Tailoring the pH dependence of glucoamylase from Aspergillus awamori by mutagenesis

Ufuk Bakir

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Tailoring the pH dependence of glucoamylase from *Aspergillus awamori* by mutagenesis

Bakir, Ufuk, Ph.D.

Iowa State University, 1993
Tailoring the pH dependence of glucoamylase from 
Aspergillus awamori by mutagenesis

by

Ufuk Bakir

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Chemical Engineering
Major: Chemical Engineering

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa
1993
I would like to dedicate this study to my parents, Guzide and Huseyin Bolukbasi, for their continuous encouragement, patience, and support during my education from the very beginning.
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GENERAL INTRODUCTION

Enzymes are protein molecules that catalyze reactions in biological systems with remarkable specificity. Although thousands of enzymes have been characterized, up to now only a small fraction of them have been used in industry, basically due to the difficulties involved in production of large quantities, as well as to low enzyme efficiencies.

Biological systems produce enzymes in small quantities for their own needs. However, for industrial purposes large amounts of pure enzymes are required. This requires that enzyme production be amplified generally by manipulation of the conditions under which the systems are operating, as well as by the choice of suitable separation and purification methods for large and pure quantities at a low cost.

Due to their biological nature, enzymes are active at physiological conditions. However, industrial conditions are often very different in order to increase enzyme productivity. Therefore, it is necessary to produce industrially more effective enzymes by increasing their stability to high temperatures, extreme pHs, and protease attack. In addition, enzymes can be made more effective by changing their kinetic properties and substrate
specificities to fit industrial requirements. A new branch of science, protein engineering, which can be described as a marriage between the disciplines of protein biochemistry and recombinant DNA technology with the common goal of altering the catalytic or structural stability properties of a given protein (Bryan, 1987; Power et al., 1989), makes preparation of better enzymes for industrial processes more efficient than before.

Protein engineering is probably an inevitable consequence of the breakthroughs in gene cloning, DNA sequencing, in vitro DNA manipulation, and efficient production of oligonucleotides of defined sequences, which can then be used to direct specific mutations into cloned DNA. Using these advances, methods of site-directed mutagenesis have progressed to a point that practically unlimited variations in gene sequences can be created given sufficient time. The ability to introduce specific mutations into a gene at will and to then express and study the altered protein has provided an essential experimental tool for studying the relationship between amino acid sequence and protein structure and function. Although many methods can be used to gain structural information about a protein, at present high quality x-ray diffraction of crystals gives the best information of
the protein structure. On the basis of the three-dimensional structure of the native protein and precise knowledge of this mechanism of action, mutations to alter a particular property can be estimated and then tested. The knowledge gained in this way should eventually allow rational protein redesign (Bryan, 1987).

**Properties of glucoamylase**

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is an exo-acting glycoenzyme that catalyzes the hydrolysis of starch, glucooligosaccharides, and glycogen molecules from their nonreducing ends to produce β-D-glucose. It hydrolyzes primarily α-(1→4) glucosidic bonds, but also α-(1→6), α-(1→3), α-(1→2) and α,β-(1→1) glucosidic bonds (Pazur and Kleppe, 1962; Nikolov et al., 1989). Although glucoamylase hydrolyzes the α-(1→6) glucosidic bonds that initiate branches in starch much slower than it hydrolyzes the α-(1→4) glucosidic bonds in the starch backbone, its debranching capacity is high enough that glucoamylase can be used in the industrial production of high-glucose syrups (Lindeman and Rocchiccioli, 1979; Linko, 1987).

Although glucoamylase is known as a microbial enzyme, produced by many molds and yeasts, it has also been found in plants and animals. However, the most important source
is filamentous fungi, namely *Aspergillus* and *Rhizopus* (Manjunath et al., 1983; Saha and Zeikus, 1989).

There are many reports on the multiple forms of the enzyme, and many of these forms vary in their function (Pazur et al., 1971; Svensson et al., 1982; Itoh et al., 1987; Saha and Zeikus, 1989). The glucoamylases from *Aspergillus niger* and *Aspergillus awamori*, which have identical sequences (Svensson et al., 1983; Nunberg et al., 1984), exist in two forms, GAl and GA2 (Lineback et al., 1969; Pazur et al., 1971, 1980; Svensson et al., 1982). Amino acid sequence analysis of glucoamylase shows that GAl has 616 amino acids and three domains: a large catalytic domain comprising the first 440 amino acids at the N-terminus, a highly glycosylated region of 72 amino acids, and a starch-binding region of 104 amino acids at the C-terminus. The GA2 form has only the first two regions with 512 amino acids (Svensson et al., 1982). The presence of GA2 is caused by either size reduction due to limited proteolysis (Svensson et al., 1986), or by splicing out of an intervening sequence from GAl mRNA into a smaller mRNA coding for GA2 protein (Boel et al., 1984). The only functional difference between GAl and GA2 is that GAl has the ability to digest raw starch and GA2 does not (Svensson et al., 1982). Dalmia (1990, 1991) determined
that GA2 from *A. niger* adsorbs to raw starch granules nonspecifically with an affinity at least 50-fold lower than that of GA1. The amino acid sequence of GA1 led to molecular weights of the polypeptide moiety and the total GA1 molecule of 65,424 and 82,000, respectively (Svensson et al., 1983).

The glycosylated region of glucoamylase contains monomeric and oligosaccharide units composed of D-mannose as the dominant monosaccharide, with D-glucose and D-galactose in small amounts. These are linked mostly O-glycosidically to serine or threonine residues (Lineback et al., 1969; Pazur et al., 1971, 1980, 1987; Svensson et al., 1983; Gunnarson et al., 1984). The presence of mannosyl-serine and mannosyl-threonine linkages is very surprising since these linkages are very rare in glycoproteins (Gunnarson et al., 1984). Structural studies showed that the monosaccharide units are connected by (1→2) and (1→6) linkages, and that only one trisaccharide, which has also an unusual branching pattern for glycoproteins, was found. On the other hand, Pazur et al. (1980) determined the presence of many di-, tri-, tetrasaccharides joined by (1→3) and (1→6) glycosidic linkages. Pazur et al. (1987) suggested a random distribution of carbohydrate side chains along the
polypeptide chain, which may be responsible for the enzyme's high resistance to proteolysis and its high stability against heat inactivation and storage at cold temperature. However, the random distribution of carbohydrates was not verified by three-dimensional structure analysis of glucoamylase from *Aspergillus awamori* var. X100 (Aleshin et al., 1992). The theories proposing possible secretion and stabilization functions of the glycosylated region are supported by the studies showing that deletions in the glycosylated region significantly affect secretion and/or activity (Evans et al., 1990; Chen et al., 1991, Libby et al., 1991).

Glucoamylase is quite stable up to 60°C, with essentially no loss of activity in 30 minutes, but approximately 80% of the enzyme inactivates between 60°C and 70°C at pH 4.5 in 90 minutes (Lineback et al., 1969). It can be stored under refrigeration over a period of several months (Pazur and Ando, 1959). The enzyme is stable between pH 3 and pH 7 at low temperatures, and the optimal pH range is between 4 and 5 for many different substrates (Pazur and Ando, 1959; Hiromi et al., 1966; Lineback et al., 1969; Saha and Zeikus, 1989).

Hiromi et al. (1966) determined the presence of two essential carboxyl groups with pKₐ's for the substrate-
enzyme complex of 2.9 and 5.9 for both maltose and panose. For the free enzyme, pKₐ₁ was 1.9 for both of the substrates, while pKₐ₂ was 5.9 for maltose and 6.4 for panose. Savel’ev and Firsov (1982) also determined the ionization constants of the catalytic groups as 2.3 and 5.75 for the maltose-enzyme complex and 2.75 and 5.55 for the free enzyme. Both of these studies showed an optimal pH in the range of 4-4.5.

Numerous kinetic modelling, chemical modification, and mutagenesis studies have been performed on glucoamylase to understand the structure of the active site, to determine the roles of amino acid residues, to find the reaction mechanism, and to change many properties of the enzyme for more efficient industrial processes.

Kinetic and subsite mapping studies on glucoamylase from different sources show an active site consisting five to seven glucosyl binding loci called subsites, with the catalytic site between subsites 1 and 2 (Hiromi, 1970; Hiromi et al., 1973; Savel’ev et al., 1982; Tanaka et al., 1983; Koyama et al., 1984; Meagher et al., 1989). The catalytic efficiency of glucoamylase increases with increasing substrate length up to six glycosyl units (Abdullah et al., 1963; Meagher et al., 1989). The similar substrate affinities of those subsites for many
different glucoamylases suggests the same configuration and catalytic behavior in the active site of the enzyme (Hiromi et al., 1973; Savel'ev et al., 1982; Tanaka et al., 1983; Koyama et al., 1984; Meagher et al., 1989).

Amino acid sequences of glucoamylases from *A. niger* (Svensson et al., 1983), *A. awamori* (Nunberg et al., 1984), *S. cerevisiae* (Yamashita et al., 1985b), *S. diastitecticus* (Yamashita et al., 1985a), *R. oryzae* (Ashikari et al., 1986), *S. fibuligera* (Itoh et al., 1987), *A. awamori* var. *kawachi* (Hayashida et al., 1989), *Neurospora crassa* (Koh-Luar et al., 1989), *A. shirousami* (Shibuya et al., 1990), *A. oryzae* (Hata et al., 1991), *S. fibuligera* KZ (Hostinova et al., 1991), *Chalara paradoxa* (Monma and Kainuma, 1992), *Hormonicus resinae* (Joutsjoki and Torkelli, 1992), *Clostridium* species (Ohnishi et al., 1992), and *Humicola grisae* var. *thermoidea* (Berka et al., 1993) have been determined. Coutinho (1992) collected and aligned most of these glucoamylase sequences. Figure 1 shows the third conserved region, suggested by Itoh et al. (1987) and aligned by Coutinho (1992). The sequences from *A. niger* and *A. awamori* are exactly the same. Figure 2 shows the entire amino acid sequence of *A. awamori* glucoamylase.

Many chemical modification studies have shown that
Figure 2. The amino acid sequence of *A. niger* glucoamylase (Svensson et al., 1983). Symbols: © - catalytic residues, bold and underlined - mutated residues, * - O-glycosylated residues, --- - raw starch binding region.
essential carboxyl groups (Inokuchi et al., 1982; Savel’ev and Firsov, 1982; Svensson, 1988). Svensson et al. (1990) found by chemical modification that Asp 176, Glu179, and Glu180 were the only inhibitor-protected carboxylic groups in the entire sequence of A. niger glucoamylase. Sierks et al. (1990) determined by site-directed mutagenesis that Glu179 and Asp176 were the catalytic acid and base groups, respectively, while Glu180 contributed to the high pKₐ values of Glu179 and was also responsible for substrate binding. Finally, the elucidation of the three-dimensional structure of the glucoamylase from A. awamori var. X100 to a resolution of 2.2 Å showed that Asp400 is the general base catalyst instead of Asp176 and confirmed that Glu179 is the general acid catalyst (Harris et al., 1993).

The secondary structure consists of mainly α-helices, with the minor structure being β-sheets (Figure 3). The thirteen α-helixes involve residues 1-20, 53-68, 72-89, 125-144, 148-168, 186-205, 211-227, 245-254, 272-288, 318-338, 345-354, 368-391, and 416-429. Twelve of the α-helices are in an α/α-barrel form. Six mutually parallel α-helices in an inner core are connected to each other through a peripheral set of six α-helices. The peripheral helices are parallel to each other, but approximately
Figure 3. Schematic of the glucoamylase (containing 471 residues). α-Helices are cylinders, sites of O-glycosylation are represented by single hexagons, and sites of N-glycosylation are symbolized by chains of three hexagons.
antiparallel to the α-helices in the inner core. The active site is in the packing void of the inner core. Only 11% of all residues belong to β-sheet structures. Residues 21-23, 36-38, 49-52, 91-93, 96-97, 100, 173-176, 181-185, and 235-238 form three three-stranded antiparallel β-sheets. On the other hand, residues 109-110, 114-116, 340-344, 361-363, 400-403, and 406-410 form three two-stranded β-sheets. The structure has three disulfide links between residues 210 and 213, 262 and 270, and 22 and 249, and three cis-peptide linkages between residues Gly23 and Ala24, Asn45 and Pro46, and Arg122 and Pro123. Pro157 and Pro218 occur in the middle of separate α-helices, creating a modest kink in the helix. Ten O-glycosylation sites involving serine or threonine residues linked to a single mannosyl residue as a separate domain were determined. The O-glycosylated domain is in an extended conformation, wrapping around the "waist" of the α/α barrel, and there are two additional N-glycosylation sites close to the belt of O-glycosylation (Aleshin et al., 1992).

Explanation of the dissertation format

This dissertation contains two different pieces of work, the major Ph. D. project in the first part, and another piece of work in the second part that was performed first.
Since both of these projects deal with glucoamylase, the preceding section introduces both main parts, which are independent of each other. A general summary of both parts is given after the second part.
PART I

TAILORING THE pH DEPENDENCE OF *Aspergillus awamori* GLUCOAMYLASE BY MUTAGENESIS
INTRODUCTION

Introduction to genetic engineering

Genetic engineering, also known as recombinant DNA technology, creates new combinations of genetic material coming from different DNA molecules. In this process the foreign DNA is inserted into a vector DNA by covalent linkages. A vector is a DNA molecule that serves as a recipient or carrier for foreign DNA. Vectors are usually plasmid or phage DNA molecules that carry an origin of DNA replication to allow them to autonomously replicate, and genetic markers, so they can be detected in host cells. Since the foreign DNA is joined covalently into the vector, it also replicates when the vector replicates itself. For covalent linkage of foreign DNA into the vector DNA, restriction endonucleases and DNA ligases are used. Restriction endonucleases recognize certain DNA sequences and make sequence-specific cuts in the double helix, leaving complementary single-stranded ends that are called sticky or cohesive ends or blunt ends, depending on whether the restriction endonuclease makes staggered or smooth cuts in the two chains. The foreign DNA is also removed from its original location with the same restriction endonuclease if the enzyme produces sticky ends, otherwise it is removed with any blunt-end producing
endonuclease. Then the two (or more) DNA fragments are ligated with the use of DNA ligase, which catalyzes the formation of new phosphodiester bonds. Afterwards, the plasmid DNA that has been formed is added into plasmid-free bacteria, generally \textit{E. coli}, in the presence of \textit{Ca}^{2+}, and the DNA is taken up to yield bacteria that will soon contain many copies of the plasmid. Since the plasmid also contains antibiotic resistance genes, the bacteria containing the plasmid can easily be selected. Then the inserted gene is replicated together with the vector during bacterial growth. If in some cases it is desirable that the foreign DNA be expressed in the host, the protein coded by this foreign DNA is synthesized by transcription and translation. If the inserted gene is not procaryotic but instead eucaryotic, then complementary DNA (cDNA) should be used in cloning, since eucaryotic genes have regions called introns whose structural information is not expressed but is lost during transcription. Since \textit{E. coli} cannot excise non-coding regions, it produces incorrect proteins from eucaryotic genes. However, since cDNA is produced from messenger RNA, it does not contain introns. Even if this cDNA is inserted via a suitable vector, bacteria generally cannot carry out post-translational modifications of eucaryotic proteins. For this reason
shuttle vectors, which are plasmids that can replicate both in *E. coli* and yeast cells, are used. Since yeast cells are eucaryotic, post-translational modifications can be achieved better.

Genetic engineering techniques are also used in protein engineering to prepare mutations at desired locations. In this way, many properties of proteins are changed to enhance their usefulness. Site-directed mutagenesis and cassette mutagenesis techniques are two ways of doing this. In the first, an oligodeoxynucleotide is synthesized that is complementary to a section of the gene containing the mutation. The oligodeoxynucleotide is then allowed to bind to the gene, and serves as a primer for the synthesis of a complementary strand of DNA by DNA polymerase. Then the circle is closed by DNA ligase and used to infect *E. coli*. Following this, the mutated DNA is selected by using the oligonucleotide as a probe. In cassette mutagenesis, two restriction endonuclease sites enclosing the mutagenized section are determined or created. A mutated oligonucleotide is synthesized between these two restriction sites. After removing the original cassette by restriction enzyme digestion, the mutated cassette is inserted with the help of DNA ligase. Finally the gene is expressed, and the product protein is studied after separation and purification steps.
Introduction to protein engineering of pH dependence of enzymes

Enzymes are protein molecules that catalyze reactions in biological systems. Even though these molecules are very large, only a small fraction of the molecule, the active site, is responsible for catalysis (Palmer, 1981). The amino acid residues in the active site might be widely separated in the primary structure but are brought together in space because of the twists and turns within the molecule. Only a few of the amino acids in the active site are responsible for substrate binding to form an enzyme-substrate complex, and only a few more are responsible for catalysis. Most of the amino acid residues in the active site do not function in binding or catalysis, but contribute to the specificity of the enzyme. The side chains must be of suitable size, shape, and character not to interfere with substrate binding, but they might interfere with the binding of other, chemically similar substances (Palmer, 1981). Since the active site contains a number of ionizable amino acid side chains in a cleft or crevice, it has its own microenvironment. Therefore the variation of many enzyme activities with pH in a way similar to simple acid and base ionization is not
surprising. Plots of rate versus pH take the form of simple single, or more generally double, ionization curves (Palmer, 1981; Stryer, 1981; Fersht, 1985).

Michaelis and Davidson in 1911 attempted to explain the characteristic bell-shaped rate versus pH curve obtained for many enzyme-catalyzed reactions. They proposed that the enzyme, which was assumed to be amphoteric, could exist in its acidic, basic, or isoelectric forms, and they suggested that it was this latter state of the enzyme that was catalytically active. Subsequent experimental studies in pH kinetics led Michaelis and Rothstein to propose in 1920 that it was the ionization state of the enzyme-substrate complex, rather than of the free enzyme, that caused changes in the rate of catalysis as pH is altered. However, it remained for Haldane (1930) some ten years later to suggest that it was the charge distribution associated with certain functional groups on the enzyme that was responsible for the observed alterations in the rates of enzyme catalysis induced by changes in hydrogen ion concentration.

In recent decades, tremendous progress has occurred in understanding protein structure and function. The recent advances in gene synthesis and genetic engineering have made it possible to construct any desired amino acid
mutation in proteins. By mutating any single amino acid residue and by using knowledge of the three-dimensional structure of the enzyme, which is obtained by X-ray crystallography, the function of that amino acid can be determined. With this knowledge, many properties of enzymes like substrate specificity, thermal and pH stability, activity, and optimal pH can be altered (Fox and Oxender, 1987). Therefore one of the goals of protein engineering is to alter the pH dependence of enzyme catalysis to optimize activity in industrial processes. Up to now many studies related to optimal pH's have been performed with glucoamylase (Sierks et al., 1990), and other enzymes like chymosin (Pitts and Mantafounis, 1990; Suzuki et al., 1990), human rennin (Yamauchi et al., 1988), subtilisin (Thomas et al., 1985; Russell et al., 1987), and pepsin and rhizopuspepsin (Lin et al., 1992).

Valenzuela and Bender (1971) showed that altering overall surface charge on an enzyme by extensive chemical modification can lead to significant changes in the pH dependence of catalysis. Rees (1980) investigated the effects of selective chemical modification of the surface lysine residues of cytochrome C to its redox potential. The consequent results were criticized by Rogers et al. (1985) because the bulky chemical reagent used for
modification could perturb the structure of the protein. The newer technique of site-directed mutagenesis avoids the problem of adding bulky groups to the side chains of amino acids, as a side chain may be replaced by an equal-sized or smaller one (Russell et al., 1987).

The enzyme chymosin (E.C. 3.4.23.4) is an aspartic proteinase that has been used extensively in cheese production since it initiates milk clotting. In chymosin, the principal catalytic residues are Asp32 and Asp215, and the enzyme has an acidic optimal pH. Human rennin, which is also an aspartic proteinase and is very similar to chymosin, differs that the residue equivalent to Asp304 in chymosin is Ala317. It is optimally active in the neutral pH range, and this residue has been implicated in affecting the optimal pH of catalysis. When Asp304 in chymosin B was substituted with Ala using site-directed mutagenesis, the pH activity profile shifted towards neutrality by 0.6 pH units, while the shape of the profile did not change (Pitts and Mantafounis, 1990). Yamauchi et al. (1988) performed a similar study with mammalian rennin. Ala317 was changed to Asp by site-directed mutagenesis, and the optimal pH shifted downward by 0.5 pH units. While the results proved the importance of this residue on the optimal pH of the two enzymes, the observed
shifts did not account completely for the higher optimal pH of rennin. Therefore this residue is not the only one responsible for determining the optimal pH (Yamauchi, 1988; Pitts and Mantafounis, 1990).

The effect of residue 304 to the optimal pH of chymosin was also studied by Suzuki et al. (1990). For this purpose, Asp304 was mutated individually to Ala and Glu. They originally expected that the loss of the negative charge of Asp304 as a result of replacement with Ala might cause an increase in the optimal pH as Pitts and Mantafounis (1990) found. They expected no change from the second mutation. However, both mutations yielded a similar shift of the optimal pH for proteolysis of acid-denatured hemoglobin toward the acidic side. Therefore, their results are contradictory to those of Pitts and Mantafounis (1990), and they explained them as probably being caused by the difference in substrates used. Suzuki et al. (1990) and Pitts and Mantafounis (1990) used acid-denatured hemoglobin and a synthetic peptide, respectively.

Dunn et al. (1987) compared pH dependence of hydrolysis of many aspartic proteinases, and reported that residue 220 in chymosin is located close to the second substrate residue and is occupied by Lys, whereas it is hydrophobic
(Leu, Phe, or Tyr) in all other related enzymes. They also showed that the activities for the cleavage by chymosin of peptides containing Glu at the second position increased at a higher pH. Taking these results into consideration, they postulated that an increased electrostatic interaction between Lys220 in chymosin and the COO\(^-\) of Glu at the second position of a substrate at a higher pH might facilitate cleavage of the substrate.

Suzuki et al. (1989, 1990) also investigated changes of optimal pH in chymosin. They produced Lys220→Leu based on the findings of Dunn et al. (1987), and determined that the optimal pH of the mutant for hydrolysis of acid-denatured hemoglobin was distinctly lower than that with the non-mutated enzyme. In contrast, no such shift by the mutation of the optimal pH for a synthetic hexapeptide containing Glu at the second position was observed.

The subtilisins are a family of extracellular serine proteases. His64 at the active site of the enzyme acts as a general base during catalysis, accepting a proton from the nucleophilic residue Ser221 as it forms a bond with the substrate carbonyl carbon. The enzyme is active at alkaline pHs when His64 is unprotonated, and catalytic activity varies with pH following the dissociation of this residue (Russell et al., 1987). A series of similar
studies was performed to change the optimal pH of the enzyme. The studies were based on results of chemical modification studies, which have shown that the pH dependence of catalysis alters with changes in the overall surface charge. Therefore, mutations were designed to alter the electrostatic environment of the active site and to change the pKₐ values of ionic catalytic groups. By using this knowledge and that of three-dimensional structure, Asp99 was mutated to Ser. No effect on either structure or catalytic properties of the enzyme other than electrostatic effects were expected from this mutation. Removal of the negative charge of Asp99 should destabilize the low-pH, positively charged form of His64 and so lower its pKₐ from the normal value of 7. The results showed that at high ionic strengths, electrostatic interactions were masked and essentially no effect on the catalytic properties that were observed. However, at low ionic strengths, the pKₐ value of His64 decreased from 7.17 to 6.88. Therefore, a significant effect on the pH dependence of the catalytic reaction was obtained with a modification of a single charge (Thomas et al., 1985; Russell et al., 1987).

The same group designed other mutations in order to investigate the effects of double mutation (Russell and
Fersht, 1987). The mutations constructed were Glu156->Lys, Asp99->Lys, either singly or together, and the Glu156->Ser/Asp99->Ser double mutation. Changing either Asp99 or Glu156 to Ser decreased the pKₐ by about 0.4 unit. Changing both simultaneously to give the double mutation with a change of two charge units lowered the pKₐ by 0.65 unit. Alternatively, making a double-charge change by mutating either Asp99 or Glu156 to Lys lowered the pKₐ of His64 by 0.6 unit. The quadruple-charge change in the double mutation to Lys gave a shift of 1.0 unit. These changes were approximately additive, and proved that the electrostatic charges are cumulative. By using different buffers at different ionic strengths and analyzing the results together with the three-dimensional structure, they showed that increasing the ionic strength of the phosphate buffer decreased the effect of the mutations. However, the effect of the Glu156->Lys mutation decreased much more rapidly with increasing phosphate concentration than did the other values. They speculated that this resulted from neutralization of the positive charge on Lys156 by the binding of phosphate dianion at higher ionic strengths. The use of different buffers did not affect the pKₐ values for Ser and Lys at position 99, but instead affected the pKₐ of the mutations at position
156. This was explained by citing the locations of the 99 and 156 residues. The latter is closer to the catalytic residue and there is only water between them. On the other hand, there is protein between the residue 99 and the catalytic group. Therefore, the positive charge on Lys156 tends to concentrate negative ions in the active site. This is far more noticeable for dianions than monoanions because there is twice the coulombic interaction energy between Lys156 and a dianion for binding, and the dianion when bound exerts twice as large a coulombic interaction energy with His64. The double Lys mutant is even more sensitive to salts (Russell and Fersht, 1987).

The pH dependence of the kinetic parameters of pepsin, rhizopuspepsin, and other active site hydrogen bond mutants has been determined by Lin et al. (1992). There is a 3 pH unit difference in the optimal pH of eucaryotic aspartic proteases and retroviral proteases even though their three-dimensional active site structures are very similar. They proposed that the difference in optimal pH may be the consequence of the hydrogen bonds to the active site aspartic acids that are present in the eucaryotic enzymes and absent in the retroviral enzymes. However, for both pepsin and rhizopuspepsin, the removal of hydrogen bonds to the active site carboxyls by mutagenesis
results in negligible changes in the pKₐ values for both free enzyme and enzyme-substrate complex. The mutations decreased k₉ₑₙₐₙ substantially and Kₐ slightly. Ido et al. (1991) studied the same phenomenon by adding a hydrogen bond to the active site of HIV-1 proteinase and succeeded in decreasing pKₐ values of the enzyme.

Sierks et al. (1990) studied the pH dependence of Aspergillus awamori glucoamylase catalysis as defined by mutagenesis of Asp176→Asn, Glu179→Gln, and Glu180→Gln. Asp176 and Glu179 appeared at the time to be the two residues responsible for catalysis, with Glu180 necessary for binding. In this study only the effects of mutations at positions 176 and 180 could be investigated, since glucoamylase with Gln179 did not have any detectable activity. The pKₐ values for the Glu180→Gln mutation decreased to 4.9 and 2.2 from the original values of 5.9 and 2.7, respectively. This result showed the electrostatic effect of Glu180 on the two catalytic residues. In contrast, the Asp176→Asn mutation affected pKₐ less strongly, changing it only to 5.3, than did the Glu180→Gln mutation, indicating that Asp176 has less effect than Glu180 on the general acid catalytic group. The pKₐ value of the Asp176→Asn mutation could not be determined because of irreversible denaturation. The
amino acid changes caused optimal pH to decrease from 4.5 to 4.0 for the Asp176→Asn mutation and to 3.0 for the Glu180→Gln mutation. Thus the cluster of acidic residues at the catalytic site creates a very highly charged environment, and removal of the acidic residues decreases the optimal pH.

Research objectives

The object of the main part of this research was to shift the optimal pH of glucoamylase with the help of mutagenesis. From the scientific point of view, the aim was just to alter the optimal pH either upward or downward with single-point mutations to check the validity of the theory on which the mutations are based on, and to have more information to design better mutations. On the other hand, from the industrial point of view, the aim was to increase the optimal pH of glucoamylase to use it simultaneously with other enzymes in starch saccharification and high-fructose corn syrup production. In the industrial starch hydrolysis process, glucoamylase is used together with pullulanase for faster hydrolysis and higher yields, but at low pullulanase activities, since the optimal pH of the latter lies in the region of 5-7 (Norman, 1979). Also, glucose isomerase, which is used in the conversion of glucose to fructose, has a neutral pH.
(Norman, 1979; Linko, 1987). If the optimal pH of the glucoamylase could be increased toward neutrality, it could be used with those enzymes simultaneously in the same batch. Another possible advantage of the simultaneous utilization of glucoamylase and glucose isomerase is to prevent the reversion reactions of glucoamylase by keeping the glucose concentration low, with the immediate conversion of the glucose to fructose in the reactor.

For this purpose, nine single amino acid mutations to change the electrostatic interactions in the active site were designed, based on the available information for this area. The absence of the three-dimensional structure of the enzyme forced us to design the mutations among those amino acid residues that are closest to the catalytic groups in primary sequence and are conserved in many glucoamylases from different sources. Even though the mutations designed were of three different types, the idea behind all of these mutations was the same, to change the pKₐ values of the general acid and general base catalyst groups either directly or indirectly to tailor the optimal pH of the enzyme.

*Type I*

Glu179 is the general acid catalyst of pKₐ 5.9. The adjacent Glu180 is likely to be negatively charged,
raising the pKₐ of the general acid catalyst, and participates in substrate binding in subsite 2. Asp176 was thought to be the catalytic base of pKₐ 2.7, which in its deprotonated form stabilizes the substrate transition state (Sierks et al., 1990). Therefore, Glu179 and Asp176 should be directly responsible for catalysis, and should be in undissociated and dissociated forms, respectively, in the active enzyme. If the pKₐ values of these groups could be changed, the optimal pH would shift. Glu and Asp have slightly different pKₐ values, with pure preparations of Glu and Asp solutions in water having pKₐ values of 4.3 and 3.9, respectively (Stryer, 1981). Therefore, changing the optimal pH might be possible by simply changing the three carboxylic acid residues from Glu to Asp or vice-versa. Based on these considerations, Glu179→Asp, Glu180→Asp, and Asp176→Glu mutations were designed.

The Glu179→Asp mutation should decrease the pKₐ value of the general acid catalyst as dissociation is increased. Since all the other dissociable groups are the same in the mutant and the wild-type enzyme, the lower pKₐ will be stabilized with a lower optimal pH. The other possible expectation of this mutation is a complete or partial loss due to possible steric influences. In other words, if the side chain of Asp cannot reach the substrate, the hyd-
The roltytic reaction cannot occur at all.

The Glu180→Asp mutation will also decrease the pKₐ of this group. The increased dissociation will increase the negative charge density around the catalytic groups and therefore decrease dissociation of the catalytically responsible groups, probably shifting the optimal pH to a higher value. On the other hand, a change in the activity is possible, if steric influences are important for this group due to its function in substrate binding.

At the time the mutations were designed, Asp176 was thought to be the general base group. With the determination of the three-dimensional structure, this group was identified as Glu400 instead (Aleshin et al., 1992). Based on the wrong assumption about Asp176, that this group should be in dissociated form, a higher optimal pH was expected, causing stabilization of the decrease in dissociation. Other possible effects expected were changes in the activity due to possible steric influences. Type II

The second category contains mutations that increase the negative charge density of the active site microenvironment by adding negatively charged amino acid residues in the vicinity of catalytically active groups. The decrease of the local pH in the active site will
decrease the dissociation of both general acid and general base groups. As a result, the optimal pH will shift to a higher value. The opposite effect has already been observed for the mutations Asp176→Asn and Glu180→Gln by Sierks et al. (1990), where a decrease in negative charge density decreased the optimal pH of the enzyme.

The mutations designed were Leu177→Asp, Trp178→Asp, Val181→Asp, and Asn182→Asp. In all these mutations, Asp was preferred to Glu because it has a higher ionization capacity, and because its smaller size decreases the possibility of perturbations in the structure of the protein. The main reason of selection for the positions 177, 178, 181, and 182 was their supposed close proximity to the catalytic site. Earlier, Sierks et al. (1993) chose the Leu177→His, Trp178→Arg, and Asn182→Ala mutations based on homologies between different glucoamylases from different sources. The Leu177→His mutation decreased both binding and the catalytic rates moderately, while Trp178→Arg decreased the catalytic rate substantially and affected the substrate selectivity. On the other hand, the Asn182→Ala mutation slightly lowered the catalytic rates without any other effect. Therefore, Asn182 seemed to be the best candidate to mutate, since the results indicated that this residue may have no important
function. On the other hand, the decreases in the reaction rates of Leu177→His and Trp178→Arg showed the possible functions of these amino acids in catalysis, substrate binding, or structural integrity. Therefore, these two mutations were designed just to determine the effect of extra negative charges added to positions 177 and 178 while expecting low but hopefully detectable activities. Obviously the mutants obtained would not be industrially superior ones, but they should show us whether this theory was correct or not. No information is available for residue 181 other than it is also in the conserved region, and appeared to be very close to the catalytic residues. Even if lower activities were obtained for these mutations, those results would give us some information about the possible functions of those amino acids, which would be also valuable by giving some more insight for new mutations to be designed. After determination of the effects of the mutations, multiple mutations could be designed to raise the optimal pH, since their effects will be cumulative.

Type III.

In the third category, two insertion mutations, 176.5Gly and 176.5Asp, were prepared by Professor P. A. Sullivan of Otago University during a visit to ISU.
Homology studies between glucoamylases, α-amylases, α-glucosidases, and transglucanohydrases from different sources (Svensson, 1988) were used to choose these mutants. A residue, usually Gly, exists between positions 176 and 177 in all of these enzymes except the glucoamylases. Therefore the insertion of a residue here can change the negative charge density in the catalytic region by insertion of an extra space, or by addition of a negative charge, in the case of 176.5Asp. Another possible effect may be total inactivation of the enzyme due to structural changes of the catalytic region.
MATERIALS AND METHODS

Genetic engineering of glucoamylase

All the mutants were prepared with the first 780-bp portion of the glucoamylase gene starting from the amino terminal, which was taken from plasmid pGAMSII by using the technique of cassette mutagenesis. The plasmid was prepared by Sierks (1988) by cloning the HindIII-BamHI cassette near the amino terminus of the glucoamylase gene from pGAC9 (Innis et al., 1985), that had been obtained from Cetus Corp. (Emeryville, Cal.) into the vector pBS(+), and creating some extra restriction enzyme sites in the glucoamylase gene by silent mutations at those sites. Sierks (1988) also prepared another plasmid, pGAMS10 (Figure 4), by removing the carboxyl HindIII site of pGAC9, which was used to express Glu179→Asp and Glu180→Asp mutant glucoamylases in S. cerevisiae.

Both plasmids, pGAMSII and pGAMS10, were produced in large scale by using E. coli TGI strain containing either plasmid, which was grown at 37°C in LB broth containing 87.5 mg/L ampicillin at 37°C (Sambrook et al., 1989). In order to increase the plasmid yield, the culture was selectively amplified by adding 120 mg/mL chloramphenicol to the partially grown culture and then incubating it at 37°C overnight with vigorous shaking. Afterward the
Figure 4. Schematic of the pGAMS10 vector.
culture was harvested by centrifugation, the cell walls and the outer membranes were broken down with 40 mg lysozyme in pH 8.0 Tris-Cl buffer and 16 mL 0.25 M pH 8.0 EDTA per 1 L of culture, and then the resulting sphero- lasts were lysed with 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS). After removing the debris and recovering the double-stranded DNA by precipitation with 2.5 volumes of 95% ethanol, plasmids were purified by equilibrium centrifugation in a cesium choride-ethidium bromide gradient. For every mL of DNA solution, 1 g of solid CsCl and 0.8 mg EtBr were added (Sambrook et al., 1989).

For Glu179→Asp and Glu180→Asp mutations the 780-bp fragment between amino HindIII and PstI sites in the glucoamylase gene containing the mutagenized region was cut and subcloned into the vector, pBS(+). For this purpose, the concentration of purified plasmid, pGAMSII, was determined spectrophotometrically at 260 nm, and then a known quantity was digested with the restriction enzymes HindIII and PstI at 37°C for 1-2 h. After isolation of the 780-bp DNA fragment with electrophoresis by using 1% agarose gel in TE buffer, this fragment was purified with the use of the Gene-Clean kit (Biol01). The vector was also cut with the same restriction enzymes that are available at the multiple cloning site, and the gluco-
amylase fragment was ligated into the vector by incubation at room temperature for 20 h in presence of the enzyme T4 ligase. The resulting recombinant plasmids were then used to transform the TG1 strain of *E. coli*. For this purpose competent TG1 cells were prepared by treating them with ice-cold CaCl₂ and MgCl₂ and heating them briefly in order to increase the efficiency of transformation. Then the transformed cells were plated on LB agar containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), a chromogenic substrate for β-galactosidase, to distinguish the plasmids containing inserted foreign DNA from those vector molecules that had recircularized without insertion of foreign DNA. The vector contains a fragment of the β-galactosidase gene at the multiple cloning site, and X-gal is a chromogenic substrate for β-glucosidase. Insertion of the DNA fragment into this polycloning site inhibits the production of β-galactosidase so that white colonies are formed; however, since β-galactosidase is produced with the vector not containing foreign genes, blue colonies are formed in the presence of the chromogenic substrate. Therefore the recombinant plasmid pUB1 was produced in large scale by isolation of a white colony and using the same protocol as used for pGAMS10.

All of the mutations were contained in a 48-bp-long
cassette between *HpaI* and *SnaBI* restriction sites, except for Asn182-Asp, which is in a 43-bp-long *HpaI*-*ApaI* cassette. Figures 5 and 6 show cassettes used in mutagenesis and the oligonucleotides prepared by the ISU Nucleic Acid Facility for all of the mutations.

The plasmid pUB1 was used in the preparation of mutants Glu179-Asp and Glu180-Asp mutations. It was first cut with *HpaI* and *SnaBI*, the wild-type cassette was removed with gel electrophoresis, and the vector, the rest of the plasmid, was purified with the Gene-Clean kit (Bio 101) as described before. Then the mutated cassette was prepared by annealing two complementary oligonucleotides containing the mutation that had been prepared at the ISU Nucleic Acid Facility. For this purpose, pure dry oligonucleotides were dissolved in water, concentrations were determined spectrophotometrically at 260 nm, and then 300 pmol of each strand were mixed in presence of the annealing buffer. After the mixture was held at 100°C in a heating block for 3 min, it was cooled slowly to room temperature. Finally the cloning was done by ligating the mutated cassette into pUB1, and transforming *E. coli* TG1 as described before. For the next step, plasmid DNA from many transformants was prepared in order to choose the right clone by restriction endonuclease digestion.
Figure 5. The SnaBI-HpaI and HpaI-ApaI cassettes used in the mutations.
MUTATION  OLIGONUCLEOTIDE CREATED BY ISU NUCLEIC ACID FACILITY

Asp176-Glu 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGAACCTGGGGAAAGATT-3′
                      5′-AACTTCTCCCAGAGTTCCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Leu177-Asp 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGATGATGAGAAGATT-3′
                      5′-AACTTCTCCCAGATCATCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Trp178-Asp 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGCTCTGAGAAGATT-3′
                      5′-AACTTCTCCCAGAGATCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Glu179-Asp 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGCTCCTGGGGATGAAGATT-3′
                      5′-AACTTCTCCCAGAGATCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Glu180-Asp 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGCTCCTGGGATGAAGATT-3′
                      5′-AACGCTTCCCAGAGATCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Val181-Asp 5′-GTAGCTCAATACTGGAGAACAGACAGGATATGCTCCTGGGAAAGAGAT-3′
                      5′-ATCTTCTCCCAGAGATCATATCCCTGCTCTGCTGGTCCAGTATTTGAGGCTAC-3′

Asn182-Asp 5′-GATGGCTCGCTTCTTTACGATTGCCTGTCGCAACACCGGGCC-3′
                      5′-CGGTTGTCACAGCAATCGTAAAGAGACGAGCCATC-3′

Figure 6. Single-stranded oligonucleotides prepared for the mutations by the ISU Nucleic Acid Facility.
Plasmid DNA was prepared according to a mini-plasmid preparation procedure (Sambrook et al., 1989). After harvesting 1.5 mL of an overnight culture, cells were lysed first with 0.8 mg/mL lysozyme in an 100 μL ice-cold solution of 500 mM glucose, 25 mM Tris·Cl (pH 8.0), and 10 mM EDTA (pH 8.0) and then with 200 μL of 0.2 N NaOH-1% SDS mixture. Then cell debris was precipitated by adding 150 μL of 3 M potassium acetate solution, proteins and organic material were extracted with an equal volumes of phenol-chloroform solution, and finally the plasmid DNA was precipitated with 2.5 volumes of 95% ethanol. After washing with 70% ethanol and drying, the DNA pellet was resuspended in 100 μL water, 4 μg/mL RNase was added to digest RNA, and the DNA was submitted to restriction endonuclease digestion. Since HpaI and SnaBI both produce blunt-ended fragments, their ends are compatible to each other. Therefore, the plasmids were cut with HpaI and SnaBI to see whether the orientation of the insert was correct or not, since HpaI and SnaBI sites could only be generated with the correctly oriented ligation. After determination of the positive clones, the single-stranded DNA was prepared from those colonies to check the mutation by sequencing. In single-stranded DNA preparation, M13 helper phage was used. After infecting the E. coli
culture with the helper phage, the culture was grown overnight in a rich medium containing 40 \( \mu g/mL \) ampicillin and 70 \( \mu g/mL \) kanamycin, which allows only phage-containing cells to grow. The single-stranded DNA secreted to the medium was separated from the cells by centrifugation and was precipitated with 20\% (v/v) polyethylene glycol 8000 in aqueous 1 M NaCl. After suspending the pellet in water, organic materials was extracted with phenol, then precipitated with ethanol and resuspended in water. The concentration of the prepared single-stranded DNA was determined electrophorethetically and given to the ISU Nucleic Acid Facility for sequencing. Once the mutation was confirmed by DNA sequencing, the remaining part of the glucoamylase gene was ligated to the mutated part. For this purpose the plasmid pRE1 containing the wild-type glucoamylase and pUB1 containing the mutation were each cut with \( PstI \). By doing so, pUB1 was opened to combine any DNA fragment having \( PstI \) sticky ends at both sides. The rest of the glucoamylase gene was separated from pRE1, which was isolated by gel electrophoresis and purified with Gene-Clean (Bio 101). This DNA fragment was ligated into pUB1, the plasmid was transformed into \( E. \ coli \), small-scale plasmids were prepared for a couple of colonies, and a positive clone was chosen by restriction
enzyme screening. Since in the correct clone the glucoamylase gene should be between two HindIII sites, the DNA was cut with HindIII, and the correct clone was determined by comparing the sizes of the DNA fragments obtained with those determined from the plasmid map. The last step in producing Glu179→Asp and Glu180→Asp mutations was the removal of the mutated part of the glucoamylase gene from pUB1 by cutting it with HindIII and BamHI and then ligating it into pGAMS10, from which the wild-type HindIII-BamHI fragment had already been removed by digestion with the same enzymes. This time the plasmids were checked for the presence of the restriction enzyme sites NotI and Sali, which were present in the mutated HindIII-BamHI fragment but not in the wild-type fragment, to confirm the presence of the mutation. Finally the mutant glucoamylase gene was transformed into S. cerevisiae to express the enzyme. For this purpose, an overnight culture of S. cerevisiae was soaked with 0.1 M lithium acetate to make the cell walls leaky, then the yeast was incubated with plasmid DNA, heat-shocked, and grown on leucine-deficient yeast minimal salts medium to enhance the growth of only plasmid-bearing yeast cells (Innis et al., 1985).

For the rest of the mutations the plasmid YEpm18,
which expresses much more glucoamylase than the plasmid pGAC9 (Cole et al., 1988), was used (Figure 7). In these mutations the same protocols were used; however, different restriction enzyme sites had to be used for cloning depending on the restriction enzyme sites available in the plasmid. All of the mutations were in the HpaI-SnaBI cassette except Asn182→Asp, which was in the Apal-HpaI cassette (Figure 5). The mutation at the position 182 destroyed the HpaI site, and this property was used to screen the recombinants for this mutant. To make the mutations, another plasmid, pUB2, was prepared by ligating the XhoI-BssHII fragment from YEpPM18 and BssHII-PstI fragment from pUBI, which together formed the first 780-bp part of the glucoamylase gene, into vector pBluescript-IIKS(+). Then the original cassettes were replaced by the mutated ones, screened, sequenced, and a three-part ligation was performed by inserting the mutated XhoI-PstI fragment and PstI-HindIII fragment containing the rest of the glucoamylase gene which had been cut from pREI into the YEpPM18 vector, from which wild-type glucoamylase gene had already been removed. An outline of the mutation protocol is shown in Figure 8. After confirming the mutations, the plasmids containing the mutations were transformed into yeast as usual. Frozen stocks of all the
Figure 7. Schematic of the YEpPM18 vector.
Figure 8. Schematics of the mutagenesis protocol: (a) for Glu179→Asp and Glu180→Asp mutations; (b) for Asp176→Glu, 176.5Gly, 176.5Asp, Leu177→Asp, Trp178→Asp, Val181→Asp, and Asn182→Asp mutations. Asterisks indicate mutated segments.
Figure 8. continued.
mutations in both *E. coli* and *S. cerevisiae* were prepared and stored at -80°C.

Before kinetic experiments, activities of all of the mutant and wild-type glucoamylases were checked by using a starch-plate technique. For this purpose, overnight liquid cultures were prepared in yeast minimal salts broth containing histidine on a 30°C shaker, cell densities were determined spectrophotometrically at 600 nm, and the same quantity of cells was plated for each type of glucoamylase on yeast minimal salts agar (Innis et al., 1985) containing 1% starch. After incubating 5 days at 30°C, the plates were incubated at 50°C overnight to maximize glucoamylase activity. An *S. cerevisiae* clone containing only the shuttle vector, but not the glucoamylase gene, was used as a negative control. Then the plates were exposed to iodine and halo diameters around the colonies were measured.

**Production and purification of mutant glucoamylases**

Mutant glucoamylases were produced by using a *S. cerevisiae* strain containing either mutated pGAMS10 or YEppM18. The four 1.5-L fermentations were performed in 3-L shake flasks containing yeast minimal salts medium without leucine for 120 h at 30°C. Glucoamylase activity was assayed using 1.8% soluble starch in 0.05 M acetate
buffer at pH 4.5 as a substrate and incubating at 35°C. The amount of glucose formed was estimated by the glucose oxidase method (Banks and Greenwood, 1971) by using the Sigma kit. This reaction also produces a stoichiometric amount of peroxide, which was subsequently reduced to water with the concomitant oxidation of o-dianisidine to an orange-colored product, which absorbs at 450 nm and whose concentration is directly proportional to the amount of glucose originally present. One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of glucose per minute at pH 4.5 and 35°C.

After fermentation, yeast cells were separated from the supernatant by means of centrifugation at 4000 x g, and then the supernatant was concentrated 50-fold and diafiltered with 0.5 M NaCl/0.1 M NaOAc buffer at pH 4.4 using an Amicon ultrafiltration unit with a molecular cutoff of 30 kDa. The protein concentration of the enzyme concentrate was calculated, and approximately 10 mg protein per mL column was applied to a 10 mm i.d. x 40 mm long acarbose-Sepharose affinity column, which had already been equilibrated with the same buffer. After loading at a flow rate of 0.75 mL/min, the column was rinsed with the same buffer until the eluent reached a low but constant absorbance at 280 nm, and then the bound enzyme was eluted
with 1.7 M Tris buffer at pH 7.6. The purified enzyme was immediately dialyzed against 0.005 M acetate buffer at pH 4.5 and freeze-dried both for storage and concentration purposes. Purity was checked by SDS-polyacrylamide gel electrophoresis before use in the kinetic experiments.

Kinetic experiments

Kinetic experiments were performed at fourteen to eighteen pH values ranging from 2.2 to 7.1 at 35°C, by using maltoheptaose (Sigma) as the substrate for the wild-type glucoamylase and all the mutants, and by using maltose (Sigma) only for wild-type glucoamylase. Citrate-phosphate buffer (McIlvane, 1921) was used to keep the pH constant; the ionic strengths of the buffer mixtures were maintained at 0.025 M by addition of potassium chloride. Reactions were performed in 2 mL liquid containing approximately 500 ng glucoamylase/mL solution. Protein concentrations of the enzyme solutions were determined by bicinchoninic acid protein assay (Smith et al., 1985) by using the Pierce reagent kit. Six samples were taken at regular time intervals during the reaction, and the reaction was stopped by addition of pH 7.0 Tris–chloride to a final concentration of 1.1 M. Then glucose concentration in the samples were determined in the quenched reaction mixtures by the glucose oxidase method using the Sigma kit.
(Banks and Greenwood, 1971). One unit of enzyme is defined as the amount of the enzyme required to produce 1 μmol/min under the assay conditions. All the experiments were duplicated and averages were used. The pH values of the reaction mixtures were checked before and after the reactions.

For each pH, reaction rates were determined at ten different concentrations, ranging from approximately 0.01 $K_m$ to 5 $K_m$, and these data were fitted to Michaelis-Menten kinetics by using non-linear regression. Then $k_{cat}$ values were calculated by dividing $V_\max$ by the molar enzyme concentration. A molecular weight of 95,000 rather than 85,000 was used, since $S. \text{cerevisiae}$ glycosylates glucoamylase to a greater extent than $A. \text{niger}$ (Innis et al., 1985). The larger molecular weight of $S. \text{cerevisiae}$-produced glucoamylases were also observed by SDS-gel electrophoresis.

After determination of the kinetic constants at different pH values, both log $k_{cat}$ vs. pH and log $k_{cat}/K_m$ vs. pH plots were prepared to observe the optimal pH ranges and to obtain $pK_a$ values of free and substrate-complexed forms of the enzymes. $K_a$ values were calculated by non-linear regression of the log $k_{cat}$ and log ($k_{cat}/K_m$) vs. pH plots with the equations
\[ \log k_{\text{cat}} = \log k_{\text{cat}0} - \log \left( 1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]^2} \right) \]  \hspace{1cm} (1) \\

and

\[ \log \left( \frac{k_{\text{cat}}}{K_H} \right) = \log \left( \frac{k_{\text{cat}}}{K_{H0}} \right) - \log \left( 1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]^2} \right) \]  \hspace{1cm} (2)

where \([H^+]\) is the hydrogen ion concentration and \(k_{\text{cat}}\) and \(k_{\text{cat}} / K_{H0}\) are maximal values, respectively (Dixon et al., 1979). Finally, to determine effects of the mutations on substrate–enzyme interactions, the change in activation energy for maltoheptaose hydrolysis calculations were performed from \(k_{\text{cat}} / K_H\) values of all mutations except 176.5Asp and Glu179→Asp by using equation 3 (Wilkinson et al., 1983; Fersht et al., 1985; Street et al., 1986)

\[ \Delta (\Delta G) = -RT \ln \frac{(k_{\text{cat}} / K_H)_{\text{mut}}}{(k_{\text{cat}} / K_H)_{\text{wt}}} \]  \hspace{1cm} (3)

where \(\Delta G\) is the activation energy, \(R\) is the universal gas constant, and \(T\) is the absolute temperature.
RESULTS

After preparation of each mutant, their activities were first checked by using starch plates. In this technique, the size of the halos formed around the mutant colonies were measured and compared with the wild-type colonies. The diameters of the halos measured are given in Table 1. Although no halos were formed for the insertion mutations 176.5Asp and 176.5Gly, and very small ones were observed for the Trp178→Asp and Glu179→Asp mutations, kinetic experiments at the wild-type’s optimal pH were performed in order to check these results, since the starch plate technique was not a very accurate method.

The kinetic constants $k_{\text{cat}}$, $K_M$, and $k_{\text{cat}}/K_M$ were determined for all the mutations with maltoheptaose as substrate at 35°C. The same measurements were obtained for wild-type Saccharomyces cerevisiae glucoamylase at the same conditions used for the mutations with maltose and maltoheptaose, and both were compared with native A. awamori glucoamylase values measured earlier (Meagher et al., 1989) (Table 2).

For studying pH kinetics, $k_{\text{cat}}$, $K_M$, and $k_{\text{cat}}/K_M$ values were determined at different pH values, and then log $k_{\text{cat}}$ and log $k_{\text{cat}}/K_M$ vs. pH plots were prepared to determine the $pK_a$ values and optimal pH’s of complexed and free forms of
Table 1. Halo sizes of mutant and wild-type glucoamylases on starch plates.

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Diameter (mm)</th>
<th>Relative diameter*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>Asp176→Glu</td>
<td>8</td>
<td>57</td>
</tr>
<tr>
<td>Glu179→Asp</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Glu180→Asp</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Leu177→Asp</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Trp178→Asp</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Val181→Asp</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>Asn182→Asp</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>Gly176.5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Asp176.5</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Colony diameter of 4 mm subtracted from both wild-type and mutant halo diameters to obtain relative diameters.
Table 2. Kinetic parameters for hydrolysis of maltoheptaose by wild-type and mutant glucoamylases at pH 4.5 and 35°C.

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$k_{cat}$ (s(^{-1}))</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s(^{-1})mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native(^a)</td>
<td>5.0±0.2(^a)</td>
<td>1.20±0.14</td>
<td>4.2</td>
</tr>
<tr>
<td>Native(^b)</td>
<td>34±1</td>
<td>0.080±0.007</td>
<td>425</td>
</tr>
<tr>
<td>Wild-type(^c)</td>
<td>8.0±0.9</td>
<td>0.90±0.08</td>
<td>8.9</td>
</tr>
<tr>
<td>Wild-type</td>
<td>40±5</td>
<td>0.092±0.014</td>
<td>435</td>
</tr>
<tr>
<td>Asp176-Glu</td>
<td>35±2</td>
<td>0.30±0.04</td>
<td>117</td>
</tr>
<tr>
<td>Glu179-Asp</td>
<td>0.0029±0.0006</td>
<td>0.094±0.001</td>
<td>0.031</td>
</tr>
<tr>
<td>Glu180-Asp</td>
<td>23±3</td>
<td>0.46±0.05</td>
<td>50</td>
</tr>
<tr>
<td>Leu177-Asp</td>
<td>8.1±0.9</td>
<td>0.094±0.018</td>
<td>86</td>
</tr>
<tr>
<td>Trp178-Asp</td>
<td>0.054±0.005</td>
<td>0.43±0.03</td>
<td>0.126</td>
</tr>
<tr>
<td>Val181-Asp</td>
<td>19±4</td>
<td>0.13±0.04</td>
<td>146</td>
</tr>
<tr>
<td>Asn182-Asp</td>
<td>22±3</td>
<td>0.082±0.009</td>
<td>268</td>
</tr>
<tr>
<td>176.5Asp</td>
<td>$3.2 \times 10^{-6} \pm 0.1 \times 10^{-6}$</td>
<td>0.21±0.03</td>
<td>$1.5 \times 10^{-8}$</td>
</tr>
<tr>
<td>176.5Gly</td>
<td>$\approx 0$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Aspergillus awamori glucoamylase (Meagher et al., 1989), with maltose.

\(^b\) Aspergillus awamori glucoamylase (Meagher et al., 1989).

\(^c\) with maltose.

\(^*\) standard deviation.
each enzyme. Based on both starch plate and kinetic results, no pH kinetic analyses were performed with the Trp178→Asp, Glu179→Asp, and insertion mutations due to either very low or undetectable activities.

Values of pK_{a1}, pK_{a2}, and pH_{opt} of free enzyme and enzyme-substrate complexes of wild-type *S. cerevisiae* for both maltose and maltoheptaose substrates, along with the same values for mutant glucoamylases with maltoheptaose substrate, are summarized in Table 3.

Values of pK_{a1} of 2.7 and 2.6 for free enzyme and 2.4 and 2.7 for substrate-enzyme complexes were determined for maltose and maltoheptaose hydrolysis by wild-type glucoamylase. pK_{a2} values were 5.8 and 6.3 for free enzyme and 6.0 and 6.7 for enzyme-substrate complexes for maltose and maltoheptaose hydrolysis. Correspondingly, the optimal pH was 4.2 for both free and complexed forms for maltose hydrolysis, while values of 4.5 and 4.7 were obtained for free and complexed forms for maltoheptaose hydrolysis (Figure 10). Generally, the pK_{a} and optimal pH values were higher for maltoheptaose than for maltose hydrolysis.

The Asp176→Glu mutation decreased k_{cat} 13% and increased K_{m} about threefold (Table 2). Accordingly k_{cat}/K_{m} was about one-quarter of the original value of the wild-type enzyme.

The effect of the Asp176→Glu mutation on the pK_{a} values
Table 3. $pK_a$ values and optimal pH of mutant and wild-type glucoamylases for hydrolysis of maltoheptaose at 35°C

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$pK_{a1}$</th>
<th>$pK_{a2}$</th>
<th>$pH_{opt}$</th>
<th>$pK_{a1}$</th>
<th>$pK_{a2}$</th>
<th>$pH_{opt}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type$^*$</td>
<td>2.7</td>
<td>5.8</td>
<td>4.2</td>
<td>2.4</td>
<td>6.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>2.6</td>
<td>6.3</td>
<td>4.5</td>
<td>2.7</td>
<td>6.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Asp176→Glu</td>
<td>2.7</td>
<td>6.3</td>
<td>4.5</td>
<td>2.5</td>
<td>7.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Glu180→Asp</td>
<td>2.4</td>
<td>6.3</td>
<td>4.3</td>
<td>2.4</td>
<td>6.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Leu177→Asp</td>
<td>2.7</td>
<td>5.9</td>
<td>4.3</td>
<td>2.4</td>
<td>6.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Val181→Asp</td>
<td>2.9</td>
<td>5.9</td>
<td>4.4</td>
<td>2.7</td>
<td>6.4</td>
<td>4.6</td>
</tr>
<tr>
<td>Asn182→Asp</td>
<td>2.6</td>
<td>6.2</td>
<td>4.4</td>
<td>2.4</td>
<td>6.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

$^*$ with maltose.
Figure 10. Effect of pH on $k_{cat}$ (s$^{-1}$) (•) and $k_{cat}/K_M$ (mM$^{-1}$s$^{-1}$) (□) at 35°C for maltose (open symbols) and maltoheptaose (closed symbols) hydrolysis by wild-type glucoamylase.
and pH\textsubscript{opt} is given in Figure 11. The pK\textsubscript{a1}, pK\textsubscript{a2} and optimal pH values of the free enzyme were not affected by this mutation. On the other hand, pK\textsubscript{a1} decreased 0.2 unit, while pK\textsubscript{a2} and the optimal pH increased 0.5 unit and 0.1 unit, respectively, for the enzyme-substrate complex.

The Glu179→Asp mutation affected only k\textsubscript{cat}, while leaving K\textsubscript{m} unchanged. A 99.99% decrease in k\textsubscript{cat} and k\textsubscript{cat}/K\textsubscript{m} occurred. These results verified the catalytic function of this residue.

Compared to wild-type glucoamylase, the Glu180→Asp mutation decreased k\textsubscript{cat} about 40% and increased K\textsubscript{m} about fivefold. As a result of these changes k\textsubscript{cat}/K\textsubscript{m} decreased 89%. While pK\textsubscript{a2} value of the free enzyme was unchanged, and that of the enzyme-substrate complex increased 0.1 unit with the Glu180→Asp mutation, pK\textsubscript{a1} was 0.2 unit lower for the free enzyme and 0.3 unit lower for the enzyme-substrate complex, respectively. As a result, the optimal pH decreased 0.2 and 0.1 unit for the free and complexed forms of the enzyme, respectively (Figure 12).

The Leu177→Asp mutation decreased k\textsubscript{cat} 80%; however, it did not affect K\textsubscript{m}. Accordingly k\textsubscript{cat}/K\textsubscript{m} decreased 80%. In addition, pK\textsubscript{a1} increased 0.1 and decreased 0.4 unit for free and complexed forms, respectively, and pK\textsubscript{a2} decreased 0.4 unit for the free enzyme and increased 0.1 unit for the
Figure 11. Effect of pH on $k_{cat}$ and $k_{cat}/K_M$ at 35°C for maltoheptaose hydrolysis by Asp176-Glu mutant glucoamylase. Units and symbols are as in Fig. 10.
Figure 12. Effect of pH on $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ at 35°C for maltoheptaose hydrolysis by Glu180→Asp mutant glucoamylase. Units and symbols are as in Fig. 10.
complexed form. The net effect of this mutation on the optimal pH were 0.1-0.2 unit decreases for both forms of the enzyme (Figure 13).

For the Trp178→Asp mutation, $k_{cat}$ decreased about 99.87% and $K_m$ increased almost five-fold. Accordingly $k_{cat}/K_m$ decreased 99.97%.

The value of $k_{cat}$ decreased about 50% and that of $K_m$ increased 50% for the Val181→Asp mutation. Therefore the decrease in $k_{cat}/K_m$ was 67%. For this mutation no change was observed for the $pK_a$ value of the free enzyme. Values of $pK_a$ and optimal pH of both forms and the value of $pK_a$ of the complex form all decreased about 0.1-0.4 unit (Figure 14).

For the Asn182→Asp mutation, $k_{cat}$ and $K_m$ decreased almost 45% and 11%, respectively. Accordingly $k_{cat}/K_m$ decreased 40%. The mutation decreased $pK_a$ 0.1 and 0.3 unit and decreased optimal pH 0.1 and 0.3 unit for the free and the enzyme substrate complexes, respectively. The values of $pK_a$ stayed unchanged for free enzyme and decreased 0.3 unit for the enzyme-substrate complex (Figure 15).

For Gly insertion between positions 176 and 177 (176.5Gly), kinetic constants could not be determined since the activity was totally lost. However, for the corresponding Asp insertion (176.5 Asp), $k_{cat}$ decreased 13 x
Figure 13. Effect of pH on $k_{cat}$ and $k_{cat}/K_M$ at 35°C for maltoheptaose hydrolysis by Leu177→Asp mutant glucoamylase. Units and symbols are as in Fig. 10.
Figure 14. Effect of pH on $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ at 35°C for maltoheptaose hydrolysis by Val181-Asp mutant glucoamylase. Units and symbols are as in Fig. 10.
Figure 15. Effect of pH on $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ at 35°C for maltoheptaose hydrolysis by Asn182→Asp mutant glucoamylase. Units and symbols are as in Fig. 10.
10^6 times, $K_m$ increased 2.5 times, and $k_{cat}/K_m$ decreased 30 x 10^6 times compared to wild-type glucoamylase with malto-heptaose as substrate.

Finally, by using the $k_{cat}/K_m$ values at three different pH values, 2.5, 4.5, and 6.5, activation energy changes for malto-heptaose hydrolysis were calculated (Table 4). Changes of pH did not significantly affect the binding strength of the substrate transition-state complex. The data suggested that hydrogen-bond breakage was not likely for the Asn182→Asp mutation, whereas one hydrogen-bond cleavage between an uncharged residue occurred for the Asp176→Glu, Leu177→Asp, Glu180→Asp, and Val181→Asp mutations. The calculations could be performed at only one pH for Trp178→Asp, and they indicated a possible hydrogen-bond cleavage from a charged residue.

Plots of $pK_m$ vs. pH for wild-type *S. cerevisiae* and other mutations are shown in Figure 16.
Table 4. Increases in activation energy of maltoheptaose hydrolysis at 35°C in mutant glucoamylases.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>pH 2.5</th>
<th>pH 4.5</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp176→Glu</td>
<td>1.6</td>
<td>3.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Glu180→Asp</td>
<td>4.7</td>
<td>5.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Leu177→Asp</td>
<td>4.1</td>
<td>4.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Trp178→Asp</td>
<td>-</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>Val181→Asp</td>
<td>3.5</td>
<td>2.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Asn182→Asp</td>
<td>0.8</td>
<td>1.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 16. $pK_a$ vs pH plots of glucoamylases for maltose (•) and maltoheptaose (●) hydrolysis at 35°C: (a) - wild-type, (b) - Asp176→Glu, (c) - Glu180→Asp, (d) - Leu177→Asp, (e) - Val181→Asp, (f) - Asn182→Asp.
DISCUSSION

The kinetic experiments performed for wild-type glucoamylase showed that expressing the enzyme in *S. cerevisiae* did not cause catalytically significant differences. Although the experimental and literature values of *k*ₐₚ and *K*ₚ are not exactly the same, they are comparable to each other (Meagher et al., 1989). In both cases, *k*ₐₚ is higher and *K*ₚ is lower for maltoheptaose than for maltose (Table 1). This result is also in agreement with the literature, in which the catalytic efficiency of glucoamylase improved with increasing substrate length (Meagher et al., 1989; Sierks et al., 1989).

Hiromi et al. (1966) determined p*K*ₐ values of free enzyme and enzyme-substrate complex as 1.9 and 2.9 for both maltose and panose for *Rhizopus delemar* glucoamylase. Corresponding values of p*K*ₐ for free enzyme and enzyme-substrate complex were both 5.9 for maltose and 5.9 and 6.4, respectively, for panose. The optimal pH was around 4.4. Later, Savel’ev and Firsov (1982) determined the dissociation constants of the catalytic groups for maltose hydrolysis as 2.3 and 5.8 for the enzyme-substrate complex and 2.8 and 5.8 for free *A. awamori* glucoamylase. The optimal pH was around 4.0. These values are comparable to the ones determined for wild-type glucoamylase in this
study, verifying similar expression of the enzyme by *S. cerevisiae*. Both pKₐ and the pHₜₒₚ values were slightly higher for maltoheptaose than for maltose, suggesting a slight influence of substrate length on the dissociations of the general acidic and basic catalytic groups. Hiromi et al. (1966) did not observe the same phenomenon, only that pKₐ of the panose-enzyme complex was higher than that of the maltose-enzyme complex. However, they worked with *R. delemar* glucoamylase and used a substrate shorter than maltoheptaose. The reason of this influence might be due to the changes in hydrogen bonds, electrostatic interactions, conformation of the enzyme-substrate complex, and/or charge density around the catalytic residues resulting from partial or complete filling of the funnel-shaped active site with substrates having different lengths. A similar effect of substrate length on pH dependence of the reaction has been observed with porcine pancreatic α-amylase (Ishikawa et al., 1991). This enzyme, which hydrolyzes α-D-(1,4) glucosidic bonds in starch, had maximal activity at pH 6.9 for substrates when the fifth subsite of PPA was occupied by a glucosyl residue, but the optimal pH shifted to 5.2 when this subsite was not occupied. Although the primary and crystal structures suggested that two carboxylic groups,
were the catalytic residues, to explain the substrate-dependent shift of the optimal pH Ishikawa et al. (1991) suggested a three-catalytic residue model containing an additional histidine residue, so that the active site could be switched from one pair of catalytic residues to another pair depending on whether subsite 5 was occupied. Human pancreatic α-amylase displays a similar substrate-dependent shift of the optimal pH (Ishikawa et al., 1990, 1991).

Before determining the values of the kinetic constants, $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$, of all the mutant glucoamylases, their activities were first checked by using starch plates. The method is not accurate enough for quantitative purposes, but was used here to gain a qualitative feel for the effect of glucoamylase mutations on activity. The same quantity of cells was plated for each type of glucoamylase. By assuming no effect of mutations on glucoamylase diffusivity and stability, sizes of halos forming around colonies of mutant yeasts could be measured and compared with those forming around colonies producing wild-type glucoamylase. An *S. cerevisiae* clone containing only the shuttle vector but not glucoamylase was used as a negative control. No halos were obtained for the insertion mutations Asp176.5 and Gly176.5, and quite small
halos were observed for the Glu179→Asp and Trp178→Asp mutations. Because the starch plate method was not very sensitive, kinetic studies were performed at the optimal pH on these almost completely deactivated mutations anyway to determine the changes in their kinetic constants.

Design of the mutations were based on primary structure, homology, chemical modification, kinetic analysis, and mutagenesis studies. A three-dimensional model was not available at this time because glucoamylase had not been crystallized. However, after preparation of the mutants, glucoamylase crystals from A. awamori var. X100 were made and the three-dimensional model was obtained (Aleshin et al., 1992), so the positions, interactions and possible functions of the mutated residues could be determined with it. The spatial arrangement of the catalytic and mutated residues in the active site is given in Figure 17, while the stereo view of the same area is given in Figure 18. Figure 19 shows schematics of deoxynojirimycin-glucoamylase complex. Table 5 shows the approximate distances of the mutated residues from the catalytic groups. The three-dimensional structure showed the closest residue to the catalytic groups was Trp178, which was 8 Å away from them. Asp176, Leu177, and Glu180 were also close to the catalytic acid, but were further
Figure 17. Ball and stick model of the catalytic and mutated residues in the active site of glucoamylase from A. awamori var. X100.
Figure 18. Stereo view of the active site of glucoamylase from A. awamori var. X100. Mutated residues were shown as bold lines.
Figure 19. Schematic of the active site of glucoamylase from A. awamori var. X100. NOJ1 and NOJ2 are 1-deoxynojirimycin at the primary and secondary subsites respectively, • denotes water molecule (Harris et al., 1993).
Table 5. The approximate distances between the catalytic groups and the mutated residues in the active site.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Catalytic oxygen of Glu179 (Å)</th>
<th>Catalytic oxygen of Glu400 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp176</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Glu179</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Glu180</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Leu177</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Trp178</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Val181</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Asn182</td>
<td>13</td>
<td>20</td>
</tr>
</tbody>
</table>
from the catalytic base. Val181 and Asn182 were the most distant residues, especially from the catalytic base. This does not mean that these mutations cannot affect dissociations of the catalytic groups, since many other studies have demonstrated the possibility of effecting dissociations of the catalytic groups by changing surface charges, even if they are very far from the active site (Valenzuela and Bender, 1971; Rees, 1980; Rogers et al., 1985).

A dramatic decrease was observed for $k_{\text{cat}}$ of the Glu179$\rightarrow$Asp mutation. However, its $K_m$ was the same as that of wild-type glucoamylase (Table 2). The large decrease in $k_{\text{cat}}$ but not $K_m$ upon mutation again shows the catalytic function of this residue. Sierks et al. (1990) obtained similar results for the Glu179$\rightarrow$Gln mutation, and similar results were reported upon mutation of the general acid catalyst of other carbohydrases (Anand et al., 1988; Bader et al., 1988). The three-dimensional structural model of the native glucoamylase could not verify the acid catalyst function of Glu179 due to the presence of two strong hydrogen bonds between Glu179 and Trp120 and Trp52, and another hydrogen bond linking Glu179 with Gln124, although it is not as strong as the other two bonds, which is not very common for general acid catalyst groups. However,
the structural information obtained with inhibitor-enzyme complexes did indeed verify that Glu179 is the general acid catalyst (Harris et al., 1993). The side chain of Gln124 makes two other hydrogen bonds with Arg54, which in turn has two hydrogen bonds to Asp126 (Figure 20). Therefore the presence of these three hydrogen bonds suggests that the second function of Glu179 is to stabilize enzyme conformation, with one of its oxygens participating in three hydrogen bonds while the other oxygen is catalytically active.

The reason for the inactivation caused by the mutation of Glu179 to Asp must be related to the length and position of the mutated residue rather than to the slight change in chemical properties between the two residues, Asp being a more acidic group with a slightly lower pK. Since the only structural difference between them is that Glu is one methylene unit longer than Asp, the loss of activity probably means that the reactive group of Asp cannot reach the glycosidic bond to be hydrolyzed. The other possible reason may be related to the structural function of Glu179, in that the shorter length of Asp may not allow it to make the correct hydrogen bonds to stabilize the conformation.
Figure 20. Close proximity of Asp179 in the active site of glucoamylase from A. awamori var. X100. Broken lines show hydrogen bonds.
Although the purpose of this study was to determine the effects of the mutations on the pH dependence of the enzyme, this could not be done with the Glu179→Asp, Trp178→Asp, 176.5Gly, and 176.5Asp mutations. To do this, it was necessary to perform kinetic determinations at different pH's. Since these mutant glucoamylases had very low activities even at their optimal pH's, it would have been almost impossible to perform reactions at other pHs where reaction rates were even lower.

The effect of the Asp176→Glu mutation on $k_{cat}$ was not very large. It affected $K_m$ more than $k_{cat}$ (Table 2). Accordingly $k_{cat}/K_m$ was about one-quarter of the original value of the wild-type enzyme. When the mutations were designed, Asp176 appeared to be the general base group. However, the three-dimensional model for native glucoamylase showed that Asp176 is buried under the loop of the polypeptide chain extending from Tyr169 to Ser185 and, in addition, Asp176 and Glu179 are not on opposite sides of a cleft or pocket as would be consistent with their putative catalytic roles (Aleshin et al., 1992). This caused Aleshin et al. (1992) to conclude that either Asp176 and the entire loop from residues 169 to 185 undergoes a major conformational displacement in response to enzyme—
substrate interactions, or that the assignment of Asp176 and/or Glu179 as the catalytic base and acid, respect­tively, was incorrect. Later the three-dimensional models of inhibitor-enzyme complexes clearly identified Glu400 as the catalytic base group instead of Asp176 (Harris et al., 1993).

According to the three-dimensional structure, Asp176 makes three hydrogen bonds with Glu180, Val181, and Trp178 (Figure 21). Although this residue does not have a direct function for either catalysis or substrate binding, it affects structural integrity of the active site. Even though this residue was mutated to one with similar properties, probably the longer length of Glu partly prevented formation of the same hydrogen bonding, resulting in lower glucoamylase activities. Previously Sierks et al. (1990) prepared the Asp176→Asn mutation and obtained a three- to fourfold increase in $K_w$ and a ten- to twentyfold decrease in $k_{cat}$ for different substrates. Since kinetic parameters of the Asp176→Asn mutation varied little with substrate length, Asp176 appeared to be in or near subsite 1 and this, along with subsite mapping studies, led to its assignment as the catalytic base. The effect of pH on the kinetic parameters of maltose hydrolysis by Asp176→Asn was determined by Sierks et al.
Figure 21. Close proximity of Asp176 in the active site of glucoamylase from A. awamori var. X100.

Broken lines show hydrogen bonds.
This mutation decreased $pK_{a2}$ and optimal pH from 5.9 to 5.3 and from 4.5 to 4.0, respectively, and they explained these decreases as due to the removal of negative charges from the region. Based on the findings of Sierks et al. (1990) a higher $pH_{opt}$ was expected for the Asp176→Glu mutation, since the slight decrease in dissociation of the general base catalyst (Asp176 at that time) was expected to be stabilized with a slightly higher $pH_{opt}$. The effect of Asp176→Glu mutation on $pK_a$ and $pH_{opt}$ is given both in Table 3 and Figure 11. Slight changes in $pK_{a1}$ and $pK_{a2}$ and no change in optimal pH were observed for the free enzyme. However, the $pK_{a1}$ of the enzyme-substrate complex decreased 0.2 unit, while $pK_{a2}$ and the optimal pH increased 0.5 unit and 0.1 unit, respectively.

Compared to wild-type glucoamylase, the Glu180→Asp mutation decreased $k_{cat}$ moderately, but its effect on $K_m$ was larger (Table 2). The Glu180→Gln mutation, prepared by Sierks et al. (1990), led to a fortyfold increase in $K_m$ and a threefold decrease in $k_{cat}$, and they explained this result as being caused by the involvement of Glu180 in formation of the enzyme-substrate complex. The smaller increase in $K_m$ for the Glu180→Asp mutation may be related to the less dramatic substitution with Asp than Gln.

From the three-dimensional structure analysis of the
native glucoamylase (Fig. 22), we can observe that the Glu180 residue hydrogen-bonds with Arg241 and Tyr306, while these two residues also hydrogen bond with each other, therefore producing a triangular hydrogen bond network. The side chain of Glu180 also interacts with the 3-OH group of the second glucosyl unit.

While $pK_a$ values of free enzyme and the enzyme-substrate complex were almost constant with the Glu180-$\text{Asp}$ mutation, $pK_{a1}$ was 0.2 unit lower for the free enzyme and 0.3 unit lower for the enzyme-substrate complex, respectively. As a result, the optimal pH decreased 0.2 and 0.1 unit for the free and complexed forms of the enzyme (Table 3 and Figure 12). Sierks et al. (1990) observed a change in both of the $pK_a$ values of the Glu180-$\text{Gln}$ mutation, with the $pK_{a1}$ shifting from 5.9 to 4.9 and $pK_{a2}$ decreasing from 2.7 to 2.2. Therefore they concluded that Glu180 affected both the catalytic residues. The results also showed the effect of this residue on both of the catalytic groups. An increase in the higher $pK_a$ of the mutant glucoamylase was expected, but a slight net decrease in optimal pH was obtained instead. The reason for this slight shift due to Glu180-$\text{Asp}$ mutation may be partly due to the small difference in the $pK_a$'s of glutamic and aspartic acids, and the changes of $pK_a$'s in the opposite direction may be
Figure 22. Close proximity of Glu180 in the active site of glucoamylase from *A. awamori* var. X100.

Broken lines indicate hydrogen bonds.
partly the result of changes in dissociation of the catalytic groups as a result of differences in the bonding network that determines dissociations of the residues in this region.

The Leu177-Asp mutation decreased $k_{\text{cat}}$ substantially, but it did not affect $K_m$. This significant decrease in $k_{\text{cat}}$ shows the importance of the Leu177 residue in the active site. According to the three-dimensional structure, Leu177 is in a highly hydrophobic region surrounded by the residues Phe187, Ile253, Val191, Ala129, Leu130, Leu59, Trp417, and Trp178, while the side chain of Leu177 is in a plane parallel to planes formed by Phe187 and Trp178 (Figure 23). The spaces that the atoms occupy overlap each other and, although there is no space for anything else in this region, Asp can easily replace Leu.

Therefore, mutation of the hydrophobic residue, Leu, to a hydrophilic one, Asp, can easily affect the hydrophobic character of the region, changing the interactions and/or conformation and decreasing the catalytic efficiency. Leu177 is approximately 2.9 Å to Arg54 and 3.1 Å to Gln124, which have important roles in the structure of the active site. Asp177 more likely makes a salt link with Arg54 and this changes the position of Gln124 accordingly. Due to the positional change of Gln124, it may not
Figure 23. Close proximity of Leu177 in the active site of glucoamylase from A. awamori var. X100. Broken lines indicate hydrogen bonds.
hydrogen-bond to Glu179 as it does in the wild-type enzyme. As a result of the absence of the Gln124-Glu179 hydrogen bond, the position of the latter may change, causing a large decrease in the activity. The oxygen in the main peptide bond of Asp177 hydrogen-bonds with the third OH group in the non-reducing-end glucosyl residue of the substrate at a distance of 2.5 Å. Due to the interaction of substrate with Leu177 and Arg54 that is evident from the three-dimensional structure, a change in the $K_m$ is also to be expected. But probably the position of the oxygen atom in the peptide bond of 177 and the position of the Arg54 did not change enough to affect the substrate binding and therefore the $K_m$. Sierks et al. (1993) mutated Leu177 to His based on homology, and obtained more than a tenfold decrease of $k_{cat}$ for isomaltose and fivefold decrease for maltose and maltoheptaose compared to wild-type glucoamylase. $K_m$ values increased nearly 50% for both maltoheptaose and isomaltose and threefold for maltose hydrolysis. They interpreted the increases in the $K_m$ values to be caused by the involvement of this residue in substrate binding, and determined by energy calculations that the binding of the main chain of Leu177, which is lost in the Leu177-His mutation, is to the 3-OH group of the first glucosyl unit of substrate.
The Leu177→Asp mutation caused pK\textsubscript{a2} to increase 0.1 unit for the enzyme-substrate complex and to decrease 0.4 unit for the free enzyme. On the other hand, pK\textsubscript{a1} increased 0.1 and decreased 0.4 unit for both free and complexed forms. The optimal pH values were 0.2 and 0.1 unit lower for the two forms of the enzyme, even though higher values were expected (Figure 13 and Table 3). This mutation was designed based on primary structure, homology studies, and the kinetic results of an earlier Leu177→His mutation. Leu177 is very close to Asp176 and Glu179 in the primary sequence, and although it is in a conserved region, the Leu177→His mutation did not cause substantial changes in the kinetic constants. Leu as a non-polar residue can also increase the dissociation of Glu179. Replacement of a non-polar residue with a negatively charged residue may have different effects depending on the dissociation of Asp177 and interactions with other residues. The increase of pK\textsubscript{a2} for the enzyme-substrate complex can be explained by comparatively higher effect of Asp than Leu on the dissociation of Glu179, as expected. A decreased pK\textsubscript{a2} for the free enzyme suggests an influence of substrate binding on the charge density in the region.

For the Trp178→Asp mutation, a very large decrease in \(k_{cat}\) but a fivefold increase in \(K_m\) was observed (Table 2).
The three-dimensional structure shows that Trp178 is in a very hydrophobic region surrounded by Leu177, Trp417, Trp317, Leu219, Leu320, Leu250, Thr246, and Leu319, as shown in Figure 24. Trp178 forms a plane between two parallel planes made by Leu177 and Arg305 side chains, with distances of 4 Å to each plane, indicating probable hydrophobic interactions among these three residues. Both the three-dimensional structure and kinetic studies with the Trp178→Arg mutation showed Trp178 is in subsite 2 and the backbone oxygen hydrogen-bonds to the 3-OH group of second glucose unit of the substrate (Sierks et al., 1993). Therefore Trp178 is an important residue for both substrate binding and structural integrity.

The mutation of this hydrophobic residue to a hydrophilic one probably damages hydrophobic interactions and consequently changes the conformation of the region, and may prevent substrate binding and catalysis depending on the degree of disturbance produced by mutation. The significant increase of $K_w$ strongly correlates with conformational changes in the region, otherwise the peptide bond oxygen could bind to the substrate. On the other hand, the large decrease of $k_{cat}$ for both free and complex enzymes also verifies conformational changes in the catalytic region, since the residue does not have any
Figure 24. Close proximity of Trp178 in the active site of glucoamylase from A. awamori var. X100. Broken lines indicate hydrogen bonds.
direct catalytic function.

The Val181→Asp mutation affected both $k_{cat}$ and $K_m$ moderately (Table 2). As this is the first mutation of this residue, it could not be compared with previous work. Although the residue is in the conserved region and is only two residues in the primary structure from the general acid catalyst, three-dimensional structure analysis showed that it is not very close to the catalytic region, approximately 12 Å from Glu79 and about 16 Å from Asp400. Val181 is the only hydrophobic residue in a very hydrophilic region, and is in a two-stranded, antiparallel $\beta$-sheet with a hydrogen bond with Asp176. It probably prevents contact of the residues around it with one another by filling the space. Therefore, this residue probably has mostly a structural function (Figure 25).

The $pK_{a1}$ value of the free enzyme increased 0.3 unit and stayed unchanged for the enzyme-substrate complex. $pK_{a2}$ decreased 0.3 and 0.4 unit for free enzyme and enzyme-substrate complexes, respectively. As a result, slight decreases in the optimal pH values of free enzyme and enzyme-substrate complex were obtained from this mutation (Table 3, Figure 14). Therefore the expected result was not obtained for Val181→Asp, since the charge density in the region was not affected.
Figure 25. Close proximity of Val181 in the active site of glucoamylase from A. awamori var. X100.

Broken lines indicate hydrogen bonds.
The Asn182→Asp mutation was prepared based on the findings from the Asn182→Ala mutation, for which kinetic parameters very similar to wild-type glucoamylase were obtained (Sierks et al., 1993; Chen et al., 1993; Part II of this dissertation). Sierks et al. (personal communication, 1992) prepared two other mutations near Asn182, Gly183→Lys and Ser184→His. The Gly183→Lys mutation increased $k_{cat}$ about 14% and decreased $K_m$ 20%, while the Ser184→His mutation increased $k_{cat}$ 8% and decreased $K_m$ 35%. The results showed that both of the mutations lead to kinetically better enzymes. Therefore Asn182 should be a reasonably good residue to mutate to change the pH dependence of the enzyme without significantly decreasing its catalytic rate. The Asn182→Asp mutation decreased $k_{cat}$ almost 45% while leaving $K_m$ unchanged (Table 2). However, the Asn182→Asp mutation had much less activity than Asn182→Ala mutation. The three-dimensional model shows that Asn182 is in a two-stranded antiparallel $\beta$-sheet and its side-chain is partially directed to the solvent. It hydrogen-bonds to a water molecule and hydrophobically interacts with Tyr175 (Figure 26). No important function could be assigned to this residue, and it did not affect dissociations in the active site. Values of $pK_{a1}$, $pK_{a2}$, and optimal pH all decreased
Figure 26. Close proximity of Asn182 in the active site of glucoamylase from *A. awamori* var. X100.  
Broken lines indicate hydrogen bonds.
unit for enzyme-substrate complex. The $pK_a$ and the optimal pH values decreased 0.1 unit, while $pK_i$ value remained unchanged, for the free enzyme. Although the purpose for this mutation was to increase the $pK_a$ values by increasing the negative charge density in the catalytic region, the opposite results were obtained.

Svensson (1988) studied the amino acid sequences for many glucoamylases, $\alpha$-amylases, $\alpha$-glucosidases, and transglucanases from different sources, and found high homology in the catalytic regions of $\alpha$-amylases, $\alpha$-glucosidases, and two transglucanases (Figure 27). Although she obtained only modest homology scores for alignment of glucoamylases, she suggested that all of the listed starch- and oligosaccharide-degrading enzymes were structurally related. Based upon her suggestion, an interesting difference between glucoamylases and the other enzymes was observed. All of the enzymes except the glucoamylases contain an extra amino acid between the positions 176 and 177. Ten $\alpha$-amylases that cleave and form only $\alpha$-(1→4)-D-glucosidic bonds contain glycine here, while the other one contains alanine. All three $\alpha$-glucosidases, which are hydrolases that can to some extent cleave and synthesize all $\alpha$-linked-D-glucosyl bonds, contain glycine in this position, and three transglucan-
Figure 27. Homologies of glucoamylases, α-amylases, α-glucosidases, and transglucanosylses (Svensson, 1988). Numbers indicate amino acid numbers. Italics show β-strands. Enzyme codes are explained below.

<table>
<thead>
<tr>
<th>Code</th>
<th>Enzyme</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>αBli</td>
<td>α-amylase</td>
<td>Bacillus licheniformis</td>
</tr>
<tr>
<td>αBst</td>
<td>α-amylase</td>
<td>B. stearothermophilus</td>
</tr>
<tr>
<td>αBam</td>
<td>α-amylase</td>
<td>B. amyoliquifaciens</td>
</tr>
<tr>
<td>αBu</td>
<td>α-amylase</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>αShy</td>
<td>α-amylase</td>
<td>Streptomyces hygroscopicus</td>
</tr>
<tr>
<td>αBl1</td>
<td>α-amylase</td>
<td>Barley isozyme 1</td>
</tr>
<tr>
<td>αBl2</td>
<td>α-amylase</td>
<td>Barley isozyme 2</td>
</tr>
<tr>
<td>αDm</td>
<td>α-amylase</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>PPA</td>
<td>α-amylase</td>
<td>Pig pancreas (rat and mouse)</td>
</tr>
<tr>
<td>HAP/S</td>
<td>α-amylase</td>
<td>Human pancreas/saliva</td>
</tr>
<tr>
<td>TAA</td>
<td>α-amylase</td>
<td>Aspergillus oryzae (Taka-amylase A)</td>
</tr>
<tr>
<td>M</td>
<td>maltase</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>I</td>
<td>isomaltase</td>
<td>Rabbit intestine</td>
</tr>
<tr>
<td>S</td>
<td>sucrase</td>
<td>Rabbit intestine</td>
</tr>
<tr>
<td>gaAn</td>
<td>glucoamylase</td>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>gaRh</td>
<td>glucoamylase</td>
<td>Rhizopus oryzae</td>
</tr>
<tr>
<td>gaSd</td>
<td>glucoamylase</td>
<td>Saccharomyces cerevisiae (diastaticus)</td>
</tr>
<tr>
<td>CGT</td>
<td>cyclodextrin</td>
<td>B. macerans</td>
</tr>
<tr>
<td>AM</td>
<td>amylomaltase</td>
<td>Streptococcus pnemoniae</td>
</tr>
<tr>
<td>BE</td>
<td>branching enzyme</td>
<td>EC 2.4.1.18</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>1. αBli</td>
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<td></td>
</tr>
<tr>
<td>2. αBst</td>
<td>225-T T N I D G F R L D A V K H</td>
<td></td>
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<tr>
<td>3. αBam</td>
<td>222-E L S L D G F R I D A A K H</td>
<td></td>
</tr>
<tr>
<td>4. αBsSu</td>
<td>167-N D G A D G F R F D A A K H</td>
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<td>5. αShy</td>
<td>165-S L G V D G F R I D A A K H</td>
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<td>6. αB1</td>
<td>171-D L G F D A W R L D F A R G</td>
<td></td>
</tr>
<tr>
<td>7. αB2</td>
<td>170-D H R L D G W R F D F A K G</td>
<td></td>
</tr>
<tr>
<td>8. αDm</td>
<td>177-D L G V A G F R V D A A K H</td>
<td></td>
</tr>
<tr>
<td>9. PPA</td>
<td>188-D I G V A G F R L D A S K H</td>
<td></td>
</tr>
<tr>
<td>10. HAP/S</td>
<td>188-D I G V A G F R I D A S K H</td>
<td></td>
</tr>
<tr>
<td>11. TAA</td>
<td>197-N Y S I D G L R I D T V K H</td>
<td></td>
</tr>
<tr>
<td>13. I</td>
<td>496-E V N Y D G L W I D M N E V</td>
<td></td>
</tr>
<tr>
<td>15. gaAn</td>
<td>172-Q T G Y D - L W E E V N G S</td>
<td></td>
</tr>
<tr>
<td>17. gaSd</td>
<td>525-S S G F D - L W E E V N G M</td>
<td></td>
</tr>
<tr>
<td>18. CGT</td>
<td>220-G M G V D G I R F D A V K O</td>
<td></td>
</tr>
<tr>
<td>19. AM</td>
<td>286-F K I Y D I V R I D H F R G</td>
<td></td>
</tr>
<tr>
<td>20. BE</td>
<td>395-R F G I D A L R V D A V A S</td>
<td></td>
</tr>
</tbody>
</table>
osylases, which synthesize α-(1→6)-D-glucosidic bonds, contain glycine, isoleucine, or alanine here. Based on these observations, insertion mutations containing either Asp or Gly between the position 176 and 177 of *A. awamori* glucoamylase were designed. The idea behind these mutants was twofold. First, it was to examine the proposed homology between glucoamylase and the others by observing the effect of these two insertion mutations, and also to change the charge density by inserting an extra space, with either Gly or Asp, and an extra charge, with Asp, into the catalytic region.

For Gly insertion between positions 176 and 177 (176.5), kinetic constants could not be determined since the activity was totally lost. This was not very surprising, considering the possible important changes in the conformation of the active site. For the corresponding Asp insertion (176.5 Asp), kinetic constants were determined, although the activity was very low. This mutation affected *k_{cat}* much more than *K_{m}*, probably by altering the conformation to change the correct orientation of the catalytic residues. The reason for the complete loss of activity for the 176.5Gly but not the 176.5Asp insertion may be the loss of the main-chain flexibility that Gly imparts, since it has only a hydrogen atom as a side chain and therefore can adopt much a wider range of conformations than the other residues (Branden and

Since glucoamylase was almost totally deactivated by the insertions between Asp176 and Leu177, a question arose about the homology suggested for this region. Therefore, the secondary structures of glucoamylase and some of the other enzymes listed in Figure 38 were compared to determine whether any possible secondary structural homology existed in this region (MacGregor, 1988; Aleshin et al., 1992). Complete primary and secondary structure homologies between all the enzymes except glucoamylases was observed. All of the enzymes but glucoamylase contain a five-residue B-strand structure corresponding to positions 175-180 of \textit{A. awamori} glucoamylase. All the other \(\alpha\)-amylases and \(\alpha\)-glucosidases have the same primary structure. However, glucoamylase from \textit{A. awamori} var. X100 does not have a \(\beta\)-sheet structure in this region; instead, it has two four-residue \(\beta\)-strands comprising residues 173-176 and 181-184, which interact with each other to form a \(\beta\)-sheet. In addition, an \(\alpha\)-helix structure involving residues 186-205 starts one residue after the second \(\beta\)-strand. But the following secondary structure element in all of the enzymes is another \(\beta\)-strand starting about twenty residues farther from that \(\beta\)-strand. Therefore the secondary structure information along with the kinetic results suggests no structural homology in this region between glucoamylases and similar enzymes. For
this reason, insertion mutations are more likely to disrupt the structure of the active site of glucoamylase.

Finally, binding activation energies were calculated at three pH values from measured $k_{catt}/K_w$ values for all of the mutations except Glu179→Asp and Asp176.5. The reasons for not including these two mutations were that Glu179 had a catalytic function, while Asp176.5 caused substantial structural change. Oligosaccharides bind to the enzyme by hydrogen bonds to form transition-state substrate-enzyme complexes. The effect of a mutation on the binding strength of the substrate transition-state complex is reflected by the increase in activation energy for substrate hydrolysis ($\Delta(\Delta G)$), calculated from values of $k_{catt}/K_w$ for mutant and wild-type enzymes (Wilkinson et al., 1983). Typical energy losses resulting from elimination of a charged and uncharged groups that hydrogen-bond to the substrate are 15-20 kJ/mol and 2-6 kJ/mol, respectively (Fersht et al., 1985; Street et al., 1986). However, these calculations are rough approximations, since mutations may have further structural consequences superimposed on the energetics of hydrogen bonding.

It should be pointed out that the mutations had not been designed to examine enzyme-substrate binding interactions. For such a purpose, mutations should be performed on the residues that are involved in binding without changing the
conformation of the protein or the mode of binding of substrate. As a result, reasonably accurate values of the contribution of that bond to the strength of substrate binding can be obtained.

The changes in the activation energies were higher at pH 4.5 than at pHs 2.5 and 6.5 for Asp176→Glu, Glu180→Asp, and Asn182→Asp mutations (Table 4). Conversely, Leu177→Asp and Val181→Asp mutations gave maximal values at pH 6.5. The minimal values were at pH 2.5 for the Asp176→Glu, Leu177→Asp, and Glu180→Asp mutations. The Val181→Asp mutations gave the lowest value at pH 4.5, while the Asn182→Asp mutation gave equally low values at both pH 2.5 and pH 6.5. The largest change with respect to pH was obtained for the Asp176→Glu mutation. The 1.9 kJ/mol change between pH 2.5 and pH 4.5 for it is quite enough to indicate a loss of a hydrogen bond between substrate and enzyme at pH 4.5 compared to pH 2.5. For the other mutations the magnitudes of the changes were not sufficiently substantial to indicate any change in hydrogen bonding. The activation energy changes of maltoheptaose hydrolysis were reported as 5.6, 5.2, and 1.2 kJ/mol for the Leu177→His, Trp178→Arg, and Asn182→Ala mutations, respectively (Sierks et al., 1993). The first and the last ones were in agreement with those values of Leu177→Asp and Asn182→Asp mutations. However, a much smaller activation energy change
was reported for the Trp178→Arg mutation than for the Trp178→Asp mutation. The same group also reported activation energy changes for Asp176→Asn and Glu180→Gln mutations as 9.1 and 12.8 kJ/mol for maltoheptaose hydrolysis (Sierks et al., 1990). These values are again higher than the ones obtained for Asp176→Glu and Glu180→Asp mutations. The differences in activation energies should be related to structural changes created by different substituted residues.

Three of the mutated residues, Leu177, Trp178, and Glu180, hydrogen-bond to the substrate, while others are not directly involved in substrate binding. However, the major problem is conformational change of the active site caused by the mutations. Therefore, the activation energy changes cannot be related only to lost hydrogen binding to that residue, but must also be related to other binding changes to other residues.

Although the Trp178 peptide bond oxygen hydrogen-bonds to the substrate, the probable loss of this bond increased the binding energy enough to indicate loss of a charged hydrogen bond. This result suggests that the structural changes caused by the Trp178→Asp mutation resulted in at least one hydrogen bond loss from a charged residue or many hydrogen bond losses from uncharged residues, since the residue itself cannot participate in charged hydrogen bonding. One possibility may
be a formation of salt bridge between Asp178 and Arg305. Since both residues hydrogen-bond to the substrate, loss of both hydrogen bonds can give this high activation energy change.

The Asn182→Asp mutation did not change the binding energy enough to indicate the possibility of broken hydrogen bonds. This result agrees with the slight decrease of $K_m$ caused by the mutation.

The other mutations, Asp176→Glu, Leu177→Asp, Glu180→Asp, and Val181→Asp, have activation energy changes consistent with a loss of one uncharged hydrogen bond. Since Asp176 and Val181 do not form hydrogen bonds with the substrate, this suggests loss of a hydrogen bond from another residue by changing the enzyme structure. The hydrogen bond lost due to the Leu177→Asp mutation could be from this residue. However, the unchanged $K_m$ value for this mutation does not support any hydrogen bond loss. The activation energy increase from the Glu180→Asp mutation was the highest after that from the Trp178→Asp mutation, but was not high enough to indicate loss of a charged residue. Most probably Glu180 is in charged form but may hydrogen bond with the uncharged oxygen, if this mutation caused the loss of this bond.
CONCLUSIONS AND RECOMMENDATIONS

Nine single amino acid mutations were prepared in the catalytic region of *A. awamori* glucoamylase with the help of cassette mutagenesis, in order to change the pH characteristics of the enzyme and to assign functions to the mutated residues. The lack of knowledge of the three-dimensional structure of the enzyme while the mutations were designed forced us either to mutate the catalytically important groups or to design the mutations among the amino acid residues that are closest to these catalytic groups and are conserved in many glucoamylases from different sources.

Before characterization of mutant glucoamylases, kinetic analysis of wild-type glucoamylase produced by *S. cerevisiae* demonstrated that its kinetic constants and pH characteristics were comparable to those of the native glucoamylase. The results showed that expression of the native *A. awamori* glucoamylase by *S. cerevisiae* did not cause changes of kinetic properties. Slightly higher pKₐ values and optimal pH values occurred with maltoheptaose than with maltose, showing that the optimal pH of the enzyme is slightly substrate-dependent.

Three groups of mutations were constructed. The first group comprised Asp176→Glu, Glu179→Asp, and Glu180→Asp mutations. When these were designed, Asp176 and Glu179 were thought to be the general base and acid catalyst groups. With
the help of the later determined three-dimensional structure, Glu179 was reconfirmed as the catalytic acid but Asp400 was identified as the catalytic base instead of Asp176.

The kinetic results showed a very large decrease in $k_{cat}$ but no change in $K_w$ for Glu179→Asp mutation, reconfirming the catalytic function of this residue. On the other hand, Asp176→Glu and Glu180→Asp mutations caused a less dramatic reduction in activity. The increase of $K_w$ constants for these two mutations implies that they possibly are involved in substrate binding or in the structural integrity of the active site. In the second group, Leu177→Asp, Trp178→Asp, Val181→Asp, and Asn182→Asp mutations were prepared. The least active one among these mutations was Trp178→Asp, which caused a very large decrease in $k_{cat}$ and increase in $K_w$, showing the importance of this residue in the active site. The Leu177→Asp mutation did not affect $K_w$, but decreased $k_{cat}$ moderately. Although the last two mutations, Val181→Asp and Asn182→Asp, decreased $k_{cat}$ values moderately, the effect of these mutations on the $K_w$ was not as substantial, a slight increase occurring with the first $K_w$ and a slight decrease occurring with the second.

The insertion mutants Asp176.5 and Gly176.5, which were prepared based on homology studies of glucoamylases with α-amylases, α-glucosidases, and transglucosylases, inactivated
the enzyme almost totally. The secondary structure of the catalytic region of glucoamylase was compared with that of some of α-amylases, but no secondary structure homology was observed, though total homology was observed among α-amylases, suggesting that insertion of one amino acid into this position likely destroyed the active site conformation.

The effect of Glu179→Asp, Trp178→Asp, 176.5Asp, and 176.5Gly mutations on pH dependence could not be investigated due to their very low or undetectable activities. For the rest of the mutations, very slight changes were observed, and none of them increased the optimal pH. The general trend of all the mutations is to cause decreased or unchanged pK$_a$ values with increasing or unchanged pK$_a$ values, and these cause either a slight decrease or no change in the optimal pH.

Change of pH did not cause much change in Δ(ΔG) for any of the mutants (Table 5). Only the Trp178→Asp mutation gave a very large increase, 20 kJ/mol, suggesting at least one charged hydrogen bond was cleaved. The Asp176→Glu, Leu177→Asp, Glu180→Asp, and Val181→Asp mutations showed that one uncharged hydrogen bond cleavage was likely, while the Asn182→Asp mutation did not show any effect.

Therefore, we can conclude that except for Asn182 and to some extent Val181, the residues used in this study are not suitable for mutation to change pH characteristics, since
their catalytic, binding, or structural functions caused large changes in $k_{\text{cat}}$ and $K_w$ upon mutation. Although smaller changes of kinetic constants occurred when Val181 and Asn182 were mutated, addition of negative charges to these positions unfortunately did not affect catalytic group dissociation. The first reason for this should be related to the dissociations of the added residues when incorporated in the protein structure. Although Asp, a negatively charged residue when in pure solution, was the substituent in all the mutated positions except 179 and 180, it may not be negatively charged at all those positions, depending on the charge density and the interactions in the vicinity. Therefore the charge density may not change as expected. Secondly, introduction of a totally different residue may change many interactions between other residues in that area and cause very unpredictable changes in the pH of that microenvironment.

Although addition or removal of charged residues may affect the optimal pH, residues to be mutated should be analyzed very carefully for possible catalytic or structural functions before preparation of mutations. The availability of the three-dimensional structure of glucoamylase will allow the choosing of correct residues for such a purpose.

To tailor the pH dependence of glucoamylase (or any other enzyme), two approaches can be used. In the first, surface
residues can be substituted with charged ones. This approach is less risky since solvent-accessible, mobile residues are not likely to affect either stability, structure, or activity. In the second approach, candidate residues can be in the active site, but the possible functions of those residues should be investigated very carefully since those buried residues can greatly change structure, stability, and activity. As an example, Gln124 seems to be an important residue to affect the dissociation of Glu179 as explained in the discussion section, and a Gln124→Glu mutation may increase its pK\textsubscript{a}. Trp120 and Trp53 are also very close to Glu179, so substitution of these residues with His may decrease the pK\textsubscript{a} of Glu179. However, the functions of the residues to be mutated should be reviewed very carefully since they might have important functions, as does Trp120 (Sierks et al., 1989). On the other hand, this study can be continued by a deeper characterization of the present mutations. Transient-state kinetic analysis can be performed to understand the effects of the mutations on the transient enzyme-substrate complex formations. In addition, the structural effects of the mutations can be determined more accurately by preparation of the crystals of the mutants and determination of their three-dimensional structures. Other methods such as nuclear magnetic resonance and circular dichroism can also be applied.
to the mutants to determine the structural changes.

The results of this study also showed a slight influence of substrate length on pH dependence. This phenomenon can be studied further by kinetic experiments with series of substrates of different length. In addition, three-dimensional studies can be performed with substrate-enzyme complexes by using analogs and/or inhibitors having different lengths.
PART II
FERMENTATIVE PRODUCTION AND HYDROLYSIS KINETICS OF

Aspergillus awamori GLUCOAMYLASE
INTRODUCTION

Starch is a polysaccharide consisting of two components, a linear glucose polymer, amylose, which contains \(\alpha-(1\rightarrow4)\) glucosidic links, and a branched polymer, amylopectin, in which linear chains of \(\alpha-(1\rightarrow4)\) glucose residues are initiated by \(\alpha-(1\rightarrow6)\) glucosidic linkages. Glucoamylase can digest both \(\alpha-(1\rightarrow4)\) and \(\alpha-(1\rightarrow6)\) linkages to produce glucose but at different rates. Linear dextrins are rapidly converted to D-glucose, whereas \(\alpha-(1\rightarrow6)\) bonds at branch points are more resistant.

An undesirable characteristic of glucoamylase is its ability to polymerize glucose by "reversion" reactions, which are formally the reverse of hydrolysis. The reversion is accompanied by the elimination of water. The main products of reversion reactions are reported as maltose and isomaltose, although on prolonged incubation at high substrate concentrations other disaccharides can be identified in addition to higher polymers (Norman, 1979; Harada, 1984; Linko, 1987, Nikolov et al., 1989). The amount of reversion sugars formed is also dependent on the substrate concentration. Norman (1979) reported that at 30% and 10% dissolved solid concentrations, hydrolyzed starch contained 85%, and 95% glucose, respectively. Lloyd and Nelson (1984) showed that the reaction ap-
proaches an equilibrium state of about DX 87 at 60°C and 30% solids, regardless of whether starch or D-glucose is used as substrate. Nikolov et al. (1989) investigated the reversion products by incubating either GA1 and GA2 with different initial glucose concentrations at different temperatures and pH values. Isomaltose, isomaltotriose, kojibiose, nigerose, maltose, α,β-trehalose, panose, and isomaltotetraose, in the order of decreasing equilibrium concentration, were the only products formed, and the rates of production increased with increasing initial substrate concentration. Maltose was produced more rapidly but to a lower level than isomaltose. No difference in the activities of GA1 and GA2, and no large changes of equilibrium concentration with different pHs, temperatures, and initial glucose concentration were observed.

Debranching enzymes that cleave α-(1→6) glycosidic bonds are used together with glucoamylase for more efficient starch hydrolysis. The two debranching enzymes used for this purpose are pullulanase and isoamylase. Pullulanase has a higher optimal temperature and pH than isoamylase (Norman, 1979). Hurst (1970, cited in Linko, 1987) patented a process for obtaining up to 99.1% D-glucose from enzyme-liquefied starch by the combined use
of *Klebsiella pneumoniae* pullulanase and glucoamylase at 50°C and pH 6.0. The high pH necessary for sufficient activity of pullulanase was also claimed to minimize reverse reactions. Harada (1984) reported that DX 95.9 and 94.2 were obtained for a glucoamylase-isoamylase mixture and for glucoamylase alone, respectively, at 55°C and pH 4.5. According to Harada (1984), the optimal pH of isoamylase is significantly lower than that of pullulanase, coinciding with that of glucoamylase. In addition, the low operational pH minimizes microbial contamination during saccharification. On the other hand, pullulanase can be used at 60°C, the temperature commonly used in saccharification, and is effective in preventing contamination (Linko, 1987). An important disadvantage of isoamylase is its lower thermostability. Inactivation starts at 45°C, while heating for 10 min at 60°C and 65°C resulted in 95% decrease and complete loss of activity, respectively (Yokobayashi et al., 1970). This might be the reason for the low utilization of isoamylase in industry. Slominska and Maczynski (1985) reported that application of pullulanase in combination with glucoamylase decreased the saccharification time and allowed an increase of glucose yield and substrate concentration. Debranching enzymes, both isoamylase and pullulanase, are
used together with glucoamylase to enhance the digestibility of raw starch (Ueda et al., 1974; Ueda and Ohba, 1976). Wankhede and Ramteke (1982) reported almost a twofold acceleration of raw starch digestion by adding pullulanase to glucoamylase.

The most important use of glucoamylase is in the production of high-glucose syrups from starch. The glucose syrups produced are either used as is or are converted to other products like ethanol and high-fructose corn syrup. The idea of simultaneous hydrolysis of starch by glucoamylase and then fermentation to ethanol has led to research on the production of glucoamylase by yeast, especially *Saccharomyces cerevisiae* (Nam et al., 1988).

Innis et al. (1985) constructed a yeast expression plasmid to produce glucoamylase in *S. cerevisiae*. The intron-free *A. awamori* GAI gene was cloned between two *HindIII* restriction enzyme sites in the expression vector pAC1, which contains an *E. coli* origin of replication, the *bla* gene from pBR322, the yeast 2μ origin of replication, and a yeast LEU2 structural gene. These features of the plasmid permit autonomous replication and selection in *E. coli* and yeast. The vector also contains the promoter and termination regions of a yeast enolase, designated ENO1. They reported that glucoamylase was efficiently secreted
into the medium, permitting growth of the transformants on starch as the sole carbon source. The secreted enzyme was glycosylated through both O-linkages at levels comparable to the native *Aspergillus* enzyme.
RESEARCH OBJECTIVES

The main object of this part of the study was to determine the maximal glucose yield for dextrin hydrolysis of wild-type and the mutant Asn182→Ala A. awamori glucoamylase produced by S. cerevisiae fermentation. For this purpose A. awamori glucoamylase was produced fermentatively by using S. cerevisiae containing the glucoamylase gene in a plasmid. Fermentations were performed selectively for cells containing the glucoamylase gene by using a special but relatively poor medium. Although a real optimization study for glucoamylase production was not performed, we attempted to maximize the enzyme yield by keeping the fermentation broth at different glucose concentrations. In the hydrolysis experiments, either pullulanase and isoamylase were also used together with glucoamylase to test their effects on maximal glucose yield. The hydrolysis experiments were also performed with native A. awamori glucoamylase, in order to determine any hydrolytic activity differences with glucoamylase produced by S. cerevisiae.
EXPERIMENTAL

Glucoamylase was produced by using an *S. cerevisiae* strain containing an autonomously replicating plasmid, YE pGAC9, in which the glucoamylase gene is subcloned. The fermentation was performed in a 19-L fermentor containing yeast minimal salts medium without leucine for 72 h at 30°C and pH 4.5, and dissolved oxygen was kept constant at 80% saturation (Innis et al., 1985). pH was controlled by adding 0.5 N ammonium hydroxide. If ammonium sulfate was added during the fermentation, 0.5 N sodium hydroxide was used instead. Samples were taken and analyzed for glucose concentration, cell growth, and glucoamylase activity during the fermentation. Glucoamylase activity was assayed using 2% soluble starch in 0.05 M acetate buffer at pH 4.5 as a substrate and incubating at 50°C. The amount of glucose formed was estimated by the glucose oxidase method (Banks and Greenwood, 1971). One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of glucose per minute at pH 4.5 and 50°C. Glucose concentrations and cell densities were detected by means of a Beckman glucose analyzer and a spectrophotometer at 680 nm, respectively.

During purification, all operations were carried out at 4°C. After fermentation, yeast cells were separated from
the supernatant by means of an Amicon hollow-fiber filter with a pore diameter of 0.1 μm, then concentrated 50-fold by using another Amicon hollow-fiber filter with a molecular cut-off of 30 kDa. Then the concentrate was freeze-dried, resuspended into a smaller volume, dialyzed against 0.05 M citrate-phosphate buffer at pH 6.0, and applied to a 15 mm i.d. x 270 mm long DEAE-Fractogel column. Before loading, the column was equilibrated with the same buffer. The column was eluted at 0.5 mL/min with a linear gradient from pH 8 to pH 3 in the same buffer. The enzyme pool was concentrated by ultrafiltration, dialyzed against 0.5 M NaCl/0.1 M NaOAc buffer at pH 4.4, and applied to a pre-equilibrated acarbose-Sepharose affinity column (10 mm i.d. x 40 mm long). After loading, the column was rinsed with the same buffer until the eluent reached a low but constant absorbance at 280 nm. Afterwards the bound enzyme was eluted by 0.7 mL/min of 1.7 M Tris buffer at pH 7.6. The purified enzyme was concentrated, dialyzed against water, and freeze-dried. Most of the purification work was performed by Hsiu-Mei Chen.

In order to determine what fraction of the enzyme produced was secreted to the medium, yeast cells were suspended in 0.05 M acetate buffer at pH 4.5 and broken open by using a Gaulin homogenizer at a pressure of 500
kg/cm². Samples were taken at various time intervals until a constant glucoamylase activity was reached, and the solution was cooled to 4°C at those intervals.

Hydrolysis kinetics were determined for the wild-type and mutant Asn182-Ala glucoamylases produced by *S. cerevisiae* fermentation and for a commercial *A. awamori* glucoamylase used as a control. For each of these three glucoamylases, three sets of experiments were performed, one with glucoamylase only, one with glucoamylase and pullulanase, and one with glucoamylase and isoamylase. The enzyme concentrations were 4.5 IU/mL for each enzyme. The substrate was 28% (w/v) Maltrin 15 in 0.05 M acetate buffer at pH 4.5, and 0.02% sodium azide was used to inhibit microbial growth in the hydrolysis mixtures. Hydrolysis was performed at 35°C and pH 4.5 for 120 h. Samples were taken at various time intervals, and reaction was stopped by addition of 2.5 M Tris buffer at pH 7.1 to the final concentration of 1.1 M. They were frozen immediately, and were analyzed for glucose concentration by a glucose oxidase method.
RESULTS AND DISCUSSION

In the first part of the research, glucoamylase was produced by fermentation in which a special *S. cerevisiae* strain containing a glucoamylase gene was used. The medium used in the fermentation was yeast minimal salts medium without leucine but with histidine. The reason for using this medium in the fermentation was its selectivity towards only plasmid-bearing cells. The plasmid containing glucoamylase also contains the leucine gene, which gives the plasmid-bearing cells the ability to grow in a leucine-deficient medium (Innis et al., 1985).

The initial glucose concentration of the medium was 2%, and under the fermentation conditions the glucose concentration dropped to zero in the first 20 h. Since the fermentation was performed for 72 h, glucose needs to be added somehow to continue the process and to obtain the maximal glucoamylase concentration possible. Therefore, we attempted to maximize both cell density and glucoamylase activity by using different glucose concentrations (Table 1). In the first run, glucose concentration was returned to 2% at 48 h. Results for glucose concentration, cell density, and glucoamylase activity are shown in Figure 1, and are the best of all five runs. Glucoamylase activity was low until 40 h and then increased
Table 1. *Saccharomyces cerevisiae* fermentations for wild-type glucoamylase production.

<table>
<thead>
<tr>
<th>Run #</th>
<th>Final optical density at 680 nm</th>
<th>Final glucoamylase activity, IU/L</th>
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<tr>
<td>1</td>
<td>7.8</td>
<td>314</td>
</tr>
<tr>
<td>2</td>
<td>6.5</td>
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</tr>
<tr>
<td>3</td>
<td>5.6</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>3.9</td>
<td>108</td>
</tr>
<tr>
<td>5</td>
<td>3.7</td>
<td>121</td>
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</table>

Figure 1. Growth curve, glucose consumption, and wild-type glucoamylase production of *S. cerevisiae*: (▲) - optical density at 680 nm, (●) - glucose concentration, g/L, (●) - enzyme activity, IU/L.
rapidly, essentially after 48 h, to a maximal value of 314 U/mL. The molar ratio of carbon to nitrogen offered to the cells through the fermentation was 9. Typical C/N values for yeast are given as 5-6 (Peppier, 1970); however, it is desirable to have higher C/N values in the medium, since a fraction of this carbon is converted to carbon dioxide.

For the second case glucose concentration was returned to 2% at 28, 50, and 60 h, leading to a C/N ratio of 18. Cell density and glucoamylase activity at the end of this fermentation were 83% and 64% of the first run, respectively. To maintain the C/N ratio at 9.5 and to hold the glucose concentration near 2%, in the third run a 40% glucose-7.5% ammonium sulfate mixture was added nine times between 14 and 60 h. However, this led to the production of ethanol through the Crabtree effect, to which S. cerevisiae is very susceptible (Polakis and Bartley, 1965; de Dekken, 1966). Cell density and glucose concentration were very low, only 72% and 31% those of the first fermentation, respectively. In the fourth and fifth runs, glucose concentrations were held constant near 0.03% and 0.3%, respectively, and the C/N ratio was held at 7 by the continuous addition of a 40% glucose-11.4% ammonium sulfate solution after 24 h. This again led to low values of
cell density, 50% and 47%, respectively, and glucoamylase activity, 34% and 39%, respectively, those of the first run.

When cells were ruptured, some further glucoamylase was released. However, 89% of the total glucoamylase activity produced by the cells was released to the medium before their rupture.

In the second part of the study, production of glucose from starch dextrin by wild-type and the mutant Asn182->Ala glucoamylases produced by S. cerevisiae and by native A. awamori glucoamylase with and without pullulanase or isoamylase was measured. The Asn182->Ala mutant was prepared by Sierks (1988), and his results showed that it had a higher selectivity toward maltose than isomaltose, without affecting the catalytic rate. Since α-(1→6) glycosidic bond formation is preferred by glucoamylase during reversion reactions, higher glucose yields were expected with this mutant. Results for the three enzyme forms are shown in Figures 2-4. Similar curves were obtained for all cases, with maximal glucose yields being obtained at about halfway through the 120 h incubation. The expected increases in maximal glucose production could not be obtained when the mutant glucoamylase was used. Although this result was surprising, later on the previous
Figure 2. Production of glucose by native glucoamylase from A. awamori: (●) - glucoamylase only, (■) - with pullulanase, (◆) - with isoamylase.
Figure 3. Production of glucose by wild-type glucoamylase from *S. cerevisiae*: (●) — glucoamylase only, (■) — with pullulanase, (◆) — with isoamylase.
Figure 4. Production of glucose by Asn182→Ala mutant glucoamylase from *S. cerevisiae*: (○) - glucoamylase only, (■) - with pullulanase, (♦) - with isoamylase.
results could not be repeated with the Asn182→Ala mutant enzyme. Slight increases were obtained when pullulanase and isoamylase, especially the former, were added to any of the native and *S. cerevisiae* glucoamylases. Almost no difference was observed between native and *S. cerevisiae* glucoamylases. The lowest rate of loss of glucose to disaccharides after the glucose peak occurred with mutant glucoamylase.
GENERAL SUMMARY

This dissertation contains two different pieces of work related to *Aspergillus awamori* glucoamylase, the major project in the first part of this dissertation, and a separate project on growth of *Saccharomyces cerevisiae* containing mutant glucoamylases and the use of these mutants to hydrolyze dextrin in the second part.

In the first part, a program of cassette mutagenesis was undertaken in the active site of *A. awamori* glucoamylase, mainly to alter the effect of pH dependence of the enzyme but also to determine possible functions of the mutated residues. For this purpose, nine single amino acid mutants were prepared and expressed in *S. cerevisiae*, and their effects were determined by using steady-state enzyme kinetics.

In the first group, Glu179→Asp, Asp176→Glu, and Glu180→Asp mutations were prepared. The second category comprised the Leu177→Asp, Trp178→Asp, Val181→Asp, and Asn182→Asp mutations. Finally, the third category contained two insertion mutations, where Asp and Gly were added between residues 176 and 177. Although the results verified the catalytic function of Glu179, the basic catalytic function of Asp176 could not be verified. Asp176, Leu177, Trp178, and Glu180 were determined to have
important functions either structurally or in substrate binding, whereas Val181 and Asn182 were of minor importance both catalytically and structurally. The complete loss of activity of the insertion mutations together with the secondary structure analysis suggested destruction of the active site structure by the insertions.

The effects of Glu179→Asp, Trp178→Asp, and the insertion mutations on the pH dependence of glucoamylase could not be determined due to very low or undetectable activities, while the others did not affect this property significantly. The Δ(ΔG) calculations for the Trp178→Asp and Asn182→Asp mutations suggested at least one charged hydrogen bond was broken in the first case and none was broken in the second case. In all other cases one uncharged hydrogen bond was probably broken.

The main object of the second part of the study was to determine the maximal glucose yield for dextrin hydrolysis of wild-type and the mutant Asn182→Ala A. awamori glucoamylase produced by S. cerevisiae fermentation. In the hydrolysis experiments, either pullulanase and isoamylase were also used together with glucoamylase to test their effects on maximal glucose yield. The hydrolysis experiments were also performed with native A. awamori glucoamylase, in order to determine any hydrolytic activity.
differences with glucoamylase produced by *S. cerevisiae*. Similar hydrolysis results were obtained for all cases, with maximal glucose yields being obtained at about halfway through the 120 h incubation. The expected increases in maximal glucose production could not be obtained when the mutant glucoamylase was used. Slight increases were obtained when pullulanase and isoamylase, especially the former, were added to any of the native and *S. cerevisiae* glucoamylases. Almost no difference was observed between native and *S. cerevisiae* glucoamylases. The lowest rate of loss of glucose to disaccharides after the glucose peak occurred with mutant glucoamylase.
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<table>
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<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>Code</th>
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<tr>
<td>Alanine</td>
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</tr>
<tr>
<td>Arginine</td>
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<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Asparagine or aspartic acid</td>
<td>Asx</td>
<td>B</td>
</tr>
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<td>Cysteine</td>
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<tr>
<td>Valine</td>
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**Figure A1. Abbreviations of amino acids (1)**
1. Amino Acids with a Nonpolar R— Group

<table>
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<tr>
<th>Amino Acid</th>
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<td>Alanine</td>
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</tr>
<tr>
<td>Valine</td>
<td>( \text{H}_2\text{C} - \text{CH} - \text{C} - \text{C} - \text{CO}_2\text{H} )</td>
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<tr>
<td>Leucine</td>
<td>( \text{H}_2\text{C} - \text{CH}_3 - \text{CH} - \text{C} - \text{C} - \text{CO}_2\text{H} )</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>( \text{CH}_3 - \text{CH}_2 - \text{CH} - \text{C} - \text{C} - \text{CO}_2\text{H} )</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>( \text{H}_2\text{C} - \text{C} = \text{C} - \text{CO}_2\text{H} )</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>( \text{H}_2\text{C} - \text{C} - \text{CO}_2\text{H} )</td>
</tr>
</tbody>
</table>

Figure A2. Naturally occurring amino acids (2).
2. Amino Acids with a Polar But Neutral R— Group

Glycine Gly

Serine Ser

Threonine Thr

**Figure A2. continued.**
1. Amino Acids with a Nonpolar \( R^- \) Group

- Alanine (Ala)
- Valine (Val)
- Leucine (Leu)
- Isoleucine (Ile)
- Phenylalanine (Phe)
- Tryptophan (Trp)

Figure A2. continued
3. Acidic Amino Acids

Aspartic acid  Asp

Glutamic acid  Glu

4. Basic Amino Acids

Lysine  Lys

Arginine  Arg

Histidine  His

Figure A2. continued.
Cysteine  Cys

Tyrosine  Tyr

Asparagine  Asn

Glutamine  Gln

Figure A2. continued.
Figure A3. Schematic of the pBS(+-) vector (3).
Figure A4. Schematic of the pBIKS(+) vector (3).