X-ray Structure of Cyclodextrin Glycosyltransferase Complexed with Acarbose. Implications for the Catalytic Mechanism of Glycosidases[†,‡]

Boris Strokopytov,[§] Dirk Penninga,[∥] Henriëtte J. Rozeboom,[§] Kor H. Kalk,[§] Lubbert Dijkhuizen,[∥] and Bauke W. Dijkstra*[,§]

BIOSON Research Institute and Laboratory of Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, and Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Groningen, The Netherlands

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ABSTRACT: Crystals of cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* strain 251 were soaked in buffer solutions containing the pseudotetrasaccharide acarbose, a strong amylase- and CGTase inhibitor. The X-ray structure of the complex was elucidated at 2.5-Å resolution with a final crystallographic *R* value of 15.8% for all data between 8.0 and 2.5 Å. Acarbose is bound near the catalytic residues Asp229, Glu257, and Asp328. The carboxylic group of Glu257 is at hydrogen bonding distance from the glycosidic oxygen in the scissile bond between the B and C sugars (residue A is at the nonreducing end of the inhibitor). Asp328 makes hydrogen bonds with the 4-amino-4,6-dideoxyglucose (residue B), and Asp229 is in a close van der Waals contact with the C1 atom of this sugar. From this we conclude that in CGTase Glu257 acts as the proton donor and Asp229 serves as the general base or nucleophile, while Asp328 is involved in substrate binding and may be important for elevating the pK_a of Glu257. On the basis of these results it appears that the absence of the C6-hydroxyl group in the B sugar is responsible for the inhibitory properties of acarbose on CGTase. This suggests that the C6-hydroxyl group of this sugar plays an essential role in the catalytic mechanism of CGTase. The binding mode of acarbose in CGTase differs from that observed in the complex of pancreatic α-amylase with acarbose where the catalytic Glu was found to be hydrogen bonded to the glycosidic nitrogen linking the A and B residues [Qian, M., Haser, R., Buisson, G., Duée, E., & Payan, F. (1994) *Biochemistry 33*, 6284−6294].

Cyclodextrin glycosyltransferases (CGTases;[1] EC 2.4.1.19) form a class of proteins which convert starch into cyclodextrins and related α-(1 → 4)-linked glucose polymers (French, 1957). Cyclodextrins (CDs) are cyclic compounds consisting of six, seven, or eight α-(1 → 4)-linked D-glucopyranose units: α-CD, β-CD, and γ-CD, respectively. CGTases generally produce a mixture of these three types of cyclodextrins, and, according to their most preferred product, they are divided into α-, β-, and γ-CGTases. The β-CGTases are especially of interest since β-cyclodextrin is most widely applied (Schmid, 1989). However, the industrially used CGTases suffer from product inhibition and their product specificity is not very high. For instance, the β-CGTase from *Bacillus circulans* strain 251, which is studied in our laboratories, produces a mixture of α-, β-, and γ-cyclodextrins in a ratio of 13:64:23 (Penninga et al., 1995). Separation of the different cyclodextrins is expensive and makes use of organic solvents. Therefore, one of our main goals is to increase the product specificity of the enzyme by means of protein engineering. Rational design of such mutants requires not only knowledge of the three-dimensional structure of the protein but also insight in the substrate binding mode and the detailed mechanism of the transglycosylation reaction. We have already elucidated the nucleotide sequence and the three-dimensional structure at 2.0-Å resolution of the CGTase from *B. circulans* strain 251 (Lawson et al., 1994). The folding of this 75-kDa enzyme is very similar to that of the CGTase from *B. circulans* strain 8 (Klein & Schulz, 1991), in accordance with the 75% identity in amino acid sequence between the two proteins. The enzyme consists of five domains, A−E. Domains A, B, and C are structurally homologous to the three domains of the α-amylases. The E domain has been implicated in starch binding (Svensson et al., 1989). The active site of CGTase is located at the N-terminal side of the $(\beta/\alpha)_8$-barrel of domain A. The catalytic residues are Asp229, Glu257, and Asp328, although their exact roles in the catalytic mechanism have not been firmly established. Site-directed mutagenesis of each of them resulted, however, in an inactive protein, indicating their importance for catalysis (Nakamura et al., 1992).

Although the presence of α-cyclodextrin or maltose is required for crystallization (Lawson et al., 1990), we did not find any α-cyclodextrin or other oligosaccharide bound in the active site of the enzyme. Density for three maltose residues was found, however, on the surface of the protein

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[‡] Coordinates of the model of CGTase complexed with acarbose have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Bernstein et al., 1977) (entry 1CXG).

* To whom correspondence should be addressed. Fax: (++31) 50 63 48 00. E-mail: bauke@chem.rug.nl.

[§] BIOSON Research Institute and Laboratory of Biophysical Chemistry.

[∥] Department of Microbiology.

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[1] Abbreviations: CGTase, cyclodextrin glycosyltransferase; CD, cyclodextrin; MPD, 2-methyl-2,4-pentanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; rms, root-mean-square.

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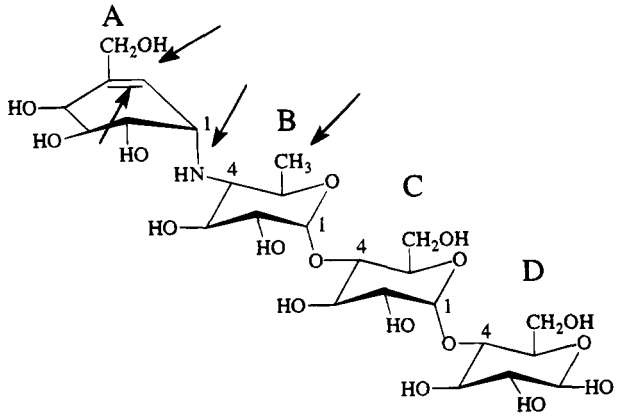

FIGURE 1: Structural formula of acarbose. Arrows mark the four differences between maltotetraose and acarbose: (i) the C6-hydroxyl group of glucose B is absent; (ii) the O-glycosidic bond between residues A and B has been replaced by an N-glycosidic bond; (iii) the O5 oxygen of residue A has been substituted by a carbon atom (C7); (iv) a double bond has been introduced in residue A between the C5 and C7 atoms.

in the C and E domains, indicating that the α-cyclodextrin had been degraded into short linear maltodextrins during the crystallization process. Therefore, to establish the binding mode of substrates in the active site cleft, we decided to use acarbose, an effective CGTase inhibitor (Nakamura et al., 1993).

Acarbose (molecular weight 645.6) is also an effective inhibitor of α-amylases, glucosidases, and sucrases (Schmidt et al., 1981). Its structure is very similar to the structure of maltotetraose (G4). It consists of one normal maltose and one pseudo-maltose residue. The pseudo-maltose residue (indicated by residues A and B in Figure 1) is essential for the inhibitory properties (Heiker et al., 1981). It consists of an unsaturated cyclitol unit (also called valienamine) and a 4-amino-4,6-dideoxyglucose unit. Acarbose is produced by fermentation of strains of *Actinoplanes* and is used as a drug for patients suffering from diabetes (Schmitt, 1987).

MATERIALS AND METHODS

Purification and crystallization of wild-type CGTase from *B. circulans* strain 251 was done according to Lawson et al. (1990). Crystals belonging to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 120.9$ Å, $b = 111.9$ Å, and $c = 65.7$ Å grew in 2−3 weeks. They contain one protein molecule per asymmetric unit, and their solvent content is 59%. The crystals were transferred to a stabilizing mother liquor containing 60% (v/v) MPD, 100 mM sodium-Hepes buffer, pH 7.55, and 0.5% (w/v) α-maltose. Soaking with the inhibitor acarbose was done at room temperature in the stabilizing mother liquor, with 0.1% acarbose and 0.5% maltose, over a 5 day period.

X-ray data were collected at room temperature from a single crystal on an Enraf Nonius FAST area detector system, with X-rays from an Elliot GX21 rotating anode generator. Data collection and processing were done with MADNES (Messerschmidt & Pflugrath, 1987) with profile fitting of the intensities according to Kabsch (Kabsch, 1988). A summary of data collection statistics is given in Table 1.

Refinement of the crystal structure was done with the TNT package (Tronrud et al., 1987) and the 2.0-Å structure of native CGTase as the starting model (Lawson et al., 1994). This model consisted of all 686 residues, two calcium ions,

Table 1: Data Collection Statistics and Quality of the Final Model

resolution range (Å)	29.0−2.5
total number of observations	59552
number of discarded observations	3009
R_{merge}[a]	0.086
completeness of the data (%)	73.9
completeness in the last resolution shell (%)	40.3 (2.58−2.50 Å)
number of protein atoms	5264
number of calcium atoms	2
number of carbohydrate atoms	113
number of solvent sites	192
overall B factor (Å^2)	20.7
final R factor[b]	0.158
rms deviations from ideality for	
bond lengths (Å)	0.011
bond angles (deg)	2.3
torsion angles (deg)	15.9
trigonal planes (Å)	0.015
planar groups (Å)	0.012
van der Waals contacts (Å)	0.028
rms difference in B for neighboring atoms (Å^2)	2.88

[a]

$$R_{merge} = \frac{\sum_{hkl}\sum_{j}|I(hkl,j) - \bar{I}(hkl)|}{\sum_{hkl}\sum_{j}|I(hkl,j)|}$$

[b]

$$R \text{ factor} = \frac{\sum_{hkl}|F_{obs}(hkl) - F_{calc}(hkl)|}{\sum_{hkl}|F_{obs}(hkl)|}$$

and three maltose molecules. All water molecules with B values larger than 40 Å^2 plus active site waters had been removed from the list of coordinates to allow an unambiguous assessment of potential carbohydrate density in the active site cleft. In the course of the refinement manual adjustments of the model were made using FRODO (Jones, 1978) running on an Evans & Sutherland PS390 computer graphics station. During the least-squares refinement, tight restraints on bond lengths, bond angles, planarity of groups, and trigonal centers were applied, but no restraints on chiral centers, torsion angles, or possible hydrogen bond distances were employed. Starting temperature factors were taken from the native protein model. Refinement statistics are summarized in Table 1. After completion of the refinement the final model was analysed with the PROCHECK package (Laskowski et al., 1993).

RESULTS

Refined Structure. Table 1 gives a summary of the final results of the crystallographic refinement of CGTase soaked with acarbose. The final R factor is 0.158 for all observed data greater than 0.0 between 8.0- and 2.5-Å resolution. A Ramachandran plot (Ramachandran & Sasisekharan, 1968) of the enzyme with bound acarbose is virtually identical to that of native CGTase (Lawson et al., 1994) (data not shown). Comparison of native and complexed structures yielded a root-mean-square (rms) coordinate difference of around 0.3 Å (all atoms included). This rms difference is of the same magnitude as the mean coordinate error derived from Luzzati plots (Luzzati, 1952) or calculated by the program SIGMAA (Read, 1986). The largest side chain differences occur at

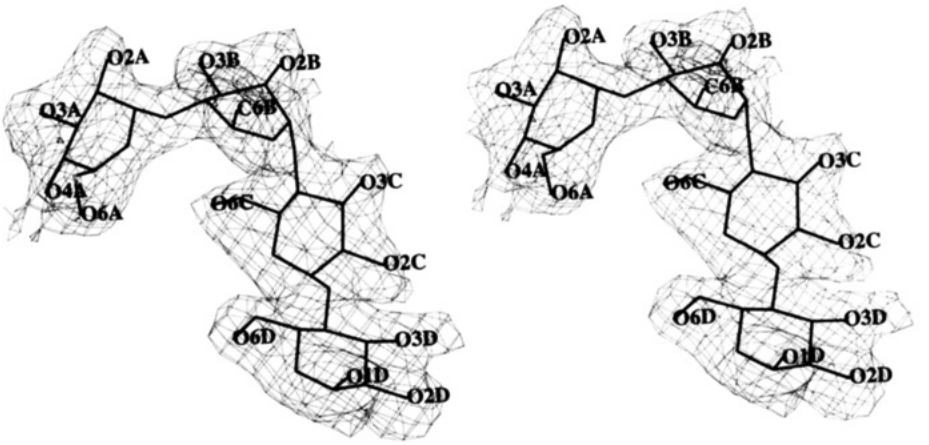

FIGURE 2: Stereodiagram showing $(2F_{obs} - F_{calc})$ $\exp(i\alpha_{calc})$ electron density of acarbose bound in the active site of CGTase. The electron density is contoured at 1.0 σ.

Table 2: Torsion Angles of the Glycosidic Bonds of Acarbose Bound in the Active Site of CGTase

glycosidic linkage between sugar residues	O_5-C_1-O_4'-C_4' torsion angle (deg)	C_1-O_4'-C_4'-C_5' torsion angle (deg)
A−B	112.2	−84.6
B−C	19.5	−148.7
C−D	100.0	−114.2
maltose[a]	116.1	−118.0

[a] Data from the crystal structure of maltose (Takusagawa & Jacobson, 1978).

flexible, solvent-exposed residues (Gln148, Asn169, Ser335) and at the active site residue Glu257 (see below). The average *B* values for the uncomplexed and complexed molecule are 26 and 21 Å^2, respectively.

Binding of Acarbose in the Active Site. After completion of the refinement the electron density for the acarbose molecule was clear with well-defined hydroxymethyl groups, especially for the A and C residues, allowing an unambiguous determination of the direction of the chain (Figure 2). The average temperature factor for the modeled acarbose (20.3 Å^2) is comparable to the average *B* factor of the protein atoms (20.4 Å^2), suggesting tight binding and an occupancy close to unity. The B and C residues are most tightly bound with average *B* factors of 13.8 and 17.3 Å^2, respectively, while the A and D sugars are somewhat more flexible with average temperature factors of 22.5 and 27.0 Å^2, respectively. Acarbose binds in an unexpectedly curved manner, making a sharp bend at the glycosidic bond between the B and C glucose residues. While the torsion angles of the glycosidic bond between glucoses C and D, and between the A and B sugar residues have values close to those found in the crystal structure of α-maltose (Takusagawa & Jacobson, 1978) (see Table 2), the $O_5-C_1-O_4'-C_4'$ torsion angle between residues B and C is rather different. Nevertheless, the conformation of the B-C glycosidic linkage is energetically not unfavorable: it is close to a second preferred conformation of maltose with $O_5-C_1-O_4'-C_4'$ and $C_1-O_4'-C_4'-C_5'$ torsion angles of +65° and −168°, respectively (Dowd et al., 1992). This bent conformation leads to differences in the internal hydrogen bonding pattern. While between the C and D glucoses and between the A and B residues the characteristic hydrogen bonds of α-(1 → 4)-linked D-glucopyranoses (Jeffrey, 1990) exist between atom pairs O2C−O3D (3.1 Å) and O2A−O3B (3.2 Å), such a hydrogen bond is not present between the B and C residues (O2B−O3C distance is 5.1 Å). The resulting acarbose structure thus may be considered to consist of two disaccharides with normal maltose-like conformations (A−B, respectively C−D), linked by a conformationally unusual glycosidic bond between residues B and C, that is not stabilized by an internal hydrogen bond.

Conformational Changes in CGTase upon Binding of Acarbose. The binding of acarbose has caused a change in conformation of a few active site residues only, the most significant occurring for the Glu257 side chain (Figure 3): the hydrogen bond existing in the native structure between the side chains of Glu257 and Asp328 (Lawson et al., 1994) has been broken and the Glu257 side chain has moved away. In its new position the $O^{\epsilon 2}$ carboxylic oxygen is at 2.9 Å from the glycosidic oxygen between the B and C residues, strongly suggesting that the $O^{\epsilon 2}$ atom is protonated. The $O^{\epsilon 1}$ atom is within hydrogen bonding distance (2.7 Å) from the O3 atom of the glucose C. The movement of the Glu257 side chain has mainly been achieved by a change in the χ_2 torsion angle (from +158° to −142°), with smaller changes in χ_1 (+174°→ −170°) and χ_3 (+10°→+16°). The Asp328 side chain has rotated by 17° about χ_1 and 11° about χ_2 (see Table 3). As a result of the movements of Glu257 and Asp328 the distance between their $O^{\epsilon 2}$ and $O^{\delta 2}$ atoms has increased from 2.5 to 3.6 Å. No significant changes occurred for the Asp229 side chain. The binding of acarbose displaces at least eight water molecules. Most of the water molecules are displaced by the A and B residues, but only a few by the C and D glucoses. Note that in the native structure less water molecules were modeled there because of disorder.

Interaction of Residue A of Acarbose with the Active Site Residues. The valienamine residue is not bound very tightly. It has hydrophobic interactions with the side chains of His98, Tyr100, Trp101, and Leu197 (Figure 4). Three hydrogen bonds are made to the active site residues Asp371 and Arg375 (see Table 4). The positions of the side chains of these residues are fixed by hydrogen bonds with neighboring amino acids. The NH1 atom of Arg375 is stabilized by an interaction with the main-chain carbonyl O atom of His327 (3.2 Å) and by a salt bridge with the $O^{\delta 1}$ atom of Asp328 (distance is 3.3 Å). The NH2 atom of Arg375 interacts with the hydroxyl group of Tyr97 (3.1 Å). Finally, the N^{ϵ} atom of Arg375 makes a hydrogen bond with the main-chain oxygen atom of His327 (3.1 Å). The Asp371 $O^{\delta 2}$ atom

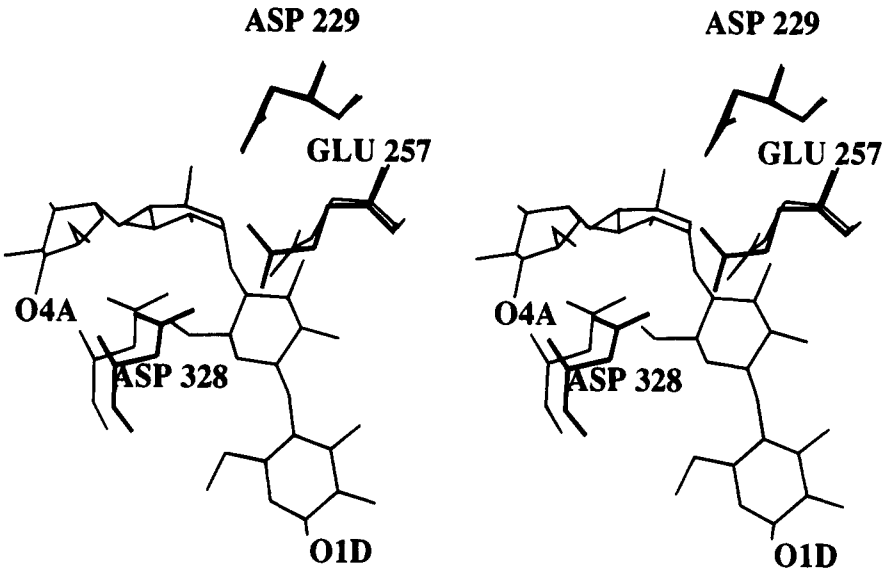

FIGURE 3: Stereofigure showing a comparison of the conformations of the catalytic residues in native CGTase (thick lines) and in the complex with acarbose (thin lines).

Table 3: Side Chain Torsion Angles for the Three Catalytic Residues

residue	torsion angles (deg) χ_1	χ_2	χ_3
Asp229			
native	171	75	
native + acarbose	−178	56	
Glu257			
native	174	158	10
native + acarbose	−170	−142	16
Asp328			
native	−81	−13	
native + acarbose	−64	−24	

makes a hydrogen bond with a water molecule (distance is 3.0 Å) which is in turn stabilized by a hydrogen bond with the hydroxyl group of Tyr97 (2.5 Å). Tyr97, His98, Tyr100, Trp101, Leu197, His327, Asp328, Asp371, and Arg375 are all absolutely conserved in CGTases, showing the importance of the interactions.

Electron density suggests that the O4 atom of the valienamine residue is hydrogen bonded to a water molecule, as is the O6 atom. The O4 atom of the A residue is located almost between the aromatic rings of Tyr89 and Tyr195 indicating that a glucose unit attached to it (as would occur with normal substrates) could be nicely positioned between two phenyl groups making stacking interactions, especially with Tyr89.

Interaction of Residue B of Acarbose with the Active Site Residues. The interactions of the B and C residues play a key role in the binding and cleavage of substrate. The B sugar residue has a full $^4C_1$ chair conformation despite the lack of the hydroxyl group at the C6-atom position. Analysis of the hydrogen bonding network (Table 4) shows that this residue makes the largest number of hydrogen bonds with the nearby active site residues, including two of the three catalytic residues (Glu257, Asp328) and the very conserved His327 residue (Svensson, 1988). The $O^{\epsilon 2}$ atom of Glu257 side chain is at 2.9 Å from the scissile bond oxygen strongly suggesting that this atom is protonated. Further binding interactions of the sugar ring come from a stacking contact with the aromatic ring of Tyr100 which is approximately parallel to the plane of the sugar ring. The average distance between the planes of the rings is about 4.2 Å. Residue B also interacts through van der Waals contacts with the very conserved His140. The distance between the C6 atom and the $C^{\epsilon 1}$ atom of His140 is 3.5 Å. This shows that His140 may influence the position of the C6-hydroxyl group which is present in natural substrates. His140 occurs in all sequences of α-amylases and CGTases (Svensson, 1988). However, it seems that His140 is not of crucial importance for catalysis, since its replacement by asparagine (Nakamura et al., 1993) resulted only in partial loss of activity. The His140 $N^{\delta 1}$ atom makes a hydrogen bond with the side chain of Asp139, which in turn is one of the ligands of the calcium ion bound close to the active site. The His140 $N^{\epsilon 2}$ atom is hydrogen bonded to the main chain carbonyl oxygen atom of Tyr100.

The $O^{\delta 1}$ atom of Asp 229 is positioned at 2.8 Å from the C1 atom of residue B. Residues in the neighborhood of Asp229 include His140, Arg227, and a water molecule. Upon binding of acarbose the Arg227 side chain moves toward Asp229 making a hydrogen bond with the $O^{\delta 1}$ atom (distance is 2.7 Å). Asp229 is further stabilized by an interaction with a nearby water molecule (distance is 2.8 Å). This water molecule is located in a pocket formed by the main chain atoms of residues 139 and 229.

Interaction of Residue C of Acarbose with the Active Site Residues. All hydroxyl oxygen atoms of the C glucose residues make hydrogen bonds with active site residues and/or water molecules. The density for this glucose is well defined, and its C6OH group could be assigned unambiguously. The sugar residue has a full $^4C_1$ chair conformation. The active site residues that directly participate in the binding of the C glucose are His233 and Glu257. A water-mediated hydrogen bond occurs between the O2C and O3C hydroxyl groups and between the carbonyl oxygen of Glu257 and the main chain NH of Trp258. Furthermore, there is a weak interaction between the hydroxyl group of Tyr195 and the O5 atom (4.1 Å). This is, however, the only interaction between Tyr195 and acarbose. Tyr195 is positioned too far from the sugars A to D for hydrophobic contacts.

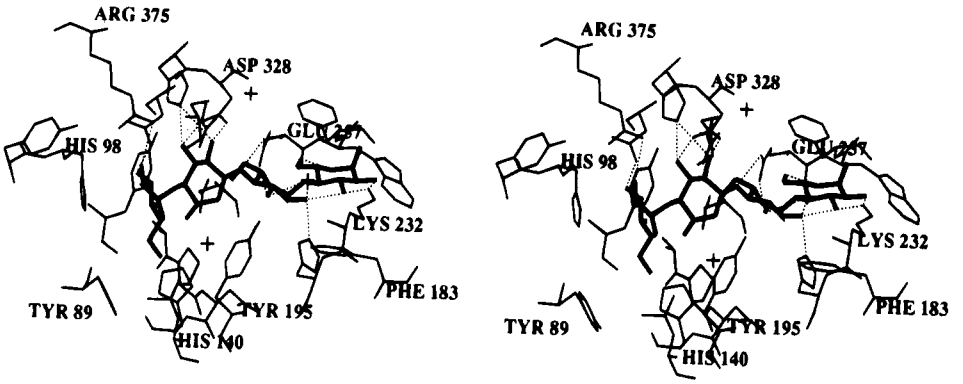

FIGURE 4: Stereopicture of the interactions of acarbose bound in the active site of CGTase. Residue A of acarbose is near His98 and the reducing end (residue D) is near Phe183.

Table 4: Possible Hydrogen Bonds Between Acarbose and CGTase

residue/ atom	protein atom or water molecule	distance (Å)	comments
residue A			
O2A	Arg375NH1	3.3	
O2A	O3B	3.2	internal H-bond
O3A	Arg375NH2	3.2	
O3A	Asp371OD2	2.8	
residue B			
O1B	Glu257OE1	3.1	
O1B	Glu257OE2	2.9	
O2B	Asp328OD2	2.7	
O2B	His327NE2	3.1	
O3B	His327NE2	3.0	
O3B	Asp328OD1	2.8	
O3B	O2A	3.2	internal H-bond
residue C			
O2C	His233NE2	2.7	
O2C	O3D	3.1	internal H-bond
O2C	WAT1133	2.9	
O3C	WAT1133	3.1	
O3C	Glu257OE1	2.7	
residue D			
O2D	Lys232NZ	3.5	
O3D	Lys232NZ	2.6	
O3D	O2C	3.1	internal H-bond

Interaction of Residue D of Acarbose with the Active Site Residues. The D glucose residue has a full $^4C_1$ chair conformation. It is sandwiched between the aromatic rings of Phe183 (at 4.2 Å) and Phe259 (at 4.1 Å). These distances are somewhat larger than the expected van der Waals distance of 3.7 to 3.8 Å, suggesting that the van der Waals interaction of the D glucose with the protein is not very strong. The side chain of Lys232 makes a hydrogen bond with the O2 and O3 hydroxyl groups (see Table 4). The O6 and O1 atoms may form hydrogen bonds with water molecules from the bulk solvent. Atom O3D forms a strong hydrogen bond with the neighboring sugar C. The hydroxymethyl group at position 6 is close to the O5 atom of the same glucose residue (the O5−C5−C6−O6 dihedral angle is 28°).

DISCUSSION

The primary structures of α-amylases and CGTases show about 30% homology, and their three-dimensional structures are quite alike. Especially the active site regions show a striking similarity with one glutamate (Glu257 in CGTase numbering) and two aspartate residues (Asp229 and Asp328) as putative catalytic residues (Buisson et al., 1987; Klein et al., 1992). The functions of these residues have, however, been under long debate. The catalytic mechanism of CGTases and α-amylases is thought to be similar to that proposed for hen egg white lysozyme. In this enzyme Glu35 acts as a proton donor while Asp52 stabilizes the oxocarbonium intermediate (Blake et al., 1967). For further details the reader is referred to Strynadka and James (1991) and Mooser (1993). Using the ideas on the lysozyme mechanism and data from soaking experiments, Matsuura et al. (1984) proposed Glu230 (Glu257 in CGTase numbering) in TAKA-amylase from *Aspergillus oryzae* to be the proton donor and Asp297 (Asp 328 in CGTase) the stabilizing acid. Later on Buisson et al. (1987) suggested on the basis of the 3D structure of pig pancreatic α-amylase that Asp197 and Asp300 were the catalytic residues (Asp229 and Asp328 in CGTase numbering). Boel and co-workers concluded that Asp206 and Glu230 (Asp229 and Glu257 in CGTase) play the catalytic role on the basis of an analysis of calcium binding sites in α-amylases (Boel et al., 1990). Several site-directed mutagenesis experiments on the presumed active site residues also did not completely clarify the situation, although it became clear that all three residues were important for catalysis (Holm et al., 1990). The difficulties in assigning specific roles to these residues were mainly caused by their close proximity. Even the high-resolution structures of CGTase could not resolve this question (Klein & Schulz, 1991; Lawson et al., 1994). Recently Qian et al. (1994) concluded on the basis of the structure of a complex of pancreatic α-amylase (PPA) with an acarbose-like inhibitor that Glu233 in mammalian α-amylase (analogous to Glu257 in CGTase) is the most appropriate candidate for the role of the general acid in the first stage of the catalytic process. Their inhibitor consisted of residues A, B, and C of acarbose (see Figure 1) with a maltose linked through an α-(1→4) bond to the O4 atom of residue A. We will refer to this inhibitor as G5-acarbose.

Our present results on binding of the inhibitor acarbose in the active site of CGTase further clarify the roles of the three carboxylates in the active site. In native CGTase a strong hydrogen bond (2.5 Å) exists between the carboxylic groups of Glu257 and Asp328 (Klein & Schulz, 1991; Lawson et al., 1994), indicating that one of the carboxylates is protonated, most probably Glu257 (Lawson et al., 1994). When acarbose is bound, Asp328 moves toward the inhibitor and hydrogen bonds are formed between the Asp328 side chain and the C2 and C3 hydroxyl groups of the B sugar residue (Table 4). The hydrogen bond between Asp328 and Glu257 is broken, and the Glu257 side chain moves to a

new position, at hydrogen bonding distance from the glycosidic oxygen of the scissile bond. From this we conclude that Glu257 is the proton donor that initiates the reaction. This result agrees with the result obtained by Qian et al. (1994) for mammalian α-amylase complexed with G5-acarbose. Movement of the Glu side chain was observed also by Schulz and co-workers when soaking crystals of a CGTase mutant in a β-cyclodextrin solution (Klein et al., 1992).

From our 3D structure of the complex of CGTase with acarbose it is clear that the Asp328 residue is involved in substrate binding, making hydrogen bonds with the O2 and O3 atoms of the B saccharide residue. An additional function of Asp328 is most likely to keep the Glu257 carboxylate group protonated at higher pHs, thereby allowing the enzyme to be active at higher pHs. Indeed, CGTase shows a broad pH optimum, ranging from pH 4.0 to 8.5 (Nakamura et al., 1993). In PPA (Qian et al., 1994) Asp300 (analogous to Asp328 in CGTase) is also used to raise the pK of the catalytic Glu233, though a chloride ion in PPA also contributes to this. In addition, Asp300 participates in inhibitor (and hence substrate) binding in the same fashion as Asp328 in CGTase. It makes two hydrogen bonds with the C2 and C3 hydroxyls of the sugar residue with the reducing end after cleavage (sugar B in CGTase; the unsaturated cyclitol unit in PPA; see below).

What is the function of Asp229? In lysozymes the active site aspartate residue has been proposed to be a general base or, alternatively, to act as a nucleophile (Blake et al., 1967; Hardy & Poteete, 1991). Asp229 is at 2.8 Å from the C1 atom and is thus close enough to act as the general base or nucleophile, while Glu257 and Asp328 are somewhat farther away. Some evidence for the formation of a covalent bond, and thus for the Asp acting as a nucleophile, has been presented for porcine α-amylase on the basis of ^{13}C NMR experiments at low temperature (Tao et al., 1989). A covalently bound intermediate offers an attractive way to stabilize an intermediate during the relatively long time that is needed for one oligosaccharide to leave the active site, and another one entering, in order to complete the cyclization and/or disproportionation reactions catalyzed by CGTases. Conclusive evidence that such a covalently bound intermediate exists in CGTase is not available, however.

Acarbose binds in the active site cleft with the nonreducing end (residue A) in the region close to Asp371, while its reducing end is positioned in between Phe183 and Phe259. The direction of the bound molecule agrees with the result described by Klein et al. (1992) on the basis of a single maltose bound in the active site. It is also in agreement with the result obtained by Qian et al. (1994) for their G5-acarbose inhibitor bound in the active site of α-amylase. The observed direction of acarbose is the same as the direction of the maltose bound at the second maltose binding site in the E domain near Tyr633 (Lawson et al., 1994). A long groove exists on the surface of the CGTase molecule running from Tyr633 toward the active site. Thus, an amylose polymer can bind in this groove and can be cleaved from its nonreducing end; five to six glucose residues are needed to span the distance between the maltose binding site in the E domain and residue D of acarbose in the active site.

Nakamura et al. (1993) have investigated the role in catalysis of three histidine residues that are conserved in α-amylases and CGTases. Individual replacement by site-directed mutagenesis of His140, His233, or His327 by an Asn resulted in mutant enzymes with somewhat reduced activity (25−40% of wild-type activity), but with considerable decreased affinity for acarbose (1400-, 320-, and 120-fold for H140N, H233N, and H327N, respectively). His140 has only one interaction with acarbose: it has a contact with the methyl group of the B residue of acarbose. His140 is, however, hydrogen bonded to the carbonyl oxygen of Tyr100 with its $N^{\epsilon 2}$ atom. Replacement of His140 by an Asn may have conformational consequences that affect the affinity for acarbose. His233 and His327 both bind directly to acarbose by their $N^{\epsilon 2}$ atoms: His233 to the O2 atom of glucose C, and His327 to the O2/O3 atoms of residue B. Substitution of His by the smaller Asn will in both cases affect the affinity for acarbose.

Why is acarbose an inhibitor of amylases and CGTases? It has been suggested that acarbose is a transition state analogue (Nakamura et al., 1993; Qian et al., 1994). Indeed, in α-amylase G5-acarbose was bound with its N-glycosidic linkage between the A and B residues (Figure 1) hydrogen bonded to the active site Glu233 (Qian et al., 1994). In glucoamylase complexed with acarbose the catalytic acid (Glu179) is also hydrogen-bonded to the nitrogen atom of the N-glycosidic linkage (Aleshin et al., 1994). Thus in these proteins the partly planar, unsaturated cyclitol unit (residue A in Figure 1) might mimick the transition state. In the complex of acarbose and CGTase, however, the nitrogen atom in the glycosidic bond between the A and B residues does not make any hydrogen bond. Instead, acarbose is bound with the O-glycosidic linkage between residues B and C hydrogen bonded to the Glu257 side chain, and the saccharide residues adjacent to the scissile bond have a normal $^4C_1$ chair conformation. Thus, none of these residues is distorted toward a planar oxocarbonium-ion-like conformation. Therefore, it seems more likely that at least in CGTase acarbose is a substrate analogue rather than a transition state analogue. Nevertheless, both in the CGTase−acarbose complex as well as in the complex of α-amylase with G5-acarbose the overall mode of binding is very similar. In CGTase a bend occurs between residues B and C; in α-amylase it is between the A and B residues. This suggests that the bend is not an inherent property of acarbose, but that in both enzymes the active site distorts substrates toward a bent conformation that is more amenable to effective cleavage and catalysis.

How can it be explained that acarbose binds in the active site of CGTase with the O-glycosidic linkage between the B and C residues near the catalytic amino acids without this bond being cleaved? The catalytic Glu257 even makes a hydrogen bond with the glycosidic oxygen. Acarbose closely resembles maltotetraose, a tetrasaccharide consisting of four α-(1 → 4)-linked glucose residues (see the legend to Figure 1). Three of the four differences between acarbose and maltotetraose are located in the A residue, away from the catalytic site. Only one difference is found in the B sugar: the absence of the hydroxyl group of the C6 atom. This suggests that the presence of a hydroxyl group at the C6 atom is essential for catalytic activity of CGTase.

At least three possible explanations can be given why a hydroxyl group at the C6 atom is required for the hydrolysis of maltooligosaccharides. The first explanation is based on the observation (Strynadka & James, 1991) that in hen egg white lysozyme the sugar near the scissile bond (in our case

sugar B) adopts a conformation distorted from the expected $^4C_1$ chair conformation. The B sugar in acarbose has a full $^4C_1$ chair conformation. However, the C6 atom of this residue is positioned near the His140 side chain. A hydroxyl group bound to this atom would come into close contact (~3.0 Å) with the His side chain. It is conceivable that as a result the B sugar becomes distorted and adopts a conformation closer to that of the transition state/oxocarbonium ion. Asp229 also could help stabilize such a conformation via a hydrogen bond with C6OH. A second possible explanation is that the O6 hydroxyl atom is needed to make an internal hydrogen bond with the O5 atom of the B sugar, thus polarizing the C1 atom, as has been observed for hen egg white lysozyme (Strynadka & James, 1991). Finally, in the soluble lytic transglycosylase from *Escherichia coli* (Thunnissen et al., 1994), an enzyme that cleaves the bacterial cell wall peptidoglycan, the O6 atom serves as the nucleophile that attacks the C1 atom of its own sugar residue, producing a covalent bond between the C1 and O6 atoms. It is therefore conceivable that the O6 atom could provide a stabilizing/polarizing interaction with C1, possibly assisted in this function by Asp229. Further research is needed to establish the precise role of the C6 hydroxyl group in the catalytic mechanism of CGTases and α-amylases. It is an interesting question whether the C6 hydroxyl group is important as well for the reactions catalysed by other glycosidases and glycotransferases, such as cellulases and muramidases.

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