the D197A and D300N enzymes of HPA were determined by the laboratory of Dr. G.D. Brayer in an attempt to elucidate a structural basis for the kinetic results (Brayer, G.D. et al. 2000). The two complexes obtained were similar and showed what appeared to be a maltose bound to the -3 and -2 subsites of the mutant enzymes (Figure 37B). Thus it appears that the binding of the valienamine moiety is disrupted in the mutant enzymes, as is any potential transglycosylation of acarbose. The bound ligand in the structures of the mutant enzymes could represent either acarbose, where reducing end maltosyl moiety is bound in the -3 and -2 subsites and the acarviosine is disordered in solution, or a degradation production of acarbose.
Figure 37 Acarbose bound to the active sites of wild-type HPA (A) and the D300N mutant enzyme (B). Although the wild-type enzyme has a pseudopentasaccharide bound after soaking with acarbose, the D197A and D300N mutants show only a maltosyl moiety bound. Clockwise from the top, the active site residues shown are D197, D300 (N300 in (B)) and E233. The active site residues have been highlighted with transparent van der Waals surfaces coloured to indicate electrostatic potential. Structural coordinates from (Brayer, G.D. et al. 2000).

Unfortunately, the kinetic and structural data for the mutant enzymes with acarbose do little to resolve issues surrounding inhibitor rearrangement or alternate binding modes in complexes with the wild-type enzyme. However, regardless of the details of the rearrangement, it seems likely that, while the wild-type enzyme is inhibited in some manner by the interaction of multiple acarbose molecules, the enzymes mutated at the catalytic carboxyl groups appear to be inhibited by the binding of only one. This difference might be rationalized if the non-competitive mode of inhibition observed for the wild-type α-amylases is an artifact resulting from the rearrangements such as those shown in Scheme 3, rather than resulting from the existence of inhibitory sites apart from the active site. Perhaps the binding of acarbose to the $-1$ to $+2$ subsites results in the non-competitive inhibition mode in a similar fashion to the inhibition of α-amylase by maltose, which is non-competitive with maltopentaose as a substrate, where the maltosyl...
moiety is bound to the +1 and +2 subsites, but competitive with an oNP-maltoside substrate, where it is bound in the –2 and –1 subsites (Prodanov, E. et al. 1984; Al Kazaz, M. et al. 1998). In any event, although this thesis has provided significant data on the inhibition of human pancreatic \( \alpha \)-amylases by acarbose, the mechanism by which the observed pseudo-pentasaccharide inhibitor is generated, remains elusive.
3.4 Conclusions

The *Pichia pastoris* expression system was successfully utilized to generate a series of active site mutants of residues D197, E233, and D300. A novel purification strategy was developed to overcome some of the unique difficulties of isolating mutant α-amylases from the yeast supernatant. Heterogeneous glycosylation at N461, which was problematic for crystallization and ESI-MS studies, was overcome by treatment with endoglycosidase F-CBD fusion protein that could be conveniently removed from the purified α-amylase by addition of cellulose. This allowed determination of the structures of the recombinant enzymes, which showed no evidence of improper folding, and also allowed kinetic analyses to be undertaken.

Kinetic analyses involved the determination of Michaelis-Menten parameters for the recombinant enzymes using α-maltosyl fluoride and α-maltotriosyl fluoride, substrates that do not require general acid catalysis, and maltopentaose, which does require general acid catalysis, as well as the determination of specific activities on soluble potato starch. In addition, product distributions and pH dependences were also studied. Finally, kinetic analyses, aided by structural determinations, were performed for the inhibition of the recombinant enzymes by acarbose.

Mutants modified at D197 are completely inactive on the natural substrates (starch and G5) and the $k_{cat}$ values of these mutants were $10^6$-fold lower than those of wild-type HPA on the α-glycosyl fluorides. This dramatic reduction in enzymatic activity, combined with sequence alignment data and labeling studies with other family 13 enzymes, suggests that D197 is the catalytic nucleophile.
The relative difference in $k_{\text{cat}}/K_m$ values for $\alpha$G3F and G5, substrates with different requirements for general acid catalysis, for E233Q when compared to wild-type HPA or D300N provide strong evidence that E233 is the general acid catalyst. The E233Q mutant had a moderately reduced specificity constant with the $\alpha$-glycosyl fluoride (12 fold), however, for G5 the $k_{\text{cat}}/K_m$ value was reduced by $2 \times 10^4$ fold. In contrast, similar reductions in $k_{\text{cat}}/K_m$ values were observed for D300N when assayed with both substrates ($10^3$ fold).

Although D300 has been suggested, on the basis of X-ray crystal structures, to play an important role in the steric and electronic distortion of the ground-state conformation of the sugar bound in the $-1$ subsite, product distribution studies for D300A and D300N with $\alpha$G3F and G5 suggest that D300, may also play a role in general base catalysis or perhaps, in binding the nucleophilic water for enzymic deglycosylation. These mutants showed a significant propensity for transglycosylation by generating numerous oligosaccharides from $\alpha$G3F. This may suggest that mutation of D300 has impaired the interaction of the enzyme with the nucleophilic water in the deglycosylation step, thus favouring a sugar acceptor instead of water. Alternatively, the double displacement reaction may be generally impaired by the mutation of D300 and the reaction can only proceed using oligosaccharide acceptors, which are inherently better acceptors than water.

An understanding of the mechanism of inhibition of $\alpha$-amylase by acarbose continues to be elusive. The kinetic and structural analyses of mutant $\alpha$-amylases only add to the confusion by demonstrating that these enzymes appear to interact differently.
with acarbose than does the wild-type enzyme. Clearly, further studies are required if the mechanism of this inhibition is to be understood.

In conclusion, the studies presented in this thesis represent a detailed mechanistic analysis of three active site carboxyl groups in human pancreatic α-amylase aided by the use of site-directed mutagenesis. A variety of kinetic and structural techniques, applied to several mutant enzymes, were utilized to build evidence for the roles of the carboxyl groups in the catalytic mechanism of HPA. The evidence supports the hypotheses that D197 plays the role of the catalytic nucleophile while E233 is the general acid catalyst. Clearly, D300 plays a significant role in catalysis by HPA, however, the precise nature of that role is still unclear. These studies, in conjunction with the analysis of acarbose inhibition, have provided substantial insight into the mechanism of α-amylase and will hopefully aid future experiments directed at the development of medically important α-amylase inhibitors.
CHAPTER 4

Experimental Methods
4.1 Generation and Isolation of Recombinant Proteins

4.1.1 General Procedures

All buffer chemicals and other reagents were obtained from Sigma Chemical Company unless otherwise noted. All chromatographic resins were purchased from Pharmacia. Endoglycosidase F was obtained from Boehringer Mannheim and an endoglycosidase F-cellulose binding domain fusion protein was a generous gift from Dr. R.A.J. Warren (Department of Microbiology, University of British Columbia). All oligodeoxyribonucleotides used in this study were acquired from the UBC NAPS (Nucleic Acid and Protein Services) unit; the oligonucleotide sequences are given in Table 11. All commercially available vectors and *Pichia pastoris* strains were acquired from Invitrogen Corp (Carlsbad, CA).

The cloning of Human pancreatic α-amylase from pancreatic mRNA into BHK cells and then into *Pichia pastoris* was performed by Drs. H. Vo and H. Côté in the laboratory of Dr. R.T.A. MacGillivray and has been described previously (Rydberg, E.H. *et al.* 1999).

4.1.2 Generation of pHICL-AMY

Digestion of the vector pHIL-S1 with SacI/SalI gave a fragment of 2949 bp and the desired 5560 bp fragment which contained an F1 origin. The 5560 bp fragment was gel purified in preparation for ligation with the fragment from pPIC9-AMY containing the amylase gene. The vector pPIC9-AMY was digested with SacI, SalI, ScaI, SphI, and DraIII in 10% NEB Buffer 3 (New England Biologicals Inc.) at 37°C overnight. The resulting 5040 bp fragment was easily gel purified from the smaller DNA fragments of
the other half of the vector, using a DNA gel-purification kit (Promega Inc.). Ligation of two of the gel purified fragments (using the Rapid-Ligation kit from Promega Inc.) yielded the desired unique vector pHICL-AMY which contained both the F1 origin and the α-factor secretion sequence of the parent vectors.

4.1.3 Mutagenesis

Generation of the D197A and D197N mutants was accomplished by Dr. H. Côté, as described in (Rydberg, E.H. et al. 1999). Briefly, this involved utilizing a standard PCR mutagenesis method (Sambrook, J. et al. 1989) with the HPA gene in a Bluescript™ vector. The mutated genes were then subcloned into the shuttle vector pPIC9, which was used to transform Pichia pastoris strain GS-115 spheroplasts (Invitrogen, 1995).

Mutagenesis was performed utilizing Kunkel’s method (Kunkel, T.A. et al. 1987). To perform site directed mutagenesis in E. coli by this method the unique shuttle vector pHICL-AMY was utilized. Briefly, single-stranded dU-DNA was generated by the transformation of E.coli strain RZ1032 (dUT −, UNG −) with the pHICL-AMY vector, followed by infection with M13 filamentous phage (strain R408). Phage were precipitated from the bacterial supernatant, after centrifugation at 10000 × g, with 3.5% (w/v) polyethylene glycol 8000, 0.4 M NaCl. After denaturation of the capsids by phenol/chloroform-isoamyl alcohol extraction, the phagemid DNA was ethanol precipitated. Mutagenesis involved annealing the desired mutagenic primer (0.5 pmol) (Table 11) to the purified single-stranded dU-DNA in annealing buffer (10 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 8.0) at 55 °C for 5 minutes followed by slow cooling to 22 °C. The extension reaction was performed by adjusting the buffer to 7.5 mM MgCl₂, 3 mM DTT, 38 mM Tris-HCl, pH 7.5 and incubating with Klenow
fragment (1 U), T₄ DNA ligase (1 U), dNTPs (0.5 mM), and rATP (0.5 mM) for 2 hours at 22 °C. Selection for the mutant strand was accomplished by the digestion of the dU containing, wild-type DNA after transformation into a dUT⁺, UNG⁺ E. coli strain (DH5α). Screening for successful mutations involved manual sequencing of the region of interest in the plasmids isolated from randomly selected colonies transformed with the cDNA from the mutagenesis reaction mixture (Sanger, F. et al. 1977). A 60-80% mutation rate was commonly achieved. Mutant cDNAs were fully sequenced using the UBC NAPS unit automated sequencing service to confirm the fidelity of the reaction. Electroporation was then used to transform the Pichia pastoris strain GS-115 with the mutant cDNA (linearized with Sac1) following the Invitrogen protocol (Invitrogen, 1995).

**Table 11** DNA Sequences of the Mutagenic Primers Used to Generate Mutants at the Positions E233 and D300

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</table>
4.1.4 Screening for Successful Transformants

Transformed *P. pastoris* were screened following a standard protocol (Invitrogen 1995), but with the addition of the "Yeastern" method (Wung, J.L. and Gascoigne, N.R.J. 1996). Briefly, screening involved patching the viable colonies from the transformation, grown on MD plates (minimal media containing dextrose), onto MM plates (minimal media containing MeOH) and, in the same pattern, onto fresh MD plates. After growing for 2 days at 30 °C the plates were replica plated, and the original MM plates were subjected to a yeastern as follows. These plates were covered with a wetted (soaked in MeOH for 5 minutes and water for 5 minutes) PVDF blotting membrane and several layers of paper towel (as in a non-electrotransfered Western blot). These plates were then incubated at 30 °C for 3 hours after which the membranes were developed by standard Western blotting methodology, with extra care taken during the washes to ensure the removal of any cells adhering to the membrane. Small BMGY cultures (10 mL) were then inoculated with the 10 colonies producing the darkest spots on the yeastern. These cultures were grown overnight at 30 °C before centrifuging (2000 rpm for 10 minutes). After decanting the supernatant, the pellets were resuspended in 2 mL of BMMY and induced overnight at 30 °C. After centrifuging (2000 rpm for 10 minutes), the supernatant was analyzed by SDS-PAGE and Western blot for the protein of interest.

4.1.5 Expression in *Pichia pastoris*

Expression of the recombinant proteins was carried out as described in the Invitrogen manual (Invitrogen Corp., 1995). Briefly, 8 x 500 mL BMGY (1% yeast extract, 2% peptone, 1% yeast nitrogen base, 1% glycerol, 100 mM potassium phosphate
pH 6.0) cultures of *P. pastoris* were incubated at 30°C for one day. After centrifugation (5 minutes, 5000 × g) the pellets were resuspended in 8 × 100 mL BMMY medium (1% yeast extract, 2% peptone, 1% yeast nitrogen base, 0.5% MeOH, 100 mM potassium phosphate pH 6.0), where expression was induced with MeOH. Following two days of expression at 30°C with batch-wise addition of 0.5% MeOH every 12 hours, the *Pichia* cells were removed by centrifugation (10 minutes, 5000 × g), leaving the secreted α-amylase in the supernatant.

### 4.1.6 Purification

Purification of wild-type α-amylase began with slow addition of cold EtOH to the supernatant with stirring at 4°C until the solution contained 40% EtOH. This solution was allowed to stir for one hour at 4°C before centrifugation (12 000 × g, 20 minutes, 4°C). Glycogen (20 mg/mL) was added to the supernatant over a period of five minutes such that 1 mg glycogen per 100 Units activity, as measured by the DNS (dinitrosalicylic acid) assay ((Somogyi, M. 1938)) , was added. The resultant solution was stirred for an additional 10 minutes at 4°C before centrifuging (13 000 × g, 5 minutes, 4°C). The supernatant was removed and the small white pellet was dissolved in 30 mL of 20 mM phosphate buffer containing 25 mM NaCl (pH 6.9). The solution was left overnight at room temperature to allow complete digestion of the glycogen. The digested sample was dialysed against 100 mM potassium phosphate (pH 7.4) at room temperature overnight. Following dialysis, the α-amylase solution was adjusted to 0.5 M NaCl and loaded onto a column (3 cm × 2 cm) of Phenyl-Sepharose CL-4B (Pharmacia) equilibrated in 100 mM phosphate buffer pH 7.4. The bound protein was eluted with distilled water and
concentrated to >2 mg/mL using a stirred-cell concentrator (Amicon). Deglycosylation of the eluted enzyme (described in §4.1.6) was performed prior to this enzyme being subjected to ion exchange chromatography on a column (2 cm × 1 cm) of Q-Sepharose (Pharmacia) equilibrated in 20 mM phosphate buffer (pH 6.9) containing 25 mM NaCl. The desired protein was collected in the column flow-through fractions.

For purification of the mutants, the supernatant was adjusted to pH 7.0 with NaOH before filtering through a 0.45 μm membrane (Gelman). Subsequently, the filtrate was loaded onto a Phenyl-Sepharose CL-4B column (3 cm × 2 cm) equilibrated in 100 mM potassium phosphate buffer pH 7.0. After washing with 100 mM phosphate buffer (pH 7.0) the bound protein was eluted with distilled water. The eluant was concentrated using an Amicon stirred cell concentrator and the buffer was adjusted to 20 mM potassium phosphate, containing 25 mM NaCl (pH 6.9) before being subjected to ion exchange chromatography on a column (2 cm × 1 cm) of Q-Sepharose (Pharmacia) equilibrated in 20 mM phosphate buffer containing 25 mM NaCl (pH 6.9). The α-amylase obtained from the clear flow-through was >99% pure as determined by SDS-PAGE and staining with Coomassie Brilliant Blue.

4.1.7 Deglycosylation

Deglycosylation was performed in different trials using endoglycosidase F from two different sources. Endoglycosidase F (20 μL, 0.05 U/μL, Boehringer Mannheim) was added to a sample of α-amylase (100 mL, 0.1 mg/mL in 100 mM phosphate buffer, pH 7.4) prior to loading onto the Phenyl-Sepharose column. Digestion was carried out overnight at room temperature, or for 4 hours at 37°C. The amylase purification was then
completed as described above. Alternatively, endoglycosidase F-CBD (10 µL, 0.5 U/µL, an endoglycosidase F-cellulose binding domain fusion protein generously donated by Dr. R.A.J. Warren) was added to a solution of purified α-amylase (20 mL, 2 mg/mL, 50 mM phosphate buffer containing 25 mM NaCl, pH 7.4) and the solution was incubated at 37°C for 4 hours or at room temperature overnight. Deglycosylation was confirmed by SDS-PAGE stained with Coomassie Brilliant Blue before cellulose (0.5 g) was added to the solution. This suspension was gently mixed for 5 minutes before centrifugation (3500 × g, 5 minutes). The supernatant contained only deglycosylated α-amylase as determined by SDS-PAGE and ESI-MS.
4.2 Structural Characterization

4.2.1 Determination of Glycosylation Site by MS

Localization of the glycosylation site by ESI-MS was carried out by Mr. S. Numao and Mr. S. He. Briefly, samples of both untreated and endoglycosidase F-treated HPA (900 μL, 1.2 mg/mL) were precipitated for 30 minutes on ice after addition of 100 μL of 100% TCA. The resulting precipitate in each case was centrifuged (5 minutes, 8000 × g), washed several times with cold acetone, and dried. The powder was resuspended in 200 μL of 8 M urea containing 0.4 M NH₄HCO₃ to which 20 μL of 45 mM DTT was added. This solution was incubated at 50°C for 15 minutes before cooling to room temperature. Iodoacetamide (20 μL, 100 mM) was then added and the reaction mix was incubated for 30 minutes at room temperature. Following incubation, the solution was diluted with deionized water (560 μL). Trypsin (5% w/w) was added to the iodoacetamide treated HPA solution (200 μL) and the digestion was incubated at 30°C overnight. The reaction was stopped by freezing this solution.

The tryptic digest was analyzed by LC-MS (as described below) and the tryptic maps of the untreated and endoglycosidase F treated α-amylases were compared. A peptide appearing in the endoglycosidase F-treated sample but not in the untreated sample was isolated, and sequenced by Edman degradation.

4.2.2 Mass Determination of Recombinant Proteins by MS

The mass determinations of the recombinant proteins and tryptic digests before and after endoglycosidase F-treatment were performed on a PE-Sciex API 300 triple
quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Protein was loaded onto a C18 column (Reliasil, 1 x 150 mm) equilibrated with solvent A (solvent A: 0.05% trifluoroacetic acid (TFA), 2% acetonitrile in water). Elution of the peptides was accomplished using a gradient (0-60%) of solvent B over 60 minutes followed by 100% solvent B over 2 minutes (solvent B: 0.045% TFA, 80% acetonitrile in water). Solvents were pumped at a constant flow rate of 50 µL/min. Spectra were obtained in the single quadrupole scan mode (LC/MS). The quadrupole mass analyser was scanned over a mass to charge ratio (m/z) range of 400-2400 Da with a step size of 0.5 Da and a dwell time of 1.5 ms per step. The ion source voltage (ISV) was set at 5.5 kV and the orifice energy (OR) was 45 V.
4.3 Kinetic Characterization

4.3.1 General Procedures

Fluoride ion concentration was measured using an Orion 96-09 combination fluoride ion selective electrode coupled to an Accumet 925 pH/ion meter (Fischer Scientific). The ion meter was interfaced with a Pentium 133 MHz personal computer for data collection. Alternatively, the electrode was fitted to a signal amplifier box connected to a personal computer and data was collected using Logger Pro™ (Vernier Software Inc). In addition, assays measuring the sugar products were performed using a Waters HPLC (model 501 pump, single port manual injector) instrument with a refractive index detector (model 410). Reaction products were separated using a Waters Dextropak 8/10 radial compression column with a Resolve C-18 guard column run in a deionized H₂O mobile phase at a 1.0 mL/min flow rate. The data were collected, and peak areas calculated, using the Baseline 810 software. Where product concentrations were desired, the peak areas were compared to those measured from a G5 standard curve. For all kinetic assays, the data were fit to the desired model using the non-linear regression program GraFit 4.0 (Erithacus Software Inc.). The error values noted in the data tables refer to the error in fitting the data to the model.

4.3.2 Measuring Specific Activities Using the Dinitrosalicylic Acid (DNS) Assay

Specific activities of crude (supernatant) and purified recombinant enzymes were measured using the dinitrosalicylic acid assay (Bernfeld, P. 1955). An enzyme sample, either from culture supernatant, or purified (up to 500 µL) was added to a solution of
soluble starch (500 μL) and incubated at 30°C. For pHPA, the incubation time was 3 minutes. The mutant enzymes were incubated for between 8 hours and 24 hours. The reactions were stopped by addition of 1.0 mL of the DNS solution (10 mg/mL 3,5-dinitrosalicylic acid and 30 mg/mL Na K tartrate in 0.4 M NaOH). Colour was developed by boiling the stopped samples for 5 minutes, followed by cooling with cold tap water and dilution with 10.0 mL distilled water. The samples were then measured spectrophotometrically at 546 nm. Concentrations of sugar reducing ends were calculated by comparison to a maltose standard curve.

4.3.3 Determining Michaelis-Menten Parameters for Malto-Oligosaccharyl Fluorides

The kinetic parameters $k_{cat}$ and $K_m$ for the recombinant proteins were determined by monitoring the release of fluoride from α-maltotriosyl fluoride (αG3F) and α-maltosyl fluoride (αG2F) as described previously, (McCarter, J. et al. 1993). The assays were performed at 30°C in 20 mM sodium phosphate buffer, pH 6.9 containing 25 mM NaCl. Stock enzyme (5 μL of 50 nM wild-type enzyme, or 50 μL of 18 μM mutant enzyme) was added to glass cells containing various concentrations of substrate in a total assay volume of 300 μL. For each Michaelis-Menten curve generated, initial rates for 6-8 substrate concentrations were used. Concentrations of αG3F were typically varied between 50 μM and 5 mM, while the range of αG2F concentration was generally from 500 μM to 20 mM. The initial rates were determined from the first 5 minutes of data. Controls were performed to compensate for the spontaneous hydrolysis of the substrates.
4.3.4 Investigating the Reactions of Recombinant α-Amylases with α-Glucosyl Fluoride

Deionized H$_2$O and αGF (1.0 M or 100 mM stock) was added to buffer (200 μL, 100 mM potassium phosphate, 10 mM NaCl, pH 7.0 for assays with wild-type enzyme and pH 6.0 for assays with mutants) in a 1 mL glass cuvette to a total volume of 280 μL (270 μL with the D197A assay). Assay concentrations of αGF were varied between 5.0 mM and 200 mM. The solution was incubated, with the fluoride electrode, at 30°C for 5 minutes before measuring the spontaneous rate of hydrolysis for 5 minutes. Enzyme (20 μL of one of - 2.0 μM [HPA-wt], 78 μM [D197N], 14 μM [E233Q], 36 μM [D300N], 160 μM [D300H], 303 μM [BSA] – or 30 μL of 110 μM [D197A]) was then added. After mixing, the rate of fluoride release was followed for 10 minutes or for less than 10% hydrolysis. For analysis, after correcting for spontaneous hydrolysis, the data were fit to a model that assumes that αGF acts as both acceptor and donor with different affinities at each site (Lougheed, B. et al. 1999).

4.3.5 Effect of Citrate and Calcium on HPA Reactions

To study the possible metal chelating ability of citrate buffer on HPA reactions, the standard assay from §4.3.2 was followed at one substrate concentration but with the addition of citrate and the absence or inclusion of calcium. A solution of 2 mM αG3F buffered with 60 mM potassium phosphate, 60 mM sodium citrate, and 15 mM NaCl at pH 7.0 was assayed with human pancreatic α-amylase (0.8 nM) that was added after a 5 minute measurement of spontaneous release of fluoride. This reaction was performed under four different conditions. 1) 60 mM sodium citrate, 2) 60 mM sodium citrate, 500
µM CaCl₂ added before the enzyme 3) 60 mM sodium citrate, 500 µM CaCl₂ added 5 minutes after the enzyme, and 4) 60 mM sodium citrate, 500 µM CaCl₂ added 10 minutes after the enzyme.

4.3.6 pH Stability

The pH stabilities of the recombinant proteins were studied using the basic assay as described in §4.3.2. However, measurements of fluoride release from αG3F were followed at 13 different pH values, from 4.0 to 10.0 in increments of 0.5 pH units. Only one concentration of αG3F (2 mM) was assayed at each pH. Assay solutions (total volume 300 µL) were buffered with 180 µL of a solution of 100 mM potassium phosphate and 100 mM sodium citrate containing 25 mM NaCl. Calcium chloride was also added to the assay mixture to a concentration of 500 µM.

4.3.7 Determining pH Dependence of \( \frac{k_{ca}}{K_m} \) (Substrate Depletion)

The pH-dependence of the second order rate constant \( \frac{k_{ca}}{K_m} \) was studied using the substrate depletion method. Measurements of fluoride release from αG3F were followed at 13 different pH values, from 4.0 to 10.0 in increments of 0.5 pH units. Assay solutions (total volume 300 µL) were buffered with 180 µL of a solution of 100 mM potassium phosphate and 100 mM sodium citrate containing 25 mM NaCl. Calcium chloride was also added to the assay mixture to a concentration of 500 µM. At each pH value the release of fluoride resulting from the reaction of enzyme with αG3F at concentrations <1/5 \( K_m \) was followed as a function of time for a minimum of 3 half-lives. Enzyme concentration was chosen so that, where possible, \( t_{1/2} \) was equal to approximately 15 minutes. The resulting pseudo first-order curve was then fit to a first-order product
accumulation model using GraFit version 4.06 (Erithacus Software Inc.). The first-order rate constant for the curve (which is equal to $V_{\max}/K_m$) was then divided by the initial enzyme concentration ($E_0$) to give the $k_{cat}/K_m$ value (see Appendix A).

### 4.3.8 Determining pH Dependence of $k_{cat}$

Determination of the pH dependence of $k_{cat}$ for the recombinant proteins was achieved using the basic assay described in §4.4.2. However, measurements of fluoride release from $\alpha$G3F were followed at 13 different pH values, from 4.0 to 10.0 in increments of 0.5 pH units. For each enzyme, the substrate ($\alpha$G3F) was assayed at a concentration that gave a rate equal to $k_{cat}$, typically in the range 2-5 mM. Assay solutions (total volume 300 µL) were buffered with 200 µL of a solution of 100 mM potassium phosphate and 100 mM sodium citrate containing 25 mM NaCl. Calcium chloride was also added to the assay mixture to a concentration of 500 µM.

### 4.3.9 Approximate $K_i$ Determination ("Rangefinder" $K_i$ values)

Approximate $K_i$ values ("range-finder" $K_i$ values) were determined using a modified version of the basic assay (§4.3.2). Assay solutions were buffered with 200 µL of 50 mM potassium phosphate containing 100 mM NaCl at the pH optimum for the enzyme being tested. Assays were performed with acarbose concentrations varying by three orders of magnitude from 10 nM to 10 µM for wild-type $\alpha$-amylase ([α-amylase] = 0.8 nM), and 1 µM to 1 mM for assays of mutant enzymes ([mutant enzymes] = 200 nM), as well as a sample with no acarbose. The $\alpha$G3F concentration used was 2 mM with one assay performed at a concentration of $\alpha$G3F giving $V_{\max}$ (5 mM or 10 mM). Since the $V_{\max}$ of an enzyme is not altered by a competitive inhibition, the intersection of the two
lines from a plot of $1/v$ vs [acarbose] (line 1 = rates with 2 mM $\alpha$G3F with various [acarbose], line 2 = a horizontal line plotted at $V_{\text{max}}$ with the data from the 5 mM or 10 mM assay), gave an estimation of the $K_i$ values (intersection = $-K_i$). To simplify the estimates of the $K_i$ values, the mode of inhibition for each enzyme was assumed to be competitive. As a comparison of estimated $K_i$ values for different modes of inhibition, the $1/v$ vs [acarbose] plots were also used to estimate the $K_i$ values assuming non-competitive inhibition. In these cases, since non-competitive inhibition cannot be overcome by increasing [substrate] and therefore $v = 0$ is the point of intersection, the intersection of the line and the x-axis ($v = 0$) gave estimates of the $K_i$ values (intersection = $-K_i$). The $K_i$ values determined for each mode of inhibition were within the same order of magnitude and therefore provided useful initial estimates, essentially independent of the inhibition type, for the full $K_i$ determinations that followed.

4.3.10 Kinetics of Inhibition by Acarbose (Dixon Plots)

The inhibition studies with acarbose were set up to follow a Dixon plot procedure. The general assay procedure described in §4.3.2 was followed but with several modifications. Assay solutions were buffered with 200 $\mu$L of 50 mM potassium phosphate containing 100 mM NaCl at the pH optimum for the enzyme being tested. For most enzymes a 2-line Dixon experiment was performed with two $\alpha$G3F concentrations (approximately 1/3 and 3 times $K_m$) and 5 acarbose concentrations (ranging one order of magnitude from the RF-$K_i$ value). For both the wild-type enzyme and D300N a “full” Dixon plot study (covering 5 concentrations each of acarbose and $\alpha$G3F) was also performed in order to assess the validity of the 2-line Dixon plots. The data was fit to a
Dixon plot ($1/v$ vs. $[\text{acarbose}]$) model by the program GraFit to determine both the mode of inhibition and the $K_i$.

4.3.11 Determination of Kinetic Parameters with Maltopentaose

An HPLC stopped-assay was used to determine the kinetic parameters of the wild-type and mutant enzymes with maltopentaose. Assays (200 µL total volume) were buffered with 10 mM potassium phosphate and 20 mM NaCl at the pH optimum for each enzyme. Enzymes were assayed at seven substrate concentrations (0.1 – 3.0 mM) of G5. Assays were incubated at 30°C and 25 µL aliquots were taken at four time points for each concentration. The reactions were stopped by removal of protein with a Waters Resolve-C18 guard column. Total incubation time for the wild-type reaction (2.5 nM enzyme) was 1 hour, for E233Q (0.7 µM) 50 hours, and D300N (1.3 µM) 10 hours. Product concentrations were determined by relative refractive index (reference cell was purged for 30 minutes with mobile phase prior to measurements) after separation from substrates by HPLC using the Waters Dextropak column. The H$_2$O mobile phase was run at a flow rate of 1.0 mL/min and retention times were: G2, 2.8 min; G3, 3.7 min; and G5 5.8 min. A maltopentaose standard curve was used for determining the substrate concentration remaining at each time point. Maltopentaose concentrations of 0.1 mM, 1.0 mM, and 10.0 mM, each measured in triplicate, were used to generate the standard curve.

4.3.12 Product Distribution Studies

Product distributions were determined following the procedure from §4.3.11. Substrate concentrations ($\alpha$G3F and G5) of 5 mM and 20 mM each were treated with
enzyme and the products formed were determined at regular intervals until the substrate was fully hydrolyzed, or for a period of five days, whichever occurred first.

**4.3.13 Azide Rescue of Alanine Mutants**

$\alpha$G3F (30 $\mu$L, 20 mM) was added to solutions of sodium azide (200 mM to 5.0 M) in buffer (250 $\mu$L, 100 mM potassium phosphate, 10 mM NaCl, pH 6.0) in a 1 mL flat-bottomed glass vial. Assay concentrations of $\alpha$GF were varied between 5.0 mM and 200 mM. The solution was incubated, with the fluoride electrode, at 30°C for 5 minutes before measuring the rate of spontaneous hydrolysis for 5 minutes. Enzyme (20 $\mu$L of 120 $\mu$M D197A, 30 $\mu$L of 710 nM E233Q, or 10 $\mu$L of 38 $\mu$M D300A) was then added. After mixing, the rate of fluoride release was followed for 10 minutes or for less than 10% hydrolysis. After correcting for spontaneous hydrolysis the data were successfully fit to the Michaelis-Menten model in all cases, although inhibition was observed at high azide concentrations (greater than 2 M) for E233A and D300A.
4.4 Synthetic Chemistry Methods

4.4.1 General Methods

a. Analytical methods

$^1$H NMR spectra were recorded on the following instruments at the indicated field strengths: a Bruker AC-200 at 200 MHz, a Varian XL-300 at 300 MHz, or a Bruker WH-400 at 400 MHz. $^{13}$C NMR spectra were recorded on a Varian XL-300 spectrometer at 75 MHz. $^{19}$F NMR spectra were recorded on a Bruker AC-200 multinuclear spectrometer operating at a frequency of 188 MHz. $^1$H NMR chemical shifts are internally referenced to CHCl$_3$ ($\delta = 7.24$ ppm) or MeOH ($\delta = 3.20$ ppm), $^{13}$C NMR spectra are proton decoupled and shifts are internally referenced to CHCl$_3$ ($\delta = 77.0$ ppm) or MeOH ($\delta = 49.0$ ppm). $^{19}$F NMR shifts are externally referenced to trifluoroacetic acid ($\delta = -76.53$ ppm).

Electrospray mass spectra were recorded on a Sciex API-300 mass spectrometer using direct flow injection. The samples were introduced in 100% MeOH by a syringe pump driven at 5 $\mu$L/min in the presence of 5 mM ammonium acetate as an ionization agent.

Thin layer chromatography (TLC) was performed using plates (silica gel 60 F$_{254}$, Merck) with visualization after charring with 10% H$_2$SO$_4$ in MeOH or ammonium molybdate-H$_2$SO$_4$ solution in MeOH. Products were purified by flash chromatography using Silica gel 60 (230-400 mesh).
b. Solvents and reagents

Solvents and reagents were either of reagent, certified, spectral, or HPLC grade. Dry solvents were prepared as follows. Dichloromethane was distilled over calcium hydride. Methanol was distilled over magnesium with iodine.

Maltose and maltotriose were obtained from Sigma. "Amberlyst" brand resin, "15 dry" (containing less than 1.5% water) was kindly donated by Rohm and Haas Canada. Chelex (200-400 mesh, Na\(^+\) form) and Dowex 50 W H\(^+\) Amberlite resin were from BioRad. All other chemicals were purchased from Aldrich Chemical Company and used without further purification.

c. Compounds synthesized and provided by colleagues

The compounds which were not synthesized in this thesis were: 4-deoxymaltose (4DG2), which was prepared by Dr. Thisbe Lindhorst, 4-deoxy-\(\alpha\)-maltosyl fluoride (4DoG2F) which was prepared by Dr. Spencer Williams, and \(\alpha\)-glucosyl fluoride which was prepared by Dr. Steven Howard and Mr. David Zechel.

4.4.1 Three step synthesis of \(\alpha\)-glycosyl fluorides

The following work was adapted from the method of Junnemann \textit{et al.}. Details are provided for the synthesis of \(\alpha\)-maltotriosyl fluoride (\(\alpha\)G3F).

a. Acetylation

\(1,2,3,6,2',3',6',2'',3'',4'',6''\)-Undeca-\(O\)-acetyl-\(\alpha\)-maltotriose (2.1). Maltotriose (10.0 g, 19.8 mmol) was dissolved in pyridine and acetic anhydride was added in excess (typical volume ratio was pyridine to acetic anhydride 3:2). The reaction mixture was stirred overnight at 50-60\(^\circ\)C. The reaction mixture was diluted into ice-water and extracted with
dichloromethane, then dried over MgSO₄. After in vacuo evaporation of the solvent, the product was purified by column chromatography or crystallized from ethyl acetate (EtOAc)/hexanes.

b. Fluorination

2,3,6,2',3',6',2'',3'',4'',6''-Deca-O-acetyl-α-maltotriosyl fluoride (2.2). Maltotriose per-O-acetate 2.1 (2.5 g, 2.59 mmol) was added directly to a plastic vial at -78°C containing 20 mL HF-pyridine and the reaction was stirred under a blanket of N₂ for 20 min. The reaction mixture was allowed to warm to room temperature and stir for a further 2 to 3 h, then diluted with CH₂Cl₂ and washed with H₂O, saturated aqueous NaHCO₃ and H₂O, and dried (MgSO₄). Evaporation in vacuo followed by column chromatography (1.5:1 EtOAc:hexanes) yielded 2.2 (1.45 g, 60%) as a white solid. ¹H NMR data (CDCl₃, 400 MHz) selected data only: δ 5.67 (dd, 1 H, J₁,F 52.9, J₁,2' 3.9 Hz, H-1), 5.39 (d, 1 H, J₁,2' 3.8 Hz, H-1'), 5.36 (d, 1 H, J₁'',2'' 3.8 Hz, H-1''). ¹⁹F NMR data (CDCl₃, 188 MHz): δ -149.2 (dd, J₁,F 52.9, J₂,F 26.0 Hz, F-1).

c. Deacetylation

α-Maltotriosyl fluoride (2.3). To compound 2.2 (1.85 g, 2.00 mmol) dissolved in dry methanol was added a catalytic amount of a freshly prepared sodium methoxide solution in dry methanol (typically 0.02 M). The reaction mixture was stirred at room temperature under N₂ until the reaction was judged to be complete by TLC (usual reaction time was 3 hours for G3 and G2). The reaction mixture was neutralized by the addition of a small amount of Dowex 50 W H⁺ Amberlite resin (Bio-Rad), then the resin removed by filtration and the solvent evaporated in vacuo. Purification (when necessary) was achieved by adsorbing the reaction mixture to silica followed by flash chromatography
(7:2:1 EtOAc:MeOH:H$_2$O) to give pure $\alpha$-maltotriosyl fluoride ($\alpha$G3F) 2,3 (630 mg, 80%) as a colourless gum. Lyophilization afforded 2,3 as a white solid. $^1$H NMR data (D$_2$O, 400 MHz) selected data only: $\delta$ 5.67 (dd, 1 H, $J_{1,F}$ 52.9, $J_{1,2}$ 3.9 Hz, H-1), 5.39 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 5.36 (d, 1 H, $J_{1'',2''}$ 3.8 Hz, H-1''). $^{19}$F NMR data (D$_2$O, 188 MHz): $\delta$ -150.4 (dd, $J_{1,F}$ 52.9, $J_{2,F}$ 26.0 Hz, F-1).


Bibliography


Bibliography


A-1 FUNDAMENTAL EQUATIONS OF ENZYME KINETICS

In 1913, Michaelis and Menten proposed a simple model to account for the relationship between the rate of catalysis and the concentration of substrate. Later, Briggs and Haldane (1925) expanded on this by introducing the concept of the steady state. The general scheme for an enzyme catalyzed reaction is shown below. Free enzyme, E, combines with free substrate, S, to form an enzyme-substrate complex, ES, which is then turned over to yield product, P.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P
\]

Under steady state conditions,

\[
\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0
\]

(1)

The total concentration of enzyme \([E]_0\), is equal to the concentration of free enzyme \([E]\), plus the concentration of the enzyme bound in the ES complex, \([ES]\).

\[
[E]_0 = [E] + [ES]
\]

(2)

Solving for \(ES\) using equations 1 and 2,

\[
[ES] = \frac{[E]_0[S]}{[S] + (k_{-1} + k_2)/k_1}
\]

(3)
Assuming that the formation of products is the rate limiting step ($k_2$), the initial velocity of the reaction, $v$, is equal to the rate of formation of product,

$$v = \frac{dP}{dt} = k_2[ES] \quad (4)$$

By substituting the expression for $[ES]$ from equation 3 into equation 4, one obtains

$$v = \frac{k_a[E]_o[S]}{\left(\frac{k_{-1} + k_2}{k_1}\right) + [S]} \quad (5)$$

The ratio of rate constants $(k_{-1}+k_2)/k_1$ is defined as $K_m$, the Michaelis constant.

The rate constant, $k_2$ is defined as the catalytic constant or $k_{cat}$ (the turnover number).

Therefore equation 5 can be expressed in a more general format, also known as the Michaelis Menten equation,

$$v = \frac{k_{cat}[E]_o[S]}{K_m + [S]} \quad (6)$$

Therefore when the initial rate of the reaction is equal to one-half the maximal velocity ($v=V_{max}/2$), the substrate concentration is equal to $K_m$. In its simplest form, the Michaelis constant is a measure of the binding affinity of an enzyme for a particular substrate. An enzyme with a high binding affinity for a substrate has a low value of $K_m$. 
A graphical representation of the Michaelis Menten equation is given below (Figure 38). It can also be plotted as a double reciprocal plot ($1/v$ vs $1/[S]$), also known as the Lineweaver-Burke plot (Figure 39).

---

**Figure 38** A typical Michaelis-Menten plot for the determination of $K_m$ and $V_{max}$

**Figure 39** A typical Lineweaver-Burke plot for the determination of $K_m$ and $V_{max}$
At high substrate concentrations ([S] >> $K_m$), $v$ approaches its maximal value, $V_{max}$, and the rate becomes independent of substrate concentration. Thus, the Michaelis Menten equation can be rewritten in the form,

$$V_{max} = k_{cat}[E]_0 \quad (7)$$

At low concentrations of substrate ([S] << $K_m$), the initial rate of the reaction is proportional to the substrate concentration,

$$v = \frac{k_{cat}[E]_0[S]}{K_m} \quad (8)$$

In this case $k_{cat}/K_m$ can be determined directly by dividing the pseudo-first-order rate constant for the reaction ($k_{cat}[E]_0/K_m$) by the total enzyme concentration ([E]$_0$).

The Michaelis Menten approach can be expanded to more complex enzyme systems in which two or more distinct reaction steps occur. Such is the case for the double displacement mechanism followed by retaining $\alpha$-glycosyl transferases (see Section 1.3). The reaction scheme for this mechanism is as follows. Free enzyme, E, combines with free substrate, S, to form an enzyme-substrate complex, ES, with a rate constant, $k_1$ (association step). The conversion of ES to EP is termed the glycosylation step ($k_2$) and the turnover of EP to P is the deglycosylation step ($k_3$).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} EP \overset{k_3}{\rightarrow} E + P$$
Assuming that a steady state concentration of both EP and ES is reached,

\[ k_2[ES] = k_3[EP] \]  \hspace{1cm} (9)

\[ \frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \]  \hspace{1cm} (10)

The total concentration of enzyme \([E]_0\) is equal to free enzyme plus all of the enzyme bound species,

\[ [E]_0 = [E] + [ES] + [EP] \]  \hspace{1cm} (11)

By substituting for [EP] using equation 9, one obtains,

\[ [E]_0 = [E] + [ES] + \frac{k_2[ES]}{k_3} \]  \hspace{1cm} (12)

By solving equation 12 for [E] and substituting for [E] into equation 10, followed by a rearrangement,

\[ [ES] = \frac{k_1[E]_0[S]}{k_{-1} + k_2 + k_1(k_2 + k_3)[S]} \]  \hspace{1cm} (13)

At steady state, the rate of product formation is equal to

\[ \frac{dP}{dt} = k_3[EP] = k_2[ES] \]  \hspace{1cm} (14)
Substituting equation 13 into equation 14, yields an expression in the form of the Michaelis Menten equation.

\[ v = \frac{k_2k_3[E_0][S]}{k_2 + k_3} \frac{k_3}{k - 1 + k_2} \frac{k_1}{[S]} \]

From the form of the Michaelis Menten equation,

\[ k_{cat} = \frac{k_2k_3}{k_2 + k_3} \]

\[ K_m = \left( \frac{k_3}{k_2 + k_3} \right) \frac{k - 1 + k_2}{k_1} \]

Therefore, it becomes evident that the values obtained experimentally for \( k_{cat} \) and \( K_m \) are composites of the individual rate constants.

**A-2 Enzyme Kinetics in the Presence of a Reversible Inhibitor**

There are three main types of reversible inhibition, competitive, noncompetitive and uncompetitive.

1. **Competitive inhibition**

A competitive inhibitor competes directly with the substrate for binding to the active site of the enzyme. The simple enzyme-catalyzed reaction must be expanded to include a second equilibrium,
$K_s$ is the dissociation constant of the enzyme-substrate complex ($K_s = [E][S]/[ES]$). $K_i$ is the dissociation constant for the enzyme-inhibitor complex ($K_i = [E][I]/[EI]$).

The total concentration of enzyme is now equal to

$$[E]_o = [E] + [ES] + [EI]$$

(18)

Using the steady state approach ($d[ES]/dt = 0$) and substituting into the Michaelis Menten equation 6,

$$v = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}$$

(19)

Therefore, a competitive inhibitor increases the apparent $K_m$ by a factor of $1 + [I]/K_i$. The value of $V_{max}$ is unaffected as at high concentrations of substrate, the inhibitor is displaced from the enzyme.
b. Noncompetitive inhibition

Noncompetitive inhibition occurs when the inhibitor and substrate can bind simultaneously to the enzyme instead of competing for the same binding site.

\[
\begin{align*}
E + S & \rightleftharpoons K_s ES \rightarrow_{k_{cat}} E + P \\
\text{Ki} + I & \quad \text{Ki} + I \\
EI & \rightleftharpoons_{K_{s'}} ESI + S
\end{align*}
\]

\[
[E]_0 = [E] + [ES] + [EI] + [ESI] \quad (20)
\]

When the dissociation constant of \( S \) from ESI is the same as that from ES (\( K_s = K_{s'} \)), by incorporating this expression into the steady state assumption for the concentration of [ES] and the expression \( v = k_2[ES] \), one obtains

\[
v = \frac{V_{\text{max}} [S]}{1 + \frac{[I]}{K_i}} \quad (21)
\]

A noncompetitive inhibitor does not change the value of \( K_m \). However, the value of \( V_{\text{max}} \) is decreased by a factor of \( (1 + [I]/K_i) \).
c. Uncompetitive inhibition

An uncompetitive inhibitor binds only to the enzyme-substrate complex.

\[ E + S \xrightarrow{K_s} ES \xrightarrow{k_{cat}} E + P + I \]

\[ [I] = \frac{[I]}{K_i} \]

\[ [E]_0 = [E] + [ES] + [ESI] \tag{22} \]

Again, using the steady state approximation of \( d[ES]/dt = 0 \) and \( v = k_2[ES] \), one obtains

\[ v = \frac{\left( \frac{V_{max}}{1 + \frac{[I]}{K_i}} \right) [S]}{\left( \frac{K_m}{1 + \frac{[I]}{K_i}} \right) + [S]} \tag{23} \]

An uncompetitive inhibitor decreases the value of both the apparent \( V_{max} \) and the apparent \( K_m \) by a factor of \( 1 + [I]/K_i \).

The three types of reversible inhibition can be distinguished graphically using a Lineweaver-Burke plot (Figure 40), or a single reciprocal (1/v vs [I]) Dixon plot (Figure 41).
Appendix A Fundamental Enzyme Kinetics

Figure 40 Graphical representation for the determination of the modes of inhibition with a Lineweaver-Burke plot. (A) Competitive Inhibition, (B) Noncompetitive Inhibition, (C) Uncompetitive Inhibition.
Figure 41 Graphical representation for the determination of the modes of inhibition with a Dixon plot. (A) Competitive Inhibition, (B) Noncompetitive Inhibition, (C) Uncompetitive Inhibition.
APPENDIX B

Graphical Representation of Kinetic Data
Figure 42 The Michaelis Menten plots for the reactions of wt-HPA with α-glycosyl fluorides. (A) gly-pHPA (0.83 nM) with αG2F, (B) gly-pHPA (0.83 nM) with αG3F, (C) degly-pHPA (4.0 nM) with αG2F, (D) degly-pHPA (0.83 nM) with αG3F
Figure 43 The Michaelis Menten plots for the reactions of mutant human pancreatic α-amylases with α-maltosyl fluoride. (A) E233Q (3.7 μM), (B) D300A (5.4 μM), (C) D300H (2.0 μM), (D) D300N (0.37 μM), (E) D300Y (18 μM), (F) E233A/D300A (4.1 μM)
Figure 44 The Michaelis Menten plots for the reactions of mutant human pancreatic α-amylases with α-maltotriosyl fluoride. (A) D197A (17 μM), (B) D197N (17 μM), (C) E233A (1.3 μM), (D) E233Q (), (E) D300A (6.4 μM), (F) D300N (0.43 μM).
Figure 45 The Michaelis Menten plots for the reactions of mutant human pancreatic α-amylases with α-maltotriosyl fluoride. (A) E233K (7.9 μM), (B) D300H (2.0 μM), (C) D300Y (18 μM), (D) E233A/D300A (4.3 μM)
Figure 46 The Michaelis Menten plots for the reactions of recombinant human pancreatic α-amylases with maltopentaose. (A) pHPA (2.5 nM), (B) E233Q (1.6 μM), (C) D300N (1.8 μM)
Figure 47 Two-Line Dixon plots representing the inhibition of recombinant human pancreatic α-amylases by acarbose using αG3F as substrate. (A) pHPA (0.68 nM), (B) E233A (81 nM), (C) E233Q (89 nM), (D) D300A (220 nM), (E) D300N (120 nM)
Appendix B  Graphical Representations of Kinetic Data

Figure 48  Plots for full $K_i$ determinations for the inhibition of recombinant human pancreatic $\alpha$-amylases by acarbose using $\alpha$G3F as substrate.  (A) Lineweaver-Burk plot for $p$HPA (0.68 nM), (B) Dixon plot for D300N (100 nM)

Figure 49  Crude “range-finder” plots for $K_i$ estimations for the inhibition of recombinant human pancreatic $\alpha$-amylases by acarbose using $\alpha$G3F as substrate.  The dashed horizontal line represents $V_{\text{max}}$.  (A) D300H (2.2 uM), (B) D300Y (15 $\mu$M)