Crystallographic Complexes of Glucoamylase with Maltooligosaccharide Analogs: Relationship of Stereochemical Distortions at the Nonreducing End to the Catalytic Mechanism[†,‡]

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ABSTRACT: Crystal structures at pH 4 of complexes of glucoamylase from *Aspergillus awamori* var. X100 with the pseudotetrasaccharides D-*gluco*-dihydroacarbose and acarbose have been refined to *R*-factors of 0.147 and 0.131 against data to 1.7- and 2.0-Å resolution, respectively. The two inhibitors bind in nearly identical manners, each exhibiting a dual binding mode with respect to the location of the last sugar residues. The reduced affinity of D-*gluco*-dihydroacarbose ($K_i = 10^{-8}$ M) relative to acarbose ($K_i = 10^{-12}$ M) may stem in part from the weakening of hydrogen bonds of the catalytic water (Wat 500) to the enzyme. Steric contacts between the nonreducing end of D-*gluco*-dihydroacarbose and the catalytic water perturb Wat 500 from its site of optimal hydrogen bonding to the active site. Interactions within the active site displace the 6-hydroxymethyl group of the nonreducing end of both acarbose and D-*gluco*-dihydroacarbose toward a more axial position. In the case of D-*gluco*-dihydroacarbose the shift in the position of the 6-hydroxymethyl group occurs with a 12° change in two dihedral angles of the glucopyranose ring toward a half-chair conformation. The observed conformational distortion of the first residue of D-*gluco*-dihydroacarbose is consistent with the generation of a glucopyranosyl cation in the transition state. Comparable distortions of stereochemistry in model compounds require approximately 2 kcal/mol, not more than 25% of the energy necessary to form the half-chair conformation in glucose. The magnitude of stereochemical distortion observed in the active site of glucoamylase suggests that favorable electrostatic interactions between the putative glucopyranosyl cation intermediate and the active site must be more important in stabilizing the transition state than mechanical distortion of the substrate.

Evidence to date is consistent with the formation of a carbocation during the enzymic hydrolysis of glycosidic bonds (Sinnott, 1990; McCarter & Withers, 1995). Effective glycosidic inhibitors, for instance, usually place a positive charge in proximity to the site normally occupied by the glycosidic linkage, as well as a covalently linked ring of atoms constrained to a planar or half-chair conformation. Clearly two factors are involved in promoting the development of the carbocation intermediate: (1) stereochemical distortion of the substrate by the enzyme and (2) electrostatic stabilization of the positive charge of the intermediate. Complexes of lysozyme with substrate analogs have demonstrated substantial stereochemical distortions in the substrate (Strynadka & James, 1991; Kuroki *et al*., 1993; Hadfield *et al*., 1994). Lysozyme, however, is an endoglycosidase; no crystallographic evidence is available regarding the extent of stereochemical distortion in a substrate analog bound to an exoglycosidase. The extent to which exo- and endoglycosidases distort the stereochemistry of a carbohydrate may differ appreciably. The endoglycosidase in principle can exert more mechanical leverage through numerous low-energy interactions at both ends of the substrate.

Glucoamylase (α-1,4-D-glucanhydrolase, EC 3.2.1.3) is an inverting exohydrolase which catalyzes the removal of β-D-glucose from the nonreducing ends of starch and maltooligosaccharides (Weill *et al*., 1954; Pazur & Ando, 1960). Several structures of the proteolytic fragment of glucoamylase from *Aspergillus awamori* var. X100, ligated with different inhibitors (Aleshin *et al*., 1992a, 1994a,b; Harris *et al*., 1993, Stoffer *et al*., 1995), reveal that the active site of glucoamylase has excess negative charge derived from three residues of subsite 1, Glu 179, Glu 400, and Asp 55 (Aleshin *et al*., 1994a,b). Glu 179 is the putative catalytic acid (Sierks *et al*., 1990; Harris *et al*., 1993; Aleshin *et al*., 1994b) and Glu 400 is the putative catalytic base (Harris *et al*., 1993; Frandsen *et al*., 1994). The observation of a secondary isotope effect in the reaction catalyzed by glucoamylase (Firsov, 1978; Matsui *et al*., 1989; Tanaka *et al*., 1994), together with the dominant role of the catalytic acid over the catalytic base (Frandsen *et al*., 1994), are consistent

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[‡] Coordinates for the structures described in this paper have been deposited with the Brookhaven Protein Data Bank (accession references 1GAH and 1GAI).

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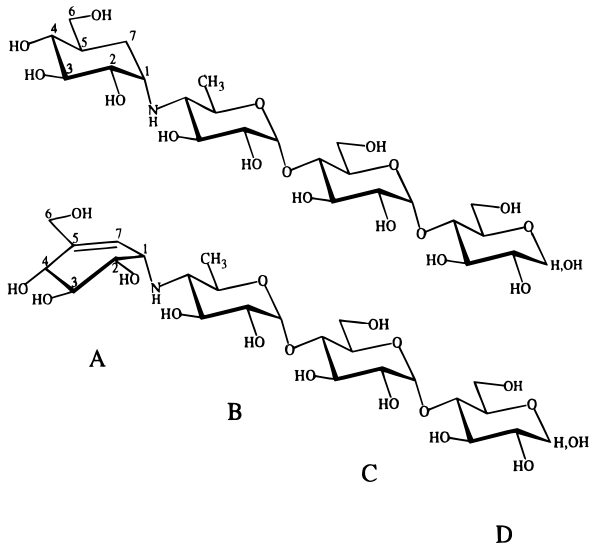

FIGURE 1: D-*Gluco*-dihydroacarbose (top) and acarbose with associated labels for residues and atoms.

with the formation of a glucopyranosyl cation during hydrolysis.

Subsite mapping investigations (Hiromi, 1970; Tanaka *et al*., 1982; Koyama *et al*., 1984; Sergeev & Firsov, 1985; Meagher *et al*., 1989) assign almost no free energy return to the substrate interaction at subsite 1 of glucoamylase. (Hydrolysis occurs at the glycosyl linkage between subsite 1 and subsite 2.) The lack of a substantial return of free energy from substrate−subsite 1 interactions, coupled with the good binding affinity of ligands constrained to a half-chair conformation, was taken as evidence for the distortion of the hexose residue bound to subsite 1. The binding affinities of a pair of related pseudotetrasaccharides, acarbose (K_d of 9×10^{-11} M) and D-*gluco*-dihydroacarbose (K_d of 3×10^{-7} M) (Svensson & Sierks, 1992; Sigurskjold *et al*., 1994), lend additional support to the premise of stereochemical distortions induced in substrates by glucoamylase. Acarbose (Figure 1) has a half-chair conformation, constrained by a double bond between C7 (corresponding to the endocyclic O5 of glucose) and C5, whereas D-*gluco*-dihydroacarbose lacks the double bond. Assuming that acarbose binds more tightly then D-*gluco*-dihydroacarbose because it mimics the putative glucopyranosyl cation intermediate, the 6 kcal/mol difference in the affinities of the two inhibitors again suggests that stereochemical distortion of the substrate is an important factor in the mechanism of glucoamylase.

Reported here are high-resolution crystallographic studies of glucoamylase, which explore the extent of stereochemical distortion as a function of pH for two related pseudotetrasaccharide inhibitors, acarbose and D-*gluco*-dihydroacarbose. Contrary to expections, distortions in the nonreducing end of ligands bound to the active site of glucoamylase are relatively small, suggesting that electrostatic charge stabilization of the transition state may be the dominant factor in promoting the formation of a glucopyranosyl cation in the active site of glucoamylase.

MATERIALS AND METHODS

Glucoamylase was prepared from *A. awamori* var. X100 as described by Neustroev and Firsov (1990). Conditions for the growth of fungus were adjusted so that glucoamylase I (M_r(app) = 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 observed heterogeneity of the protein, however, did not prevent the formation of good quality crystals.

Glucoamylase II(471) was crystallized from buffered poly(ethylene glycol), as described earlier (Golubev *et al*., 1992; Aleshin *et al*., 1992). Crystals belonging to the space group $P2_12_12_1$ grew over a period of 1−2 months to a size of 0.2−0.4 mm. Before soaking with ligands, the crystals used here were transferred to a solution containing 0.1 M sodium acetate (pH 4.0) and 30% poly(ethylene glycol) 6000 (Hampton Inc.).

D-*Gluco*-dihydroacarbose (kindly provided by K. Bock and M. Meldal) was produced from acarbose by reduction of the double bond (Bock *et al*., 1991). Thus, traces of acarbose (K_d 10^{-4}-fold less than that of D-*gluco*-dihydroacarbose) could be present in the sample of D-*gluco*-dihydroacarbose (K. Bock and M. Meldal, personal communication). In order to avoid artifacts due to an acarbose impurity, we performed an additional step of purification. Glucoamylase II from *A. awamori* was dissolved in a solution containing 5×10^{-4} M D-*gluco*-dihydroacarbose and 0.1 M sodium acetate, pH 4.0, so that its concentration was equal to half that of the inhibitor. After 1 day, this solution was filtered through an Amicon concentrator, using a membrane with a size exclusion limit of M_r = 30 000. Any initial contaminant of acarbose is reduced approximately by a factor of $(10^{-4})C_{ac}/C_{gac}$, where C_{ac} and C_{gac} are the initial concentrations of acarbose and D-*gluco*-dihydroacarbose, respectively, and the factor 10^{-4} is the ratio of their dissociation constants. The K_d (1.6×10^{-8} M) of the initial D-*gluco*-dihydroacarbose solution, measured by the method of Svensson and Sierks (1992) was the same as that of the filtrate. The K_d value agrees well with published results (Svensson & Sierks, 1992; Sigurskjold *et al*., 1994), suggesting that the initial acarbose impurity was well below 1 part in 10 and that the purified D-*gluco*-dihydroacarbose should have no more than 1 part acarbose in 10^5. The crystals were soaked 2 days in a solution containing 0.12 mM purified D-*gluco*-dihydroacarbose, 0.1 M sodium acetate, pH 4.0, and 30% poly(ethylene glycol) 6000. The volume of the solution (about 50 μL) was such that 3 equiv of ligand molecules were present for 1 equiv of glucoamylase in the crystal. Under these conditions, no more than 1 molecule of glucoamylase in every 10 000 will have acarbose, instead of D-*gluco*-dihydroacarbose, at the active site. Crystals were soaked with acarbose under the same conditions [0.12 mM acarbose, 0.1 M sodium acetate, pH 4.0, and 30% poly(ethylene glycol) 6000] but using a larger volume of soaking solution.

Data collection was carried out at Iowa State University on a Siemens area detector. The X-ray data for both complexes were collected at room temperature from single crystals. Data reduction was done with XENGEN (Howard *et al*., 1985). Data collection statistics are in Table 1.

A Silicon Graphics 4D25 and the program TOM (Cambillau & Horjales, 1987) were used for model building. The starting model for each complex was the 2.2-Å resolution structure of native glucoamylase (Aleshin *et al*., 1994a), including 214 water molecules of the native structure that

Table 1: Statistics of Data Collection and Refinement for the Complexes of D-*gluco*-Dihydroacarbose and Acarbose with Glucoamylase

	D-*gluco*-dihydroacarbose complex	acarbose complex
cell parameters ($P2_12_12_1$) (Å)		
a	116.9	116.8
b	104.4	104.1
c	48.4	48.4
resolution limit (Å)	1.69	2.0
no. of measurements	298 712	87 952
no. of unique reflcns	62 713	34 394
completeness of data set (%)	96	85
completeness of data in the last resolution shell (%)	75 (1.8−1.7 Å)	54 (2.1−2.0 Å)
R_{sym}	0.055	0.057
refinement range (Å)	10−1.7	10−2.0
no. of reflcns in refinement[b]	61 302	34 143
total no. of atoms	4631	4537
total no. of solvent sites	571	544
R factor[c]	0.147	0.131
mean *B* (Å^2) for protein and glycans	11.7	11.5
mean *B* (Å^2) for inhibitor		
residue A	5.2	4.3
residue B	6.1	5.6
residue C	12.5 (9.6)[d]	13.8 (7.2)[d]
residue D	23.8 (24.3)[d]	22.7 (21.6)[d]
conformer occupancies	0.63 (0.37)[d]	0.53 (0.47)[d]
RMS deviations		
bond lengths (Å)	0.008	0.008
bond angles (deg)	1.33	1.35

[a] $R_{sym} = \sum_j\sum_i|I_{ij} - \langle I_j\rangle|/\sum_i\sum_{ij}$, where *i* runs over multiple observations of the same intensity and *j* runs over all crystallographically unique intensities. [b] Only reflections with $|F_{obs}| > 1\sigma|F_{obs}|$ were used in refinement. [c] *R* factor $= \sum||F_{obs}| - |F_{calc}||/\sum|F_{obs}|, |F_{obs}|\rangle$ 0. [d] Value for the low-weight (pH 6-type) conformers.

had thermal parameters below 30 Å^2 and deviated in position by less than 0.4 Å from corresponding water molecules of the acarbose−glucoamylase complex at pH 6 (Aleshin *et al*., 1994b).

We used XPLOR (Brunger, 1992) in the refinement of both complexes of glucoamylase. Constants of force and geometry for the protein came from Engh and Huber (1991) and for the carbohydrates from Weis *et al*. (1990). Geometry and force constants for *O*-glycosylated serine and threonine and residues C and D of D-*gluco*-dihydroacarbose and acarbose were drawn from the above sets as well, using atoms of amino acids and sugars of corresponding type. The constants of force and geometry for residue A of D-*gluco*-dihydroacarbose were as those for glucose with the substitution (in residue A) of the endocyclic oxygen by a carbon of the same type as other carbons of glucose. The constants of geometry for the valienamine moiety of acarbose were obtained by averaging two models, one of which was generated by using QUANTA (Molecular Simulations, Inc.) and the other by replacing the appropriate hydrogens with hydroxyls in the X-ray structure of a methylcyclohexane derivative (Beckhaus *et al*., 1978). The positions of corresponding atoms in the two models differed by less than 0.1 Å.

The force constants for the valienamine moiety were largely the same as for glucose. Force constants for the torsion angle C4−C5−C7−C1 and improper angles C4−C5−C7−C6 and C5−C7−C1−H7, however, were set to 300 kcal mol^{-1} deg^{-1}, identical to the value for improper angles of chiral centers in glucose. A test refinement was made, in order to determine the sensitivity of the conformation of the valienamine moiety to the above force constant. For the purpose of the test only, the force constants for the angles C4−C5−C7−C1 and C4−C5−C7−C6 of acarbose were increased to 750 kcal mol^{-1} deg^{-1} (as in the phenylalanine side chain), and a cycle of simulated annealing with a starting temperature of 450 K was performed. The refined models differed insignificantly from each other regardless of whether a force constant of 300 or 750 kcal mol^{-1} deg^{-1} was used.

A test of the influence of force constants on observed stereochemistry was performed on the D-*gluco*-dihydroacarbose complex of glucoamylase. In this case, all force constants were reduced by 3-fold. After refinement the conformation of residue A of D-*gluco*-dihydroacarbose changed insignificantly.

All refinement was done on a 4D-35 Silicon Graphics workstation and proceeded over the course of 5 cycles, in the case of the D-*gluco*-dihydroacarbose complex, and 4 cycles, in the case of the acarbose complex. Each cycle included steps of simulated annealing, refinement of positional and individual isotropic *B*-parameters, and refinement of occupancy parameters for specific disordered residues. Initial cycles employed a slow-cool protocol with a starting temperature of 2000 K, whereas final cycles used a temperature of 350 K and harmonic restraints (50 kcal/mol) for the positions of oxygens in water molecules. The simulated annealing step with positional restraints on the oxygen atoms of water molecules allowed new water molecules to relax by adjustments in orientation. The conjugate gradient refinement of positional parameters after slow cooling was made without harmonic restraints on the positions of water molecules.

Criteria for the building of the water structure and side chains in alternative conformations were the same as in previous studies (Aleshin *et al*., 1994a,b). Any sites for water molecules within a distance of 2.45 Å of each other were considered a mutually exclusive set of sites for a single water molecule. In these cases, pairs of water molecules were refined together without nonbonded interactions. The alternative conformers for D-*gluco*-dihydroacarbose and acarbose were included in the refinements beginning with the second cycle. Two approaches were used to model and refine the disorder of the ligand: (i) two copies of the entire inhibitor molecule were used and (ii) sugar residues A and B were common for the two alternative conformations and only copies of residues C and D were used. As the two treatments of inhibitor disorder gave indistinguishable results, we report here the model that resulted from the second approach. Only the occupancies for residues with alternative conformations were refined and then normalized to unity.

In order to estimate the extent to which glucoamylase alters the conformation of residue A of pseudotetrasaccharides when they bind to the active site, reliable models for the "relaxed" inhibitors are essential. We developed a model for the relaxed conformation of residue A of acarbose and D-*gluco*-dihydroacarbose by averaging, respectively, the crystal structures of five different compounds containing the methylcyclohexane moiety and eight compounds containing the methylcyclohexane moiety, two of which contained oxygens linked to the C1, C2, C3, and C4 atoms. Structures of the cyclohexane moiety with the identifiers VERKAE, FOBMAK, DEPCGUA, DEPGUA01, DCYHCH, CUF-

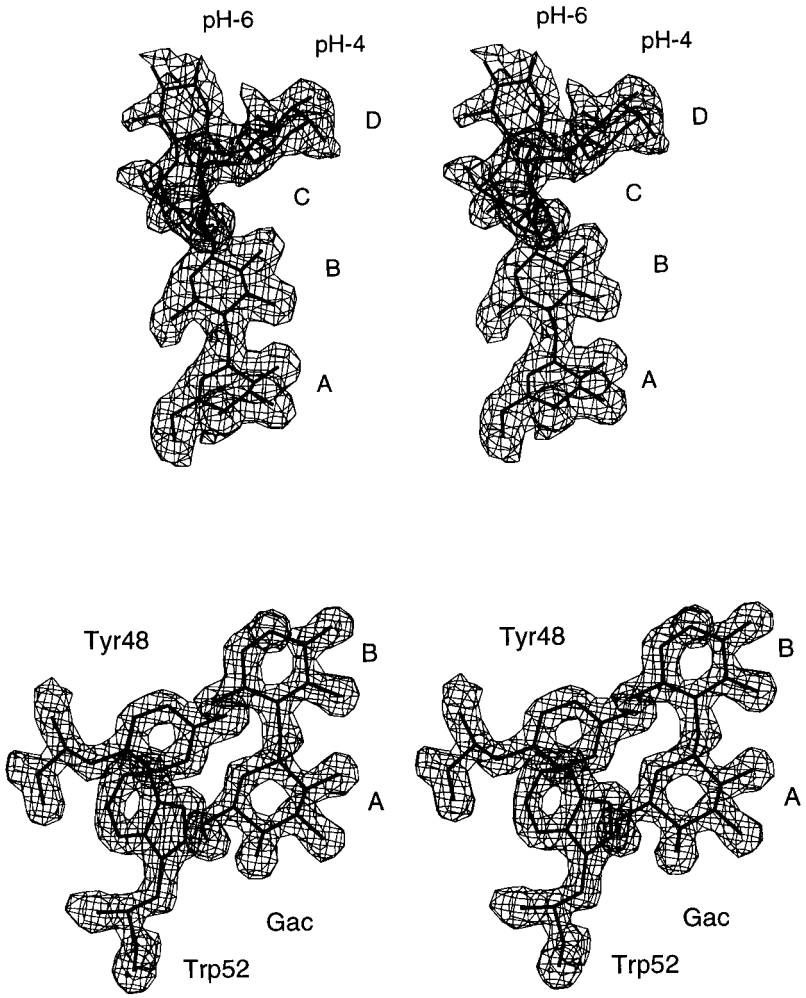

FIGURE 2: Stereoview of ($F_{obs} - F_{calc}$)exp($i\alpha_{calc}$) electron density associated with (top) two conformers of acarbose at a contour level of 2.5σ and (bottom) a selected region of the active site of glucoamylase containing residues A and B of D-*gluco*-dihydroacarbose (labeled as Gac) at a contour level of 12σ.

WUV, CUDHEO, and CUFWEF were taken from the Cambridge Structural Database. Structures of the cyclohexane moiety came from the Cambridge Structural Database (DCHXYKO1, CYHBUO, DMCYBA, and DAMDUQ) and from Beckhaus *et al.* (1978). All dihedral angles deviate from their mean values by less than 4°. The ideal models of residue A used in our refinement deviate from these averaged structures to the same extent.

RESULTS AND DISCUSSION

General Features of the Refined Structures. Models for the complexes of glucoamylase with D-*gluco*-dihydroacarbose and acarbose have been deposited with the Protein Data Bank, Brookhaven National Laboratory. In conformance with prior submissions (Aleshin *et al*., 1992, 1994a,b; Harris *et al*., 1993), we have adopted the amino acid numbering of glucoamylase from *Aspergillus niger* (Svensson *et al*., 1983; Boel *et al*., 1984). Thus, the sequence numbers skip from 101 to 103 in the deposited coordinate sets.

The amino acid sequence used in the refinement of the D-*gluco*-dihydroacarbose complex had two additional residues on the C-terminus, in comparison to previous models of glucoamylase (Aleshin *et al*., 1992, 1994a,b; Harris *et al*., 1993). The additional electron density at the C-terminus of the acarbose complex, however, permitted the addition of only one residue. The final *R*-factor for the D-*gluco*-dihydroacarbose complex is 0.147 against all data between 10.0- and 1.7-Å resolution. The *R*-factor for the acarbose complex is 0.13 for all data between 10.0- and 2.0-Å resolution. Refinement statistics for the complexes of glucoamylase with D-*gluco*-dihydroacarbose and acarbose are in the Table 1. A Luzzati analysis (1952) infers an overall root-mean-square uncertainty in atomic coordinates of 0.15 Å. Analysis of both models with PROCHECK (Laskowski *et al*., 1993) indicates stereochemistry that is significantly better than expected for structures of 1.7- and 2.0-Å resolution. Electron density in the region of the active site is presented in Figure 2.

Thermal parameters for the refined structures are low, but not aberrant. A Wilson plot of the diffraction data for the D-*gluco*-dihydroacarbose complex (not shown) is explained well by a linear fit over the resolution range of 10−1.7 Å. The slope of the Wilson plot predicts an overall thermal parameter that is within 0.5 Å^2 of the average thermal parameter reported from refinement (Table 1). The low thermal parameters are indicative of a well-ordered crystal, rather than a systematic error in the data. The low *R*-factor, which includes essentially all the data from 10 to 1.7 Å coupled with the superb stereochemistry of the model, is a consequence of statistically reliable measurements of intensity out to 1.7-Å resolution.

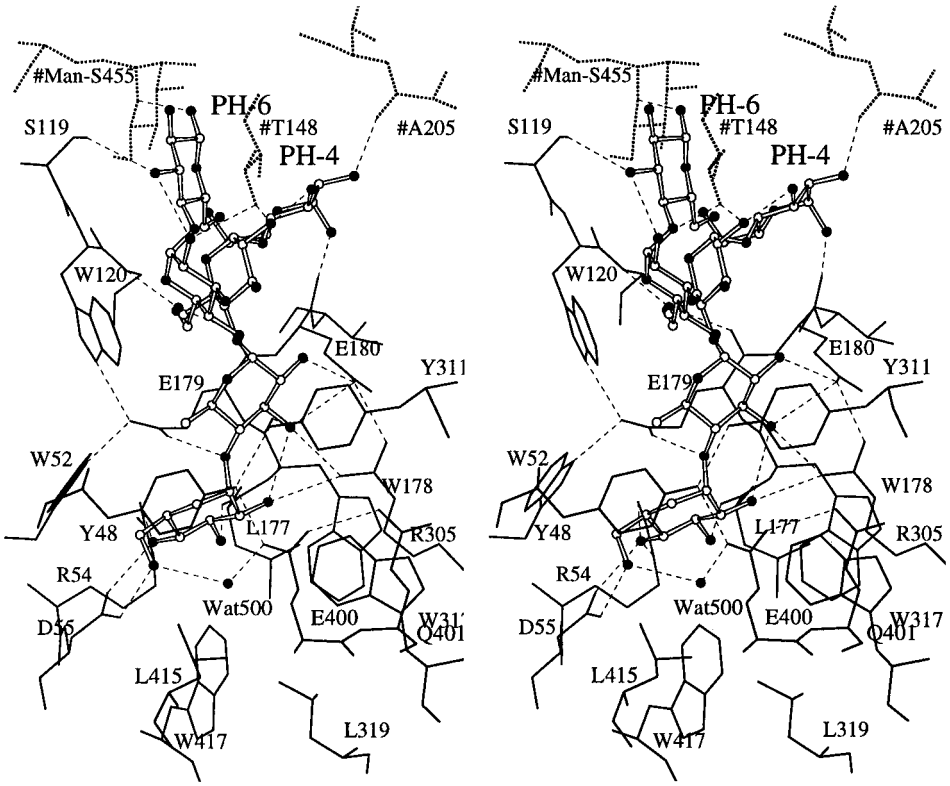

FIGURE 3: Stereoview of the two conformations of D-*gluco*-dihydroacarbose in its complex with glucoamylase. D-*Gluco*-dihydroacarbose is represented as a ball-and-stick model, with carbons shown as open circles and oxygens and nitrogens as filled circles. Residues from symmetry-related molecules of glucoamylase are shown in dotted lines. Hydrogen-bonded contacts are shown in dashed lines. This figure and Figures 4 and 5 were prepared using MOLSCRIPT (Kraulis, 1991).

In general, glucoamylase undergoes little conformational change in response to the binding of the pseudotetrasaccharide inhibitors, consistent with previous results (Harris *et al*., 1993; Aleshin *et al*., 1994b; Stoffer *et al*., 1995). The root-mean-square deviation in the position of all atoms between the D-*gluco*-dihydroacarbose complex and the native enzyme is about 0.2 Å. The 14 side chains which have at least one atom within 3.5 Å of residues A, B, C, and D of the inhibitors have root-mean-square deviations in atomic positions of 0.23 Å against the native enzyme (pH 4 and 6) and 0.13 Å against the acarbose-ligated enzyme at pH 6. The two new structures at pH 4 deviate from each other by 0.11 Å for all atoms and by 0.07 Å for residues of the active site. Similarities between the two new models extend to the solvent structure. Ninety-two percent of the water molecules of the D-*gluco*-dihydroacarbose complex fall within 1.0 Å of the solvent sites for the acarbose complex with an overall root-mean-square deviation of 0.32 Å and a root-mean-square deviation in their *B*-parameters of 8.0 Å^2.

pH-Dependent Binding of Pseudotetrasaccharide Inhibitors to the Active Site. Residues A and B of acarbose bind in the same locations in its pH 6- and pH 4-complexes, exhibiting root-mean-square deviations for coordinates of residues A and B equal to 0.14Å and 0.08Å, respectively. In the pH 4 complex, however, residues C and D exhibit a dual binding mode (Figure 3, Tables 2 and 3). The weights of the two conformers of acarbose, as determined by refined occupancies, are 0.53 and 0.47, the low-weight conformer corresponding to the observed conformation of acarbose in its pH 6 complex (Aleshin *et al*., 1994b). The high-weight conformer exhibits a shift in the center of mass of residue D of about 5 Å relative to that of residue D of the minor conformer (Figure 3). Differences in the conformation of the high-weight conformer at pH 4 and the single conformer, observed at pH 6, begin with the dihedral angles between residues B and C (Table 4). The large rotation about the dihedral angle ϕ in the linkage between residues B and C, however, is offset by a change in the angle ψ of the same linkage, so that the position and interactions of residue C with the protein change only to a small extent. The observation of a single conformer for the acarbose complex at pH 6 (Aleshin *et al*., 1994b), coupled with the observed disorder phenomenon for the acarbose complex at pH 4, is compeling evidence for a pH-induced conformational change in the ligand. The p*K* governing the equilibrium between the two conformations is closer to 4 (the pH optimum of the enzyme) than to 6. In further discussions we name the conformers according to the pH at which they appear with maximum weight, namely, the pH 4-type and pH 6-type conformers.

D-*Gluco*-dihydroacarbose exhibits almost an identical disorder phenomenon in its complex with glucoamylase at pH 4; the positions of residues C and D of both conformers of D-*gluco*-dihydroacarbose deviate from those of acarbose by less than 0.2 Å. The weights of conformers in the D-*gluco*-dihydroacarbose complex are 0.63 and 0.37. The striking similarity in the structures of the two inhibitor complexes at pH 4 indicates that the disorder phenomenon, first observed by Stoffer *et al*. (1995), is not due to differences in the covalent structures of acarbose and D-*gluco*-dihydroacarbose.

Both the pH 4- and pH 6-type conformers have lattice contacts between their C and D residues and a neighboring glucoamylase molecule (Table 2). Nonetheless, each conformation is probable for the binding of maltotetraose to glucoamylase in solution. The dihedral angles of the

Table 2: Contacts Less than 3.3 Å Involving Polar Atoms of Acarbose and D-*gluco*-dihydroacarbose[a]

atom of the inhibitor	bridging water[b]	contact atom	distance (Å): D-*gluco*-dihydroacarbose complex (high/low-weight conformer)	distance (Å): acarbose complex (high/low-weight conformer)
O2A		Arg 305 NH1[c]	3.05	2.99
		inhibitor O3B[d]	3.16	3.09
O3A		carbonyl 177 O	2.71	2.73
O4A		Asp 55 OD1	2.68	2.80
		Arg 54 NH2	2.93	2.86
		Arg 54 NE	3.09	3.12
O6A		Asp 55 OD1	2.81	2.77
		Wat 500	3.01	2.91
	Wat500	Glu 400	2.92	2.87
O2B		Glu 180 OE2	2.73	2.68
O3B		carbonyl 178 O	2.68	2.69
		Arg 305 NH1	2.81	2.74
		Glu 180 OE2[e]	3.15	3.12
		inhibitor O2A[d]	3.16	3.09
N4B		Glu 179 OE1	2.66	2.66
O2C		Thr 148 OG1[f]	2.50/2.92	2.53/2.95
		inhibitor O3D	2.99/3.36	2.81/3.35
O5C		Wat 758[g,h]	3.02/−	−/−
	Wat 758[g,h]	inhibitor O6D	2.86/−	−/−
	Wat 758[g,h]	mannose-Ser 455 O6[f]	2.98/−	−/−
O6C		carbonyl 179	2.82/2.88	2.88/2.81
		amide 121	2.82/2.85	2.76/2.80
O1D[g]		carbonyl 180	2.71/−	2.81/−
		mannose-Ser 455 O5[f]	−/3.02	−/3.13
		Wat 739	−/2.66	−/2.88
	Wat 739	mannose-Ser 453 O3[f]	−/2.85	−/2.97
	Wat 739	mannose-Ser 455 O5[f]	−/3.19	−/3.00
O2D		carbonyl 205[e]	2.89/−	2.94/−
		Wat 739	−/2.90	−/3.00
	Wat 739	mannose-453 O3[f]	−/2.85	−/2.97
	Wat 739	mannose-455 O5[f]	−/3.25	−/3.00
O3D		inhibitor O2C	3.02/3.36	2.81/3.35
		Ser 119 OG[f]	−/3.19	−/2.95
O6D		mannose-Ser 454 O6[e]	2.75/−	2.77/−

[a] Values listed represent donor−acceptor distances between hydrogen-bonded atoms unless noted otherwise. [b] Only waters with *B*-parameters less than 30 Å^2 are listed. [c] Poor hydrogen-bond angle. [d] Hydrogen bond unlikely because of a close contact between hydrogen H41 of the imino linkage and hydrogen atoms on O2A or O3B. [e] Hydrogen bond possible only if Glu 180 is protonated. Atom O3B donates its proton to carbonyl 178. [f] Crystal lattice contact. [g] Atom partially occupied in both crystal structures. [h] Wat 758 present only in the D-*gluco*-dihydroacarbose complex, pH 4-type conformer.

glycosyl linkages between residues B and C and between residues C and D in the pH 6-type conformer are close to the minimum energy values for the corresponding linkage in maltose (Table 4). The same glycosyl linkages in the pH 4-type conformer deviate from the two preferred conformations of maltose but still correspond to a low-energy conformation (Dowd *et al*., 1992).

The pH-dependent interaction of acarbose and D-*gluco*-dihydroacarbose with glucoamylase is an unanticipated phenomenon, suggesting the influence of an ionizable group with a pK_a near 4. Possible candidates for this ionizable group are the imino group of the inhibitor and/or side chains of the protein in proximity to residues C or D. The strong interaction between the imino group and Glu 179 at pH 4 and at pH 6 (Table 2) suggests that the imino group is protonated throughout the pH range 4−6. On the other hand, neither residue C nor residue D of the bound inhibitors is in direct contact with a carboxylate or a histidine. In fact, the closest ionizable side chains are Glu 180, which is involved in the binding of residue B, and Glu 168 of a symmetry-related molecule of glucoamylase. Glu 180 is 7 and 11 Å from the centroids of residues D of the pH 4- and pH 6-type conformers, respectively, whereas Glu 168 is 12 and 9 Å from the same centroids. The pH-dependent conformational change in the inhibitors may stem from the perturbation on the solvent structure in the vicinity of residues C and D, caused by the ionization/protonation of Glu 180 and/or Glu 168. The solvent structure in the vicinity of residues C and D of the ligands is essentially disordered and, thus, does not allow an atomistic interpretation of the relationship between the states of ionization of Glu 180 and/or Glu 168, the solvent structure, and the conformation of the ligands at different pH. Although subsite mapping indicates approximately six subsites for the active site of glucoamylase, perhaps only the first two subsites are common to all maltooligosaccharides and their chemically inert analogs. The location of additional subsites is subject to relatively modest variations in the physical conditions under which the enzyme is studied.

Structural Basis for the Decreased Binding Affinity of D-*Gluco-dihydroacarbose Relative to Acarbose*. In spite of the large discrepancy in affinity, both acarbose and D-*gluco*-dihydroacarbose have nearly identical interactions at subsites 1 and 2 (Table 2 and 3, Figure 4). The root-mean-square deviation in the positions of all carbon and oxygen atoms in residue A of acarbose and D-*gluco*-dihydroacarbose, excluding C5A and C7A, is 0.15 Å, and the root-mean-square deviation of atoms of side chains in contact with residue A is 0.08 Å. The 0.39-Å shift of C5A in the D-*gluco*-dihydroacarbose complex from its corresponding position in the acarbose complex is relatively large, then, but causes no

Table 3: Nonbonded Contacts Involving Wat 500 and Pseudotetrasaccharide Inhibitors of Glucoamylase Complexes at pH 4[a]

atom of the ligand	atom of the enzyme	distance (Å): D-*gluco*-dihydroacarbose complex (high/low-weight conformer)	distance (Å): acarbose complex (high/low-weight conformer)
		Wat 500	
O	inhibitor C7A	3.41	3.43
	Trp 317 CZ3	3.37	3.39
	Leu 415 CD2	3.54	3.59
	inhibitor C1A	3.82	3.72
		Inhibitor	
C7A	Wat 500	3.41	3.43
	Thr 48 OH	3.46	3.56
	inhibitor C6B	3.40	3.23
C1A	Wat 500	3.82	3.72
	Glu 400	3.98	4.02
C5A	Wat 500	3.83	3.23
C6A	Trp 52 CE2	3.56	3.58
C3B	carbonyl 178 O	3.21	3.22
C5B	Glu 179 OE1	3.29	3.27
O2C	Thr 148 CG2[b]	3.22/−	3.19/−
O5C	Trp 120 CE3	−/3.01	−/3.09
	Trp 120 CZ3	−/3.19	−/3.34
	Trp 120 CD2	−/3.28	−/3.35
C2C	Thr 148 OG1[b]	3.12/−	3.06/−
C6C	carbonyl 179 O	−/3.29	−/3.26
	Glu 179 CB	−/3.42	−/3.38

[a] All contacts between carbon and oxygen atoms less than 3.3 Å and between carbon atoms less than 3.5 Å are listed as well as selected distances of significance to the catalytic mechanism of glucoamylase. [b] Crystal lattice contact.

Table 4: Values for Conformational Parameters of Linkages in Acarbose and D-*Gluco*-dihydroacarbose in Their Complexes with Glucoamylase[a]

linkage	ϕ_H H1−C1−O1−C4′ (deg)	ψ_H C1−O1−C4′−H4′ (deg)	τ C1−O1−C4′ (deg)	O3−O2′ (Å)
		D-*Gluco*-dihydroacarbose Complex, pH 4		
A−B	−17	5	115	3.16
B−C	−21 (−69)	−67 (−50)	123 (120)	4.06 (4.83)
C−D	−11 (−30)	9 (−18)	118 (117)	3.02 (3.36)
		Acarbose Complex, pH 4		
A−B	−6	7	115	3.09
B−C	−25 (−71)	−62 (−47)	122 (120)	4.09 (4.94)
C−D	−5 (−25)	3 (−19)	118 (118)	2.81 (3.35)
		Acarbose Complex, pH 6[b]		
A−B	−6	7	114	2.97
B−C	−66	−53	126	4.99
C−D	−25	−17	117	3.18
		α-Maltose[c]		
conformer I	−23	−22		
conformer II	−55	−48		

[a] Values in parentheses are for the low-weight (pH 6-type) conformer. [b] Aleshin *et al*. (1994b). [c] Dowd *et al*. (1992).

bad van der Waals contacts with the protein. The 0.27-Å shift of C7A toward Wat 500 is the more likely cause for the reduction in binding affinity of D-*gluco*-dihydroacarbose relative to acarbose. However, it alone cannot explain the 10 000-fold drop in affinity, because Wat 500 relaxes its repulsion with C7A by a compensating movement of 0.20 Å.

The significant difference in the binding affinity of D-*gluco*-dihydroacarbose and acarbose, however, may lie with the interactions of two hydrogens which appear in D-*gluco*-dihydroacarbose after the reduction of the double bond in ring A of acarbose. In particular, the additional hydrogen on C7A of D-*gluco*-dihydroacarbose lies on a line between the oxygen of Wat 500 and C7A at a distance of 2.4 Å from the oxygen. The minimum van der Waals distance between hydrogen and oxygen atoms before repulsive forces dominate attractive forces can be as small as 2.2 Å (Melberg *et al*., 1979). However, the hydrogen atoms of Wat 500, if aligned optimally in linear hydrogen bonds with atom O6A of the inhibitor and OE1 of Glu 400, make close contacts (2.0 and 2.2 Å, respectively) with the additional hydrogen atom on C7A. According to *ab initio* calculations of nonbonded interactions in glucose (Melberg *et al*., 1979), two hydrogen atoms separated by 2.0 Å generate an energy of repulsion of approximately 20 kcal/mol. Thus, the presence of the additional hydrogen on C7A of D-*gluco*-dihydroacarbose should perturb Wat 500 by repulsive interactions with its hydrogen atoms. In fact, in the refined D-*gluco*-dihydroacarbose complex Wat 500 has been displaced (a shift of 0.2 Å), with its distance to O6A of the inhibitor increased by 0.1 Å. More significantly, the thermal parameter of Wat 500 increases dramatically from 5.0 Å^2 in the acarbose complex to 12.9 Å^2 in the D-*gluco*-dihydroacarbose complex, implying that Wat 500 is less tightly held

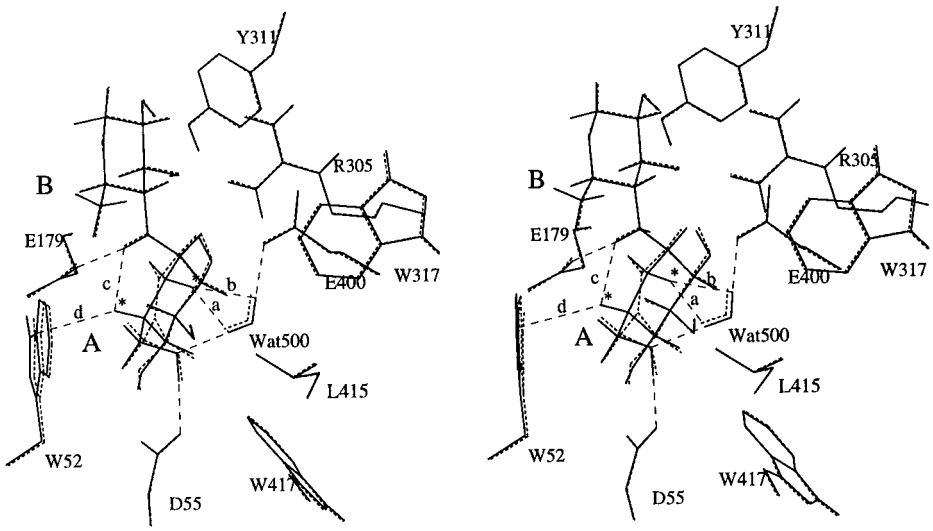

FIGURE 4: Stereoview of the acarbose complex (dotted lines) superimposed on the D-*gluco*-dihydroacarbose complex in the region of residues A and B of the ligands. Hydrogen atoms of the ligands and hydrogen atoms of polar groups of the protein are shown. Hydrogen atoms of D-*gluco*-dihydroacarbose that are absent in acarbose are marked with asterisks. Hydrogen bonds mentioned in the text are shown as dashed lines with associated letters that correspond to the following distances in angstroms: $a = 2.0$, $b = 2.2$, $c = 2.4$, and $d = 3.1$.

Table 5: Comparison of Selected Torsion Angles

dihedral angle	lysozyme complex residue D[a]	glucose	Gac[c] complex residue A	Gac relaxed[d] residue A	acarbose complex residue A	acarbose relaxed[d] residue A
C1−C2−C3−C4	−61	−53	−64	−56	−63	−61
C2−C3−C4−C5	64	53	60	55	46	47
C3−C4−C5−C6		−175	−172	180	177	163
C3−C4−C5−O5/C7	−47	−55	−49	−55	−12	−16
C4−C5−O5/C7−C1	24	61	44 (42)[e]	56	−5	−2
C5−O5/C7−C1−C2	−22	−62	−47 (−46)[e]	−58	−12	−13
O5/C7−C1−C2−C3	43	56	56	57	45	43

[a] Residue D of the lysozyme complex of Hadfield *et al*. (1994). [b] Arnott and Scott (1972). [c] Gac = D-*gluco*-dihydroacarboase. [d] Relaxed conformations of acarbose and D-*gluco*-dihydroacarbose are derived from model structures as described in the text. [e] Values in parentheses result from refinement with force constants reduced by a factor of one-third.

in the D-*gluco*-dihydroacarbose complex than in the acarbose complex. Nor is the increase in thermal parameter an artifact of refinements against data sets of differing resolution. The thermal parameter for Wat 500 in the D-*gluco*-dihydroacarbose complex, when refined against data to 2.0-Å resolution (the same resolution as the acarbose complex), increases marginally to 13.5 Å^2. Furthermore, well-defined water molecules (*B*-parameters less than 15 Å^2) common to both the acarbose and D-*gluco*-dihydroacarbose complexes exhibit a root-mean-square deviation in thermal parameters of only 1.5 Å^2. Thus, the comparison of the two structures suggests that the perturbation of Wat 500 from its site of optimal hydrogen bonding, caused by the shift of C7A and amplified by the addition of a hydrogen atom in D-*gluco*-dihydroacarbose, is the most apparent cause for the reduced affinity of D-*gluco*-dihydroacarbose.

Conformational Distortion of Residue A by the Active Site. The comparison of dihedral angles of residue A of bound D-*gluco*-dihydroacarbose to the angles of its relaxed conformer reveals a decrease in the absolute values of the dihedral angles C5−C7−C1−C2 and C4−C5−C7−C1 (Table 5). The superposition of the relaxed model of D-*gluco*-dihydroacarbose onto its refined model demonstrates that the decrease in the dihedral angles, named above, is associated with the shift of atoms C6A and O6A toward a more axial position with respect to ring A. The relaxed model of D-*gluco*-dihydroacarbose, positioned in the active site of glucoamylase (Figure 5), makes close contacts with Trp 52 (carbon-to-carbon distance of 3.0 Å) and lacks the hydrogen bond to Wat 500 (donor−acceptor distance of 3.5 Å). These two interactions are apparently the major cause for the shift of the 6-hydroxymethyl group of residue A. A similar shift in the 6-hydroxymethyl group, observed for acarbose, is caused by the distortion of a different dihedral angle, C3−C4−C5−C6 (Table 5).

Deformation of the pyranose ring as a means of promoting a carbocation intermediate/transition state has been debated for a quarter of century (Phillips, 1967; Blake *et al*., 1967; Vernon, 1967; Thoma, 1968; Imoto *et al*., 1972; Ford *et al*., 1974; Warshel & Levitt, 1976; Post & Karplus, 1986; Strynadka & James, 1991) but has been observed directly only in the crystal structure of lysozyme complexed with oligosaccharides (Strynadka & James, 1991; Kuroki *et al*., 1993; Hadfield *et al*., 1994). In spite of differences in the mechanisms of glucoamylase and lysozyme (inverting vs retaining) and their substrates (α-1−4-linked glucosides vs *β*-1−4-linked muramic acid and *N*-acetylglucosamine), the observed distortions in stereochemistry have similar features, the most notable of which is the displacement of the 6-hydroxymethyl group toward a more axial position in the D-*gluco*-dihydroacarbose and acarbose complexes. Moreover, in both enzymes the interactions of the 6-hydroxymethyl group within the active site are putatively the main cause of the distortion. Although the distortions are qualitatively similar, they differ quantitatively. The dihedral angle C5−C7−C1−C2 of the D-*gluco*-dihydroacarbose complex

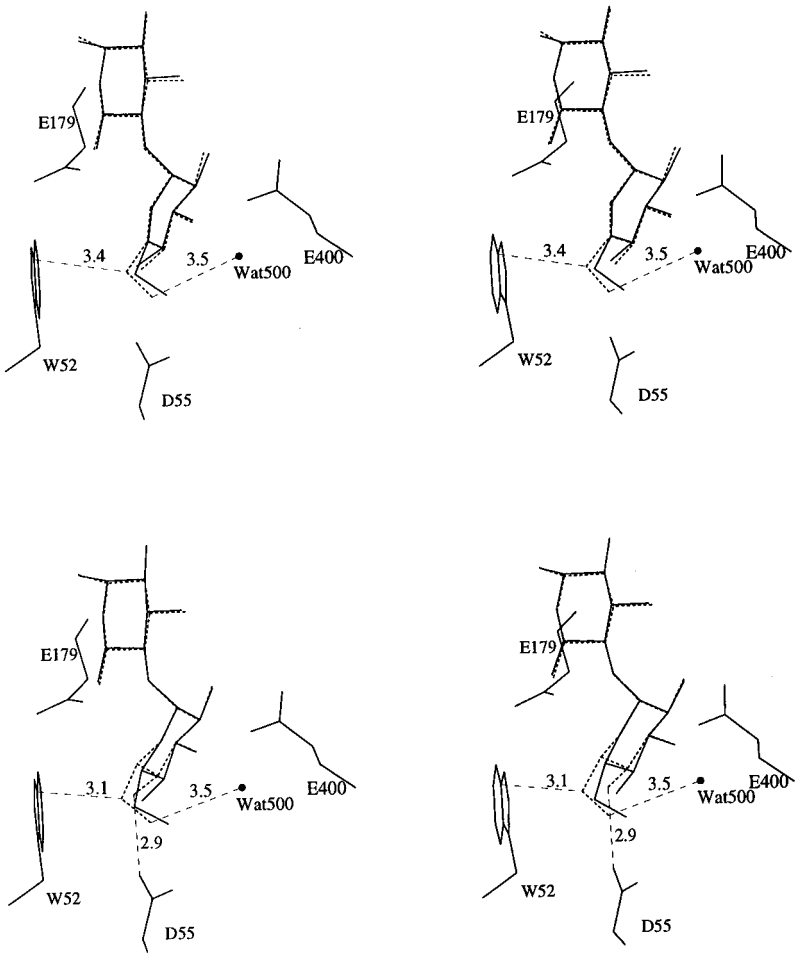

FIGURE 5: Stereoview of the "relaxed" models (dotted lines) of (top) acarbose and (bottom) D-*gluco*-dihydroacarbose superimposed on corresponding models (solid lines) from their refined complexes. Only residues A and B are shown. Dashed lines (with distances in angstroms) indicate contacts responsible for the deformation of residue A.

changes from its relaxed conformer by only 12° to a value of −47°, whereas its value in the lysozyme complex is −22°. The same angle in the glucopyranosyl cation, on the basis of the structure of δ-gluconolactone (Hackert & Jacobson, 1971), should approach −15°. Even when stereochemical restraints are relaxed, as described in the Materials and Methods section above, the torsion angle C5−C7−C1−C2 changes to −46°, indicating that stereochemical restraints have little influence on the refined model in the region of the active site.

Torsion angles of residue A of the D-*gluco*-dihydroacarbose complex differ significantly from those observed in oligosaccharide crystals (Arnott & Scott, 1972) and are characterized by 12° decreases in the absolute values of torsion angles C4−C5−C7−C1 and the adjacent C5−C7−C1−C2. The calculation of relative energies associated with different glucopyranosyl conformations (Joshi & Rao, 1979; Dowd *et al*., 1994), supported by experimental data (Squillacote *et al*., 1975), demonstrates that the half-chair conformation of glucose occurs at the transition between the chair and boat conformers with a relative energy of 9−12 kcal/mol. The energy of deformation of glucose into the conformation of residue A of the D-*gluco*-dihydroacarbose complex is approximately 2 kcal/mol and into the conformation of δ-gluconolactone approximately 8.0 kcal/mol (M. K. Dowd, personal communication). The deformation in the ring of the D-*gluco*-dihydroacarbose complex in energy terms then is at best 25% of the way toward a half-chair conformation, the putative conformation of the transition state. Lysozyme apparently deforms the pyranose ring more effectively than does glucoamylase (Table 5), because it perhaps exercises greater leverage over the ligand by interacting with the 2-acetamide and glycan extensions from ring D.

Of the 6 kcal/mol difference in the binding energies of D-*gluco*-dihydroacarbose and acarbose, only approximately 2 kcal/mol can be attributed to the diversion of binding energy to the distortion of the inhibitor. Perhaps as much as 4 kcal/mol is lost, then, by poor steric interactions between the hydrogen atom on C7A of D-*gluco*-dihydroacarbose and the catalytic water. These unfavorable steric interactions should be reduced in the case of a true substrate, as there is no atom analogous to the hydrogen on C7A in maltooligosaccharides. Thus, the D-*gluco*-dihydroacarbose complex may represent an upper limit in the level of "steric stress" imposed by glucoamylase on a real substrate.

As the energy associated with the observed distortion represents only $^1/_4$ of the energy necessary to achieve a half-chair conformation, electrostatic interactions may be the most significant factors in stabilizing the transition state. As previously noted (Aleshin *et al*., 1994a,b), Glu 400, Asp 55, and Glu 179 introduce uncompensated negative charge into the active site of glucoamylase. The uncompensated negative charge probably stabilizes a partially or completely formed carbocation at the active site. The dominant role of electrostatic interactions in hydrolysis by glucoamylase is

consistent with observations of Berland *et al*. (1995), who compared the effect of mutations in the active site of glucoamylase on the binding affinity of acarbose and the rate of hydrolysis of maltooligosaccharides. Changes in K_d for acarbose correlate with changes in k_{cat}/K_m of maltooligosaccharides. The mutation of ionizable residues in the vicinity of the active site produces the greatest effect on both the affinity of acarbose and the rate of hydrolysis of maltooligosaccharides by glucoamylase.

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