

## Refined Structure for the Complex of Acarbose with Glucoamylase from *Aspergillus awamori* var. X100 to 2.4-Å Resolution\*

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The three-dimensional structure of the pseudotetraraccharide acarbose complexed with glucoamylase II(471) from *Aspergillus awamori* var. X100 has been determined to 2.4-Å resolution. The model includes residues corresponding to 1-471 of glucoamylase I from *Aspergillus niger*, a single molecule of bound acarbose, and 535 sites for water molecules. The crystallographic *R* factor from refinement is 0.124, and the root-mean-squared deviation in bond distances is 0.013 Å. Electron density for a single molecule of bound acarbose defines what may be the first four subsites in the binding of extended maltooligosaccharides. Hydrogen bonds between acarbose and the enzyme involve Arg<sup>54</sup>, Asp<sup>55</sup>, Arg<sup>305</sup>, carbonyl<sup>177</sup>, main chain amide<sup>121</sup>, Glu<sup>179</sup>, Glu<sup>180</sup>, and carbonyl<sup>179</sup>. Glu<sup>179</sup> forms a salt link to the imino linkage between the first and second residues of acarbose. This buried salt link probably contributes significantly to the unusually tight association ( $K_d \sim 10^{-12}$  M) of acarbose with glucoamylase. In addition, a significant hydrophobic contact between the third residue of acarbose and the side chain of Trp<sup>120</sup> distorts the three-center angle of the glucosidic linkage between the second and third residues of acarbose. A water molecule (water<sup>500</sup>) hydrogen bonds to Glu<sup>400</sup> and the 6-hydroxyl of the valienamine moiety of acarbose and is at an approximate distance of 3.7 Å from the "anomeric" carbon of the inhibitor. The relevance of the acarbose-glucoamylase complex to the mechanism of enzymic hydrolysis of oligosaccharides is discussed.

Glucoamylase ( $\alpha$ -1,4-D-glucan glucohydrolase, EC 3.2.1.3) catalyzes the removal of  $\beta$ -D-glucose from the non-reducing ends of starch and other related poly- and oligosaccharides (Weill *et al.*, 1954; Pazur and Ando, 1960; Manjunath *et al.*, 1983; Saha and Zeikus, 1989). The enzyme is used widely in industry for the conversion of starch to glucose syrups. The glucose in turn is used in the production of fructose sweeteners, ethanol, and light beer (Saha and Zeikus, 1989). Consequently, efforts are underway to engineer new glucoamylases with properties more amenable to various industrial applications. Our

objective here, however, is a better understanding of the relationship of the structure of glucoamylase to its function.

Glucoamylase cleaves the  $\alpha$ -1,4-glucosidic bond and, at a slower rate, the  $\alpha$ -1,6-glucosidic bond (Abdullah *et al.*, 1963), thus allowing it to completely hydrolyze starch (Takahashi *et al.*, 1985). The enzyme also shows a preference for maltooligosaccharides of at least 6 residues. The rate of hydrolysis rises and the  $K_m$  decreases as the chain length of maltooligosaccharides increases to six (Abdullah *et al.*, 1963; Meagher *et al.*, 1989). Several investigators (Hiromi *et al.*, 1970; Tanaka *et al.*, 1983; Koyama *et al.*, 1984; Sergeev and Firsov, 1985; Meagher *et al.*, 1989) have partitioned the free energy of substrate-enzyme interactions among the subsites of the active site of glucoamylase. The common feature of these free energy assignments is a near zero free energy return for substrate-enzyme interactions at subsite 1, a large free energy return at subsite 2, and diminishing returns for each subsite following subsite two. Finally, investigations in fast kinetics reveal a rapid initial step followed by a much slower step in the binding of substrates or substrate analogues. The slow step has been attributed to a conformational change in the enzyme (Olsen *et al.*, 1992) or to the slow diffusion of the substrate from an initial encounter complex to the catalytically productive complex with the enzyme (Tanaka *et al.*, 1982a; Hiromi *et al.*, 1983).

Species of *Aspergillus* produce at least two major forms of glucoamylase (Hayashida, 1975; Svensson *et al.*, 1982; Saha and Zeikus, 1989). These forms include the parent protein (called glucoamylase I) of 615 or 616 amino acid residues and other forms which result from the limited proteolysis of the parent protein (Svensson *et al.*, 1986a; Hayashida *et al.*, 1989a, 1989b). Glucoamylase I from *Aspergillus* possesses three functional domains (Svensson *et al.*, 1983, 1989): (i) an N-terminal catalytic domain of approximately 440 residues; (ii) a highly O-glycosylated linker domain of approximately 70 residues; and (iii) a C-terminal starch binding domain of approximately 100 residues. The catalytic domain consists of 13  $\alpha$ -helices, 12 of which are arranged in a polypeptide fold that is a variation on the  $\alpha/\beta$ -barrel (Aleshin *et al.*, 1992). This fold has been dubbed an  $\alpha/\alpha$ -barrel (Aleshin *et al.*, 1992) or a twisted  $\alpha$ -barrel (Juy *et al.*, 1992). The O-glycosylated region protects the protein from proteolysis and thermal denaturation (Takegawa *et al.*, 1988) and may also play a role in the transport of the enzyme to the extracellular environment (Yamashita, 1989). Hayashida *et al.* (1989a, 1989b) also attribute a starch binding function to the O-glycosylated domain. However, Williamson *et al.* (1992) have questioned whether the O-glycosylated domain plays a significant role in the binding of glucoamylase to raw starch. Instead, the starch-binding domain apparently confers upon glucoamylase the ability to adsorb to and digest raw starch (Svensson *et al.*, 1982; Dalmia and Nikolov, 1991; Williamson *et al.*, 1992; Belshaw and Williamson, 1990, 1991).

Previous crystallographic work has focused on a proteolytic fragment of glucoamylase I from *Aspergillus awamori* var.

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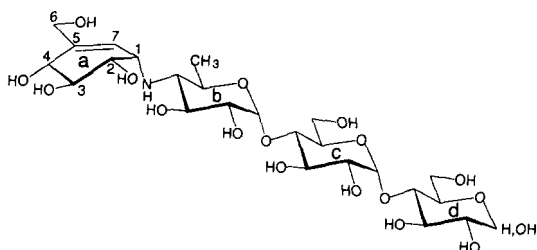


Fig. 1. Acarbose molecule with associated labels for residues and atoms.

*X100*, which contains residues corresponding to 1–471 of glucoamylase I from *Aspergillus niger*. Currently, structures of the enzyme at pH 6 (Aleshin *et al.*, 1992, 1994) and at pH 4 (Aleshin *et al.*, 1994), as well as the enzyme at pH 6 ligated to a single-residue inhibitor, 1-deoxynojirimycin, (Harris *et al.*, 1993) are in the literature. The studies above provide no information regarding the interaction of an extended substrate with the enzyme. We have pursued, therefore, an investigation of the complex of the pseudotetrasaccharide acarbose, with glucoamylase. Acarbose stands as the most effective known inhibitor of glucoamylase, having a  $K_d \sim 10^{-12}$  M (Svensson and Sierks, 1992). The inhibitor differs from maltotetraose in two respects (Truscheit *et al.*, 1981; Fig. 1): 1) it contains a valienamine residue at its “nonreducing” end and 2) it contains 4-amino-4,6-dideoxy- $\alpha$ -D-glucopyranose in place of the second glucose residue.

We present here the structure of the complex of glucoamylase II(471) with acarbose. Below is a description of the enzyme-inhibitor interactions and the relevance of those interactions to the mechanism of hydrolysis of substrates by glucoamylase.

#### MATERIALS AND METHODS

Glucoamylase II(471) was prepared from *A. awamori* var *X100* as described by Neustroyev and Firsov (1990). Conditions for growth of the fungus were adjusted so that the glucoamylase I ( $M_r = 100,000$  by SDS-polyacrylamide gel electrophoresis), produced initially, was reduced in size by the action of fungal acid proteases to a mixture of glucoamylases of average  $M_r = 72,000$ . This mixture of proteins migrated as two partially resolved bands on SDS-polyacrylamide gel electrophoresis.

The apparent heterogeneity of the protein did not prevent the reproducible growth of crystals of glucoamylase II(471) by vapor diffusion. A solution consisting of 15 mg/ml protein, 50 mM potassium phosphate, pH 5.95, and 13% (w/v) polyethylene glycol 6000 (Fluka) was equilibrated against a solution of 50 mM phosphate, pH 5.95, and 20–30% (w/v) polyethylene glycol 6000. Crystals of space group  $P2_12_12_1$  appeared in 1–2 weeks and grew slowly to a size of 0.2–0.3 mm. Crystals of glucoamylase II(471) are stable in 50 mM potassium phosphate, pH 5.95, and 28% (w/v) polyethylene glycol 6000. The protein used in the crystallization was not exposed to the action of  $\alpha$ -mannosidase, as suggested by Golubev *et al.* (1992). Crystals of glucoamylase have the same chemical composition regardless of the  $\alpha$ -mannosidase treatment (Aleshin *et al.*, 1994). The acarbose complex was formed by soaking crystals for 2–3 days in a solution containing 1 mM acarbose (kindly provided by Dr. Peter Reilly, Dept. of Chemical Engineering, Iowa State University), 33% (w/v) polyethylene glycol 6000, 50 mM potassium phosphate, pH 6.0.

Data collection for the acarbose complex was carried out at Argonne National Laboratory, Chicago, IL on a Siemens area detector in the laboratory of Dr. Ed Westbrook. A total of 51,528 reflections were recorded from a single crystal. Of the 26,797 possible unique reflections to 2.3-Å resolution, 21,212 were actually collected with an  $R$  merge of 0.032. Of those, 20,286 had  $|F| > 2\sigma(|F|)$ . The data were 85% complete to 2.4-Å resolution. Crystals of the inhibitor complex diffracted as well as native crystals (Aleshin *et al.*, 1992), exhibiting an identical fall-off in average intensity as a function of resolution. Only constraints on time of the investigators prevented the collection of complete data to 2.0-Å resolution. Data from the crystal of the complex were scaled against native data collected previously (Aleshin *et al.*, 1992) by a process similar to that of Matthews and Czerwinski (1975).

Two models of acarbose, the  $\alpha$ - and  $\beta$ -anomers at residue D (Fig. 1), were generated using software from the POLYGEN package (Molecular Simulations, Inc.). The model was developed directly from the structure of four  $\alpha$ 1–4-linked D-glucopyranose molecules replacing or deleting the appropriate atoms. The initial model for acarbose was optimized by energy minimization using CHARMM. During the energy minimization process the dihedral angle defined by the atoms C4A, C5A, C7A, and C1A was restrained to 0 °C. The coordinates for residues C and D (the glucose units of acarbose) deviated by less than 0.1 Å from coordinates gotten by averaging different x-ray structures of sugars (Arnott and Scott, 1972).

All two-, three-, and four-center distances were restrained in the two models for acarbose, except for the four-center distances involving the atoms O6 and the linkages between the residues of acarbose. Restraints on chiral volumes associated with atoms C2, C3, C4, and C5 (excluding atom C5A) were also applied during refinement. The combination of distance and chiral restraints essentially fixed the conformation of the ring in each of the residues of acarbose. All possible intramolecular nonbonded contacts were considered between atoms of acarbose.

The high mannose glycosyl chain (Einsphar, 1989) was used as the basis for modeling the *N*-glycosyl chains of glucoamylase, whereas the density of *O*-glycosyl residues was represented by  $\alpha$ -D-mannose, in accordance with the findings of Gunnarsson *et al.* (1984). Standard groups for  $\alpha$ -D-mannose and *N*-acetyl-D-glucosamine were based on the coordinates of monosaccharides obtained by x-ray diffraction studies (Chu and Jeffrey, 1968; Longchambon *et al.*, 1976). The glycosidic bond angles were set initially to 115°. Standard groups were developed separately for serine-mannose, threonine-mannose, and asparagine-glycosyl chains. Glycosyl residues were restrained to the C1 chair conformation by specification of all two-, three-, and four-center distance restraints, except for those four-center distance restraints involving the O6 atom of each glycosyl residue or atoms of the *N*-acetyl group of *N*-acetyl-D-glucosamine. All four-center distance restraints which influence the dihedral angles associated with the oxygen atoms of the glycosidic bonds were omitted also. In addition to the distance restraints, the chiral volumes were restrained for atoms C1, C2, C3, C4, and C5 of each residue in all the glycosyl chains. The *N*-acetyl group of *N*-acetyl-D-glucosamine residues was subject to planar restraints. Restraints on all possible nonbonded contacts between atoms of different residues of the *N*-glycosyl chains were in force during the refinement, as well as restraints involving nonbonded contacts between the O6 atom and atoms O5 and C4 of the same residue.

Initial phases for the complex of acarbose with glucoamylase II(471) were calculated from the model for the native enzyme at pH 6.0 (Aleshin *et al.*, 1992a). The model for the free enzyme at pH 6.0 also represented the initial conformation of glucoamylase II(471) in the inhibitor complex. A Silicon Graphics 4D25 and the program TOM (Cambillau and Horjales, 1987) were used for model building. Initially the  $\alpha$ -conformer of acarbose was built into the electron density for the inhibitor and refined by restrained least-squares (Hendrickson and Konnert, 1980). Refinement proceeded over the course of 35 cycles and involved the interpretation and building of the solvent structure and the modeling of specific side chains in multiple conformations. The coordinates ( $x$ ,  $y$ , and  $z$ ) and an isotropic thermal parameter were adjusted for each atom of the model. The final three cycles of refinement were done with a model for acarbose which had residue D in the  $\beta$ -conformation.

Initially, 350 sites for water molecules were taken directly from the native model at pH 6 (Aleshin *et al.*, 1994). After the first 12 cycles of refinement, the solvent structure was compared with that of the 1-deoxynojirimycin-glucoamylase complex (Harris *et al.*, 1993). Corresponding water molecules, that differed by more than 0.7 Å in position or significantly in their thermal parameters, were removed from the model. Only 44 of the initial 350 sites for water were rejected by these criteria. Additional water molecules were added to the model provided that 1) electron density was present at a level of at least  $3.5\sigma$  in maps based on the Fourier coefficients  $(|F_{obs}| - |F_{calc}|)e^{i\phi_{calc}}$  and  $5\sigma$  in maps based on Fourier coefficients  $(2|F_{obs}| - |F_{calc}|)e^{i\phi_{calc}}$ ; 2) at least one hydrogen bond with an associated donor-acceptor distance of 2.4–3.2 Å could be made between the new site and an existing atom of the model; 3) no close nonbonded contacts (less than 3.0 Å) could be made between the new site and existing atoms of the model; and 4) the appearance of the electron density was approximately spherical. As a final criterion, sites for waters were omitted if their associated  $B$  parameters exceeded 70 Å<sup>2</sup> after refinement. Water occupancies were not refined because of the high correlation between occupancy and thermal parameters for data of a nominal resolution of 2.4 Å. Thus, solvent sites with  $B$  values between 50 and 70 Å<sup>2</sup> probably represent water molecules with occupancy parameters below 1.0 and true thermal parameters substantially

TABLE I

Agreement between target and observed stereochemistry for the complex of acarbose with glucoamylase II(471)

Target root-mean-square deviations used in refinement are in parentheses.

|                                                                    |              |
|--------------------------------------------------------------------|--------------|
| Total no. of atoms                                                 | 4,417        |
| Total no. of solvent sites                                         | 535          |
| No. of structure factors used in refinement <sup>a</sup>           | 20,601       |
| R factor <sup>b</sup>                                              | 0.124        |
| Mean B (Å <sup>2</sup> ) for protein                               | 10.7         |
| RMS deviation (in Å) for distances <sup>c</sup>                    |              |
| Two-center                                                         | 0.013 (0.02) |
| Three-center                                                       | 0.029 (0.30) |
| Four-center                                                        | 0.039 (0.05) |
| RMS deviation (in Å) for planar groups <sup>c</sup>                | 0.012 (0.02) |
| RMS deviation (in Å <sup>3</sup> ) for chiral volumes <sup>c</sup> | 0.137 (0.15) |
| RMS deviation (in Å) for nonbonded contacts <sup>c</sup>           |              |
| Determined by single torsion angles                                | 0.224 (0.25) |
| Determined by multiple torsion angles                              | 0.153 (0.25) |
| Hydrogen bond                                                      | 0.192 (0.25) |
| RMS deviation (in degrees) for torsion angles <sup>c</sup>         |              |
| Planar peptides ( $\omega = 180^\circ$ )                           | 2.3 (3)      |
| Staggered side chains                                              | 13.8 (15)    |
| Orthonormal                                                        | 28.7 (20)    |
| RMS $\Delta B$ (in Å <sup>2</sup> ) <sup>c</sup>                   |              |
| Main chain bond                                                    | 0.643 (1.0)  |
| Main chain angle                                                   | 1.000 (1.5)  |
| Side chain bond                                                    | 1.972 (2.0)  |
| Side chain angle                                                   | 2.864 (3.0)  |

<sup>a</sup> Comprises all observed moduli from 10 to 2.3-Å resolution, with  $|F| \geq \sigma(|F|)$  and  $|F| \geq 3.0$ .

<sup>b</sup>  $R \text{ factor} = \sum |F_{\text{obs}}| - |F_{\text{calc}}| / \sum |F_{\text{obs}}|$ .

<sup>c</sup> Root-mean-square.

lower than those reported from the refinement.

Side chains were screened for multiple conformations on the basis of the following criteria: 1) electron density must be present at a level of at least  $3\sigma$  in maps based on the Fourier coefficients  $(|F_{\text{obs}}| - |F_{\text{calc}}|)e^{i\phi_{\text{calc}}}$  and  $(2|F_{\text{obs}}| - |F_{\text{calc}}|)e^{i\phi_{\text{calc}}}$ ; 2) alternative conformers must have reasonable contacts with nearby atoms; 3) each conformer must adopt or be close to a conformation with staggered  $\chi$  angles.

## RESULTS AND DISCUSSION

**General Features of the Refined Model**—The model for the complex of acarbose with glucoamylase II(471) has been deposited with the Protein Data Bank, Brookhaven National Laboratory. In keeping with prior work (Aleshin *et al.*, 1992, 1994; Harris *et al.*, 1993), we have adopted the amino acid numbering of glucoamylase from *A. niger* (Svensson *et al.*, 1983; Boel *et al.*, 1984). Thus, the sequence numbers skip from 101 to 103 in the deposited coordinate set. The amino acid sequence used in refinement is identical to that of Aleshin *et al.* (1992). A description of the secondary structure appears in Aleshin *et al.* (1992, 1994). Refinement statistics for the complex of acarbose with glucoamylase II(471) are in Table I. A Luzzati (1952) analysis infers an overall root-mean-square uncertainty in atomic coordinates of between 0.1 and 0.2 Å. Fig. 2 is a schematic of the acarbose glucoamylase complex.

The unit cell parameters of the glucoamylase-acarbose complex ( $a = 116.7$  Å,  $b = 103.9$  Å, and  $c = 48.34$  Å) are nearly identical to those reported for the complex of glucoamylase II(471) with 1-deoxynojirimycin ( $a = 116.6$  Å,  $b = 103.6$  Å, and  $c = 48.3$  Å) and slightly smaller than those reported for the native enzyme at pH 6 ( $a = 116.7$  Å,  $b = 104.3$  Å, and  $c = 48.49$  Å). The difference in size probably stems from the dehydrating

effect of the soaking buffer, which has an elevated concentration of polyethylene glycol relative to that used in the growth and mounting of native crystals (33% as opposed to 28% polyethylene glycol).

The conformations of the acarbose-ligated, 1-deoxynojirimycin-ligated, and native enzymes are almost identical. The root-mean-square deviation in the positions of all atoms between the acarbose-ligated and native enzymes is 0.2 Å and between the acarbose-ligated and 1-deoxynojirimycin-ligated enzymes, 0.16 Å. The 13 side chains which have at least one atom within 3.7 Å of residues A and B of acarbose have a root-mean-square deviation in atomic positions of 0.23 Å against the native enzyme and 0.14 Å against the 1-deoxynojirimycin-ligated enzyme. The differences in thermal parameters between the three models are also within the uncertainty levels for these parameters (2–3 Å<sup>2</sup>). The glycosylation and the conformation of glycosyl chains in the acarbose complex are identical to that of the native, pH 4 structure, which has been described in detail by Aleshin *et al.* (1994). Eighty % of the sites for water molecules in the acarbose complex fall within 1.2 Å of a water molecule in the native, pH 6 structure or the 1-deoxynojirimycin structure. The root-mean-square deviation between corresponding water molecules of the acarbose-ligated and native enzymes is 0.48 Å and for the acarbose-ligated and 1-deoxynojirimycin-ligated enzymes, 0.3 Å. The remaining sites for water molecules in the acarbose complex which cannot be assigned unambiguously to a site for water in the native or 1-deoxynojirimycin-ligated enzymes have thermal parameters that average to 48 Å<sup>2</sup> and generally do not have hydrogen bonds to the protein.

The first 2 residues at the N terminus and the last 6 residues at the C terminus have thermal parameters above 30 Å<sup>2</sup>, which, as noted previously (Aleshin *et al.*, 1992, 1994), may be due to proteolytic degradation. The distribution of  $\phi, \psi$  angles (Ramachandran *et al.*, 1963) for amino acid residues of the acarbose complex is similar to previous structures (Aleshin *et al.*, 1992; Harris *et al.*, 1993). Only residues Asn<sup>313</sup> and Ser<sup>411</sup> have  $\phi, \psi$  angles which lie significantly outside of the allowed regions, their conformations being enforced by local hydrogen bonds.

Four side chains (Asp<sup>25</sup>, Asp<sup>44</sup>, Thr<sup>246</sup>, and Lys<sup>378</sup>) have alternative conformations in the acarbose complex. These residues also exist in alternative conformations in the native enzyme and the 1-deoxynojirimycin complex. Made conspicuous by its absence is the disorder in the native enzyme for the side chain of Asp<sup>55</sup>. As noted by Aleshin *et al.* (1994), the side chain of Asp<sup>55</sup> is near the beginning of a channel, filled with water molecules, that connects the active site to the bulk solvent. In the native structure one conformation of Asp<sup>55</sup> leaves the channel open, whereas the alternative conformer blocks access to the channel from the active site. In the acarbose complex (and the 1-deoxynojirimycin complex, as well) Asp<sup>55</sup> assumes only the conformation which blocks access to the channel.

The only unambiguous conformational difference in the acarbose complex and the free enzyme involves atom O6 of Ser<sup>mannosyl</sup><sup>455</sup>. Atom O6 moves to a new staggered conformation, which allows it to hydrogen bond to the main chain of Trp<sup>120</sup>. The conformational change, however, may not be significant to the mechanism of glucoamylase. If atom O6 were to remain in its observed position in the native enzyme, it would make a close contact with a bound acarbose molecule related by crystal symmetry.

**The Acarbose Molecule and Its Interactions with the Active Site**—Residues A, B, C, and D of acarbose (Fig. 1) have electron density of approximately 16 $\sigma$ , 15 $\sigma$ , 11 $\sigma$ , and 10 $\sigma$ , respectively (Fig. 3). The mean thermal parameters for atoms of residues A, B, C, and D are 8, 10, 20.5, and 34 Å<sup>2</sup>, respectively. Thus, the density and atomic positions are well defined for the entire acarbose molecule.

FIG. 2. Stereoview of the acarbose-glucoamylase complex. Structures with open circles represent glycosyl chains. The structure represented by filled circles represents the bound molecule of acarbose. The enzyme is represented only by the trace through its  $\alpha$ -carbons. Illustration prepared by MOLSCRIPT (Kraulis, 1991).

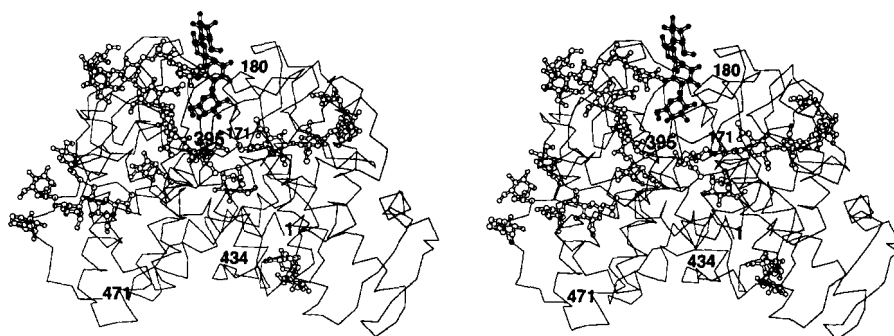
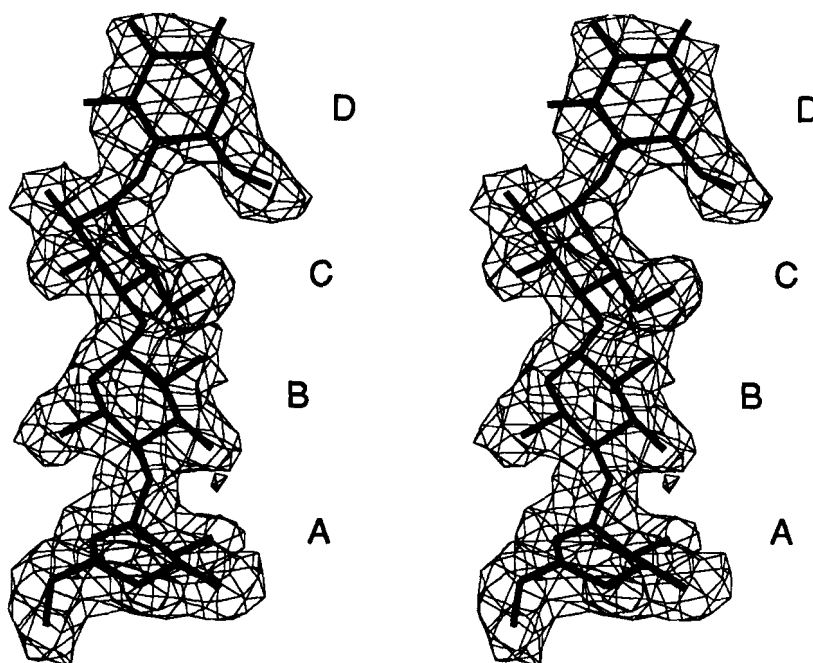


FIG. 3. Stereoview of the acarbose molecule with associated electron density.



By far, interactions involving residue A of acarbose are most numerous (Table II; Figs 4 and 5) and are similar to those exhibited in the complex of 1-deoxynojirimycin and glucoamylase (Harris *et al.*, 1993; Fig. 6). Arg<sup>54</sup> binds to the 4-hydroxyl of residue A, Asp<sup>55</sup> binds to both the 4- and 6-hydroxyls, and carbonyl 177 binds to the 3-hydroxyl. In addition water<sup>500</sup> hydrogen bonds to the putative catalytic base, Glu<sup>400</sup>, and to the 6-hydroxyl of residue A so that its dipole moment is oriented away from the "anomeric carbon" (atom C1A). Water<sup>500</sup> makes numerous contacts with the carbon atoms in the ring of residue A. Except for water<sup>500</sup>, residue A is sequestered from solvent. Hydrophobic contacts are contributed by Trp<sup>178</sup> and Trp<sup>417</sup>, consistent with observations that the binding of substrate analogues at subsite 1 perturbs the fluorescence of tryptophan residues (Hiromi *et al.*, 1982). The  $\alpha$ -anomer of glucopyranose has hydrophobic and hydrophilic faces (Johnson *et al.*, 1988) with the hydrophilic face defined by the positions of hydroxyls 1, 2, and 4. The nonreducing end of a maltooligosaccharide, assuming it binds as does acarbose, would position its hydrophobic face toward water<sup>500</sup>.

Several significant differences are apparent between the 1-deoxynojirimycin complex and the acarbose complex. First, water<sup>500</sup> is approximately 0.6 Å farther away from atom C7A than the corresponding atom N5 of the 1-deoxynojirimycin complex (Table II). The relative shift in the position of water<sup>500</sup> arises from perhaps at least three competing factors: 1) water<sup>500</sup> cannot form a stable hydrogen bond to atom C7A as it does to the protonated N5 of 1-deoxynojirimycin (Harris *et al.*, 1993), 2)

the double bond between atoms C5A and C7A of residue A forces C7A away from water<sup>500</sup>, and 3) the presence of an imino linkage between residues A and B, as well as the presence of interactions between the enzyme and residues B and C, influences the position and orientation of residue A. The second difference between residue A of acarbose and 1-deoxynojirimycin is the presence of an additional hydrogen bond between atom O2A of acarbose and NH1 of Arg<sup>305</sup>. Factors which contribute to the increased distance between water<sup>500</sup> and C7A of acarbose also contribute to the formation of this hydrogen bond.

Glu<sup>179</sup> probably forms a salt link with the imino linkage of acarbose (atom N4B). The imino group of acarbose has a  $pK_a$  of approximately 5 (Truscheit *et al.*, 1981). At the pH of the current study (pH 6), we would expect Glu<sup>179</sup> to be ionized (Aleshin *et al.*, 1994). Thus, the proximity of Glu<sup>179</sup> to the imino linkage should elevate the  $pK_a$  of the linkage, so that even at pH 6, atom N4B should bear two protons and a net positive charge. Indeed, the short donor-acceptor distance (2.58 Å) between atom OE2 of Glu<sup>179</sup> and atom N4B of acarbose suggests an unusually strong hydrogen bond, one that is enhanced by the columbic interaction of ionized groups. The salt link that putatively exists between acarbose and Glu<sup>179</sup> is inaccessible to solvent. A buried salt link would contribute significantly to the tight binding of acarbose to glucoamylase ( $K_d \sim 10^{-12}$  M). The stability of a buried salt link should be insensitive to pH changes, and indeed, acarbose binds tightly to glucoamylase from pH 4.5 to 8 (Iwama *et al.*, 1985; Håkansson and Svensson, 1989). The positively charged amino alcohol Tris can displace

TABLE II

Nonbonded contacts between glucoamylase II(471) and acarbose

Only distances less than or equal to 3.5 Å in the acarbose complexes are listed.

| Atom of the inhibitor | Atom of the protein        | Contact distance (Å) <sup>a</sup> |
|-----------------------|----------------------------|-----------------------------------|
| O6A                   | Water <sup>500</sup>       | 2.87 (2.97)                       |
| C7A (N5)              | Asp <sup>55</sup> OD1      | 2.73 (2.57)                       |
|                       | Water <sup>500</sup>       | 3.42 (2.82)                       |
| O2A                   | Tyr <sup>48</sup> OH       | 3.47 (3.54)                       |
|                       | Arg <sup>305</sup> NH1     | 2.94 (3.23)                       |
| O3A                   | Trp <sup>178</sup> CA      | 3.54 (3.43)                       |
|                       | Carbonyl <sup>177</sup>    | 2.61 (2.65)                       |
| O4A                   | Arg <sup>54</sup> NE       | 3.54 (3.27)                       |
|                       | Trp <sup>417</sup> CZ3     | 3.49 (3.41)                       |
|                       | Asp <sup>65</sup> OD2      | 2.75 (2.73)                       |
| C1A                   | Arg <sup>54</sup> NE       | 3.34 (3.32)                       |
|                       | Arg <sup>54</sup> NH2      | 2.99 (2.91)                       |
| C2A                   | Water <sup>500</sup>       | 3.87 (3.28)                       |
| C3A                   | Water <sup>500</sup>       | 3.62 (3.39)                       |
| C4A                   | Carbonyl <sup>177</sup>    | 3.51 (3.46)                       |
| C5A                   | Water <sup>500</sup>       | 3.48 (3.60)                       |
| C6A                   | Water <sup>500</sup>       | 3.37 (3.49)                       |
| O2B                   | Asp <sup>55</sup> OD1      | 3.59 (3.41)                       |
|                       | Glu <sup>180</sup> OE2     | 2.73                              |
| O3B                   | Glu <sup>180</sup> OE2     | 3.43                              |
|                       | Water <sup>1183</sup>      | 3.16                              |
|                       | Carbonyl <sup>178</sup>    | 2.67                              |
| N4B                   | Arg <sup>305</sup> NH1     | 2.71                              |
|                       | Acarbose O2C               | 2.73                              |
| O5B                   | Glu <sup>180</sup> OE2     | 3.28                              |
| C3B                   | Glu <sup>179</sup> OE1     | 2.58                              |
| C4B                   | Water <sup>1309</sup>      | 3.08                              |
| C5B                   | Carbonyl <sup>178</sup>    | 3.16                              |
| C6B                   | Glu <sup>179</sup> OE1     | 3.35                              |
| O2C                   | Glu <sup>179</sup> OE1     | 3.14                              |
| O3C                   | Glu <sup>179</sup> OE1     | 3.41                              |
|                       | Acarbose O3D               | 3.21                              |
| O5C                   | Water <sup>1102</sup>      | 2.73                              |
| O6C                   | Water <sup>1309</sup>      | 3.03                              |
|                       | Trp <sup>120</sup> CE3     | 3.30                              |
| C2C                   | Trp <sup>120</sup> CD2     | 3.48                              |
|                       | Carbonyl <sup>179</sup>    | 2.76                              |
|                       | Amide <sup>121</sup>       | 2.82                              |
| C6C                   | Gly <sup>121</sup> CB      | 3.34                              |
|                       | Glu <sup>179</sup> CB      | 3.40                              |
| O1D                   | Water <sup>1102</sup>      | 3.40                              |
|                       | Carbonyl <sup>179</sup>    | 3.28                              |
| O2D                   | Glu <sup>179</sup> CB      | 3.41                              |
|                       | Ser-mannose <sup>455</sup> | 2.95 <sup>b</sup>                 |
| O3D                   | Water <sup>1301</sup>      | 2.98                              |
|                       | Water <sup>825</sup>       | 3.14                              |
| O4D                   | Water <sup>825</sup>       | 2.84                              |
| O5D                   | Water <sup>1191</sup>      | 3.07                              |
| O6D                   | Acarbose O2C               | 3.21                              |
| C2D                   | Water <sup>1310</sup>      | 3.50                              |
|                       | Water <sup>1347</sup>      | 3.47                              |
| C5D                   | Water <sup>1347</sup>      | 2.89                              |
|                       | Water <sup>1317</sup>      | 3.15                              |
|                       | Water <sup>1340</sup>      | 3.31                              |
|                       | Water <sup>825</sup>       | 3.13                              |
|                       | Water <sup>1340</sup>      | 3.46                              |

<sup>a</sup> Distances in parentheses are for the chair conformer of 1-deoxynojirimycin.<sup>b</sup> Crystal lattice contact.

acarbose from glucoamylase, but only at a pH above 7.5 and only by using elevated concentrations of the amino alcohol (Clarke and Svensson, 1984b).

The  $\phi$  angle of the  $\alpha$ -glucosidic linkage is the dihedral angle defined by atoms H1, C1, O4, and C4, and the  $\psi$  angle by atoms C1, O4, C4, and H4. Studies by NMR and force field calculations indicate two energy minimum conformations for maltose with average  $\phi, \psi$  angles of  $-25^\circ, -20^\circ$ , and  $-60^\circ, -45^\circ$  (Lemieux and Bock, 1983; Shashkov *et al.*, 1986; Tvaroska and Perez, 1986; Dowd *et al.*, 1992). The two favored conformations for maltose are separated by a low energy barrier of 0.5 kcal/mol (Dowd *et al.*, 1992). The linkage between residues A and B of

acarbose has two conformations in solution with  $\phi, \psi$  angles of  $-51^\circ, -10^\circ$  and  $-30^\circ, 178^\circ$  (Bock and Pedersen, 1984). The  $\phi, \psi$  angles observed for acarbose in its complex with glucoamylase are  $-16^\circ, 6.5^\circ$ , being close to one of the favored conformations for maltose in solution. Furthermore, acarbose still retains a hydrogen bond between atoms O2A and O3B (Table II), an interaction that contributes to the stability of the preferred conformations of maltose (Dowd *et al.*, 1992). Goldsmith *et al.* (1987) found the same intramolecular hydrogen bond in acarbose in its complex with glycogen phosphorylase.

Residue B of acarbose binds to the approximate location of the secondary binding site for 1-deoxynojirimycin, but in an orientation that is completely different. The second molecule of 1-deoxynojirimycin cannot adopt the same binding interactions as residue B of acarbose because of unfavorable contacts that would occur between the 4-hydroxyl of the second molecule of 1-deoxynojirimycin and atom C1 of the first molecule of 1-deoxynojirimycin. The hydrophilic side of residue B faces Glu<sup>179</sup>, Glu<sup>180</sup>, and Arg<sup>305</sup>, whereas its hydrophobic side faces Tyr<sup>311</sup> and Tyr<sup>48</sup>. The principal hydrogen bonds between residue B and the enzyme involve Glu<sup>180</sup> with atom O2B and Arg<sup>305</sup> and carbonyl<sup>178</sup> with atom O3B. The interaction of atom O2B with Glu<sup>180</sup> was suggested by Sierks and Svensson (1992). Except in the vicinity of atom C6B, water molecules do not come into contact with residue B. However, atom C6B does project into a solvent cavity, which would easily accommodate the 6-hydroxyl group of a maltooligosaccharide.

The angle defined by atom C1B, O4C, and C4C (the glucosidic link between residues B and C) deviates significantly from the target value used in the refinement (observed value,  $125.6^\circ$ ; target value,  $115.5^\circ$ ). The angle of  $125.6^\circ$  is significantly larger than angles of similar structures, which average to  $117^\circ$  (Arnott and Scott, 1972). The enlarged angle is not due to nonbonded contacts within the acarbose molecule. The  $\phi, \psi$  angles of the linkage ( $-66^\circ, -53^\circ$ ) are near the preferred values for maltose, representing an increase of 1.5 kcal/mol in energy relative to the preferred conformation (Dowd *et al.*, 1992). Interactions between residues A, B, and C of acarbose with the enzyme are the principal causes for the observed distortion in the linkage (see below).

The only hydrogen bonds between residue C and the protein have atom O6C interacting with both carbonyl<sup>179</sup> and the backbone amide<sup>121</sup>. In contrast, the hydrophobic face of residue C stacks against the side chain of Trp<sup>120</sup>. Atom CE2 of Trp<sup>120</sup> moves 0.34 Å relative to its position in the native structure and in the 1-deoxynojirimycin complex. Presumably the hydrogen bonds acting on residues A and B pull the inhibitor into the active site. However, the composite force of the hydrogen bonds is opposed by the nonbonded interaction between residue C and Trp<sup>120</sup>. The result is the observed distortion in the angle of the glucosidic linkage between residues B and C. The strain placed on the acarbose molecule by the active site of glucoamylase has clear implications, as discussed below, for the catalysis of extended maltooligosaccharides.

The  $\phi, \psi$  angles for the linkage between residues C and D of acarbose have values  $-25^\circ, -17^\circ$ , respectively, in good agreement with the preferred values for maltose. Furthermore, there is no evidence for angular strain in the glucosidic linkage. A weak hydrogen bond probably exists between atoms O2C and O3D (donor-acceptor distance of 3.21 Å), as expected for the preferred conformation of maltose.

The reducing end of acarbose, residue D, has been modeled in both the  $\alpha$ - and  $\beta$ -conformation. The O1D atom of the  $\beta$ -anomer hydrogen bonds to atom O5 of Ser-mannosyl<sup>455</sup> from a glucoamylase molecule related by crystallographic symmetry. For the  $\alpha$ -anomer atom O1D does not hydrogen bond to the enzyme. Aside from the position of atom O1D, we observe no significant

FIG. 4. Stereoview of the interaction of bound acarbose with glucoamylase. The acarbose molecule is represented in open lines, ionized residues in bold lines, and water molecules as filled circles. Illustration prepared by MOLSCRIPT (Kraulis, 1991).

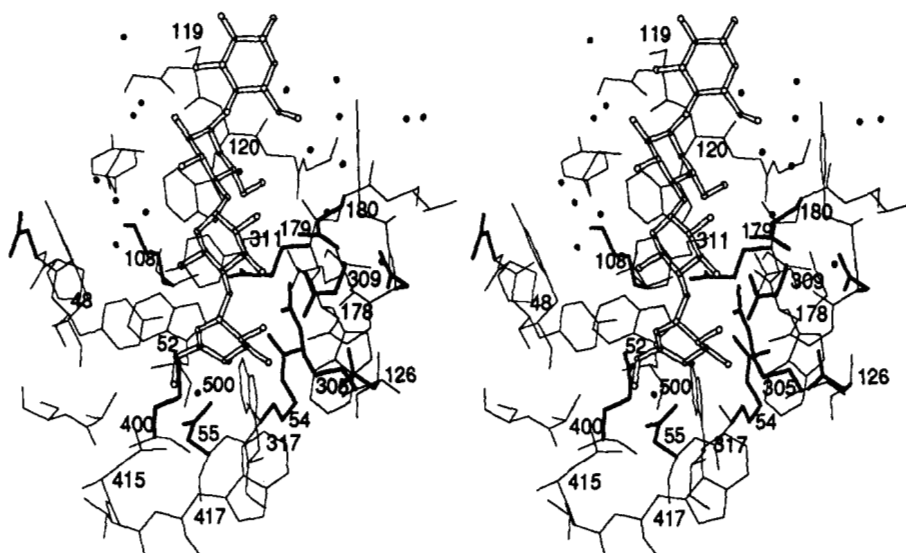
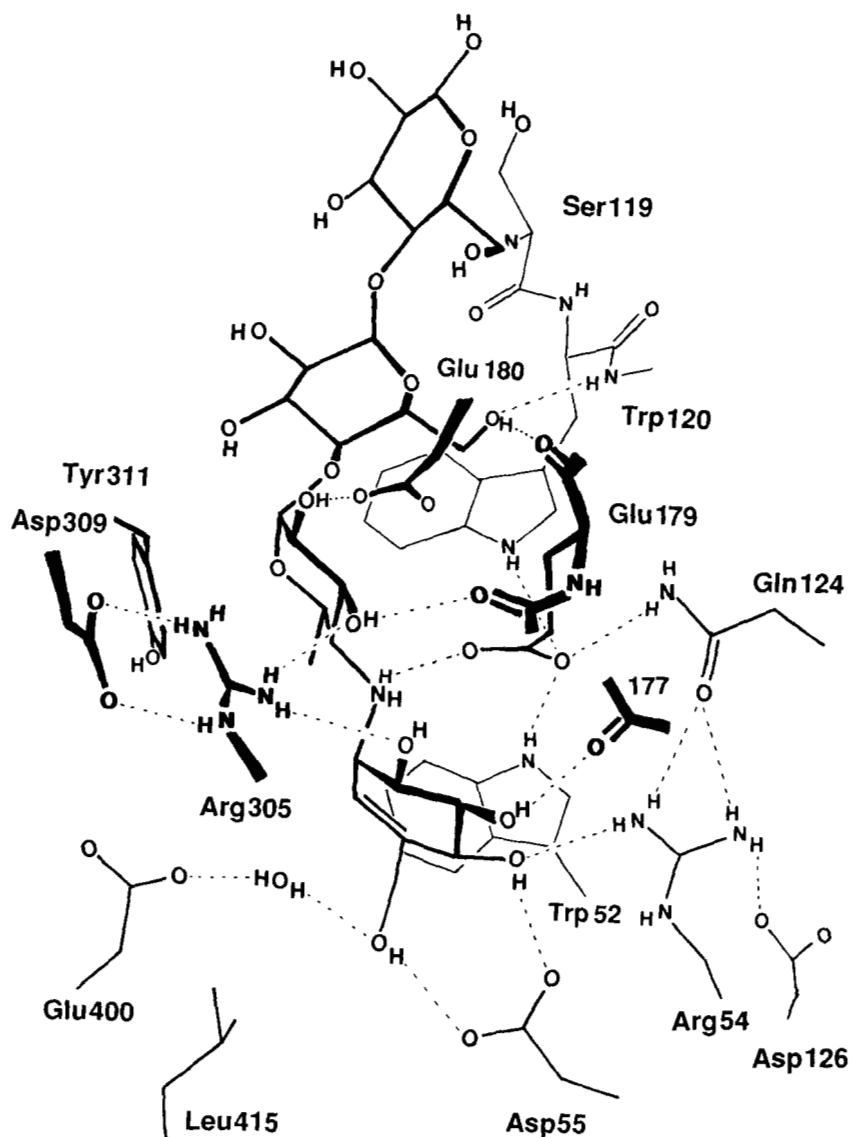


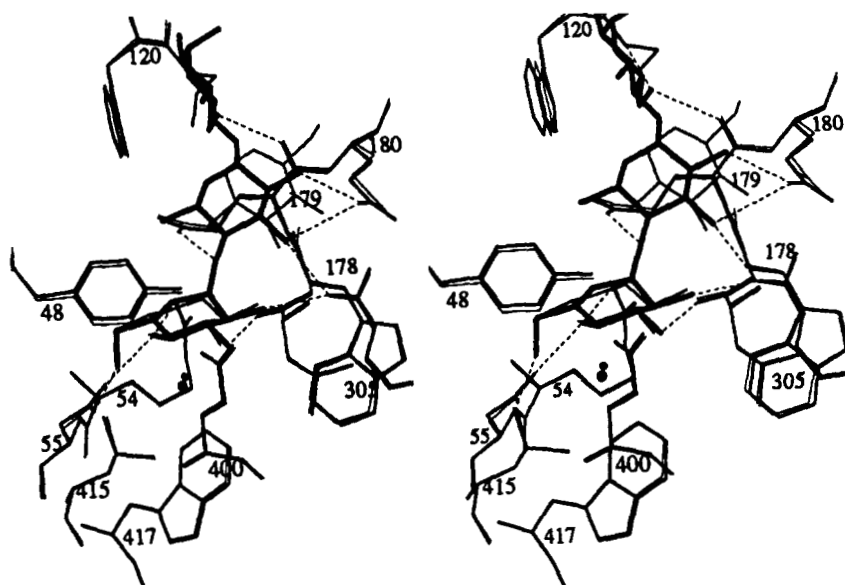
FIG. 5. Schematic of the acarbose-glucoamylase complex. Dotted lines represent hydrogen bonds.



difference in the refined coordinates for the  $\alpha$ - and  $\beta$ -conformers of acarbose. The hydrophobic face of residue D contacts a segment of the main chain from residues 119 to 121. Although

residue D of acarbose does participate in a lattice contact, we believe the acarbose-enzyme complex is a faithful representation of the acarbose-glucoamylase complex in solution. The  $\phi, \psi$

FIG. 6. Stereoview of the structure of the acarbose-glucoamylase complex superimposed on the 1-deoxynojirimycin-glucoamylase complex in the region of residues A and B of acarbose. Bold lines represent the structure of the acarbose complex. The filled circles represent the positions of water<sup>500</sup> in the two complexes. Illustration prepared by MOLSCRIPT (Kraulis, 1991).



angles of the linkage between residues C and D are essentially equal to the values anticipated for maltose in solution.

**Relevance of the Acarbose-Glucoamylase Structure to Substrate Specificity and the Mechanism of Hydrolysis**—The putative mechanism of hydrolysis has Glu<sup>179</sup> cast as a catalytic acid (Sierks *et al.*, 1990; Harris *et al.*, 1993) and Glu<sup>400</sup> as a catalytic base (Harris *et al.*, 1993). The complex of acarbose with glucoamylase is consistent with this proposal. Yet, beyond this general outline for the catalytic mechanism, many details remain uncertain. For instance, the state of ionization of Glu<sup>179</sup> is in doubt. The putative mechanism requires Glu<sup>179</sup> to be protonated at the onset of the reaction, yet the local environment of Glu<sup>179</sup> favors the ionized form of the side chain (Aleshin *et al.*, 1994). Specifically, the proximity of Arg<sup>54</sup> to Glu<sup>179</sup> and the presence of three hydrogen bonds to atom OE1 of Glu<sup>179</sup> should depress the  $pK_a$  of the acid side chain rather than elevate it. As the enzyme undergoes no conformational change in response to the binding of acarbose, the only available mechanism by which one can raise the  $pK_a$  of Glu<sup>179</sup> is through electrostatic interactions. Indeed, uncompensated negative charge exists in the immediate vicinity of the active site. The positive charge of Arg<sup>54</sup> is offset by its salt link to Asp<sup>126</sup> and that of Arg<sup>305</sup> is offset by its interaction with Asp<sup>309</sup>. However, Glu<sup>400</sup>, Asp<sup>55</sup>, and Glu<sup>179</sup> each introduce uncompensated negative charge into the active site. In the absence of inhibitors, these side chains project into the active site and are solvated. However, as the substrate replaces water of the active site with a less polar medium, the  $pK_a$  of Glu<sup>179</sup> should rise, thus favoring its protonated state.

Investigators have assigned a  $pK_a$  of 5.8–5.9 to the essential catalytic acid (Hiromi *et al.*, 1966; Savel'ev and Firsov, 1982), which is putatively Glu<sup>179</sup> (Sierks *et al.*, 1990). However, the practice of assigning a  $pK_a$  to a specific functional group on the basis of pH-dependent kinetics is scientifically unsound (Knowles, 1976). From the standpoint of the catalytic mechanism, a proton need be present on Glu<sup>179</sup> only at the onset of a catalytic turnover. Prior to the association of substrate with the enzyme, the proton in question may exist over time in partial occupancy at many different locations. The uncompensated electrostatic charge would stabilize, for instance, a hydronium ion at subsite 1 in the absence of substrate. Thus, for a brief time any one of the water molecules in subsite 1 could bear an additional proton, which would end-up on Glu<sup>179</sup> as the solvent is displaced by the substrate.

The transfer of electrostatic charge from a polar to nonpolar environment is thermodynamically unfavorable. Yet, this is one

of the processes that occurs when the substrate binds spontaneously to the active site of glucoamylase. Clearly, some favorable thermodynamic process must occur to offset the penalty of burying electrostatic charge. We suggest that the favorable thermodynamic process is the release of water molecules, bound to the substrate and to the active site, resulting in a significant increase in entropy. Indeed the binding of 1-deoxynojirimycin to glucoamylase is associated with a large increase in entropy (Tanaka *et al.*, 1982b). Inhibitors such as 1-deoxynojirimycin, acarbose, *D*-gluco-dihydroacarbose, and methyl acarviosinide are so effective as inhibitors, because, unlike the substrate, they introduce a positive charge to the active site, which eliminates the thermodynamic penalty associated with burying uncompensated negative charge. The only way a substrate can eliminate or reduce the thermodynamic penalty is by generating a complete or partial glucopyranosyl cation.

As in the case of the 1-deoxynojirimycin complex with glucoamylase, the dipole moment of water<sup>500</sup> is directed away from atom C1A of the inhibitor. The orientation assigned here to water<sup>500</sup> assumes that Glu<sup>400</sup> and the 6-hydroxyl of residue A of acarbose both serve as proton acceptors in hydrogen bonds with water<sup>500</sup>. Asp<sup>55</sup> hydrogen bonds to the proton on the 6-hydroxyl of residue A, thus forcing the 6-hydroxyl to serve as a proton acceptor in its hydrogen bond with water<sup>500</sup>. As long as Asp<sup>55</sup> and Glu<sup>400</sup> are ionized, water<sup>500</sup> should bind with its lone pair orbitals directed toward the anomeric carbon of the substrate. In the mutation of Asp<sup>55</sup> to glycine (Sierks and Svensson, 1993) substrates bind to the enzyme with only a modest change in  $K_m$ , yet the mutant enzyme is inactive. Furthermore, the mutation of Asp<sup>55</sup> to asparagine (Itoh *et al.*, 1989) in a glucoamylase, closely related to the enzyme from *A. niger*, is inactive. We suggest that the loss of electrostatic charge and the misorientation of water<sup>500</sup> are principal factors in the absence of activity in these mutants.

A hydrogen bond between water<sup>500</sup> and the endocyclic oxygen of the nonreducing end of a true substrate is unlikely. Harris *et al.* (1993) noted that the chair conformation would place the endocyclic oxygen closer to the oxygen of water<sup>500</sup>, and thereby lead to an unfavorable nonbonded contact. This unfavorable nonbonded contact could be relieved by several mechanisms, one of which is a transition of the nonreducing end of the substrate to a half-chair conformation.

The observation of strain on the glucosidic linkage between residues B and C is also relevant to the catalytic mechanism.

The strain could be used in three ways to promote a catalytic turnover: 1) in the case of the true substrate, the strain could be transferred to the glucosidic linkage between residues A and B, thus promoting hydrolysis, 2) the strain may remain in the linkage between residues C and D, and be used to promote the release of products, and 3) a requirement for a strained linkage would screen against tight unproductive binding of extended substrates, as the interactions at subsite 1 would be necessary to offset the unfavorable energetics of the strain. The active site should not strain the glucosidic linkage of maltose, representing perhaps the origin of discrepancies in the kinetic parameters of maltose relative to other maltooligosaccharides of 3 or more residues (Sergeev and Firsov, 1985). Finally, on the basis of studies in chemical modification, kinetics, and directed mutation, investigators (Savel'ev and Firsov, 1983; Clarke and Svensson, 1984a, 1984b; Sierks *et al.*, 1989) have suggested that Trp<sup>120</sup> lies outside of the first two subsites of the active site and plays a significant role in the stabilization of the transition state. The interaction of the third residue of acarbose with Trp<sup>120</sup> and the observed strain in the linkage between residues B and C are entirely consistent with the findings of the above investigators. As pointed out by Aleshin *et al.* (1992), Trp<sup>120</sup> also interacts indirectly with subsites one and two by virtue of its hydrogen bond to Glu<sup>179</sup>.

Several investigators have partitioned the free energy of substrate binding among the various subsites of glucoamylase (Hiromi *et al.*, 1970; Tanaka *et al.*, 1983; Koyama *et al.*, 1984; Sergeev and Firsov, 1985; Meagher *et al.*, 1989). As noted by Hiromi *et al.* (1983) for glucoamylases from several sources, the binding of a glucose residue to subsite 1 returns essentially no free energy, and the binding of a glucose residue to subsite 2 returns a large negative free energy. These analyses tacitly assume the independent binding of the individual residues of the substrate to each of the subsites of the active site. Thus, the interaction of a glucose residue at subsite 3, for instance, should not influence the interactions felt by the residues occupying subsites 1 and 2. Our observations of uncompensated electrostatic charge (which represent long range columbic interactions) and the distortion of the linkage between residues B and C of acarbose (which clearly exacts a penalty on the stability of hydrogen bonds formed by residue A) contradict the assumption of independence. Thus, we regard the assignment of no free energy to the interaction of the substrate with subsite 1 and a large negative free energy to the interaction of the substrate with subsite 2 with caution.

Investigations in kinetics reveal a fast step followed by a slow step in the hydrolysis of maltose and in the binding of the single residue inhibitor gluconolactone. Tanaka *et al.* (1982a) suggest that the fast step reflects the weak binding of maltose to subsites 2 and 3, followed by a slow process whereby the substrate slides into subsites 1 and 2. Olsen *et al.* (1992) have suggested instead that the binding is initially to subsites 1 and 2, followed by a slow conformational change. The suggestion of a slow conformational change stems from the observation that gluconolactone also interacts with glucoamylase by way of a slow and a fast step. If the partitioning of free energy is correct, gluconolactone should bind and remain in subsite 2, thus necessitating another explanation (a conformational change) for the slow kinetic process.

Of the two explanations for the observed kinetics, we favor the interpretation of Tanaka *et al.* (1982a). First, we question the validity of partitioning the free energy among the various subsites for reasons stated above. Second, all crystallographic investigations thus far point to subsite 1 as the primary site of substrate-enzyme complexation. Third, we have yet to observe any significant conformational change in glucoamylase as a consequence of inhibitor binding. Finally, seven water molecules

bind firmly to subsite 1, six of which must be displaced by substrate. Aleshin *et al.* (1994) suggest that these water molecules exit the active site by way of a narrow channel. The expulsion of water to the bulk solvent by way of that channel should be a relatively slow process and could account for the observed slow step in the kinetics of substrate and inhibitor binding.

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