Refined Structure for the Complex of 1-Deoxynojirimycin with Glucoamylase from *Aspergillus awamori* var. X100 to 2.4-Å Resolution†

E. M. S. Harris,‡ A. E. Aleshin,§ L. M. Firsov,§ and R. B. Honzatko*,‡

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011, and Department of Molecular Biology, Leningrad Nuclear Physics Institute, St. Petersburg, Russia

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ABSTRACT: The three-dimensional structure of the complex of 1-deoxynojirimycin with glucoamylase II-(471) from *Aspergillus awamori* var. X100 has been determined to 2.4-Å resolution. The model includes residues corresponding to residues 1–471 of glucoamylase I from *Aspergillus niger*, two molecules of bound 1-deoxynojirimycin and 605 sites for water molecules. The crystallographic *R* factor from refinement is 0.119, and the root-mean-squared deviation in bond distances is 0.012 Å. The inhibitor complex confirms the location of the active site in the packing void of the α/α-barrel as proposed by Aleshin et al. [Aleshin, A., Golubev, A., Firsov, L., & Honzatko, R. B. (1992) *J. Biol. Chem. 267*, 19291–19298]. One inhibitor molecule is associated with strong electron density and represents the principal site of interaction of 1-deoxynojirimycin with the enyzme. The other 1-deoxynojirimycin molecule is associated with weak electron density and, therefore, probably represents a binding site of low affinity. Interactions of 1-deoxynojirimycin with the enzyme at its principal site involve Arg 45, Asp 55, Arg 305, and carbonyl 177. In addition, a water molecule (water 500) hydrogen bonds to Glu 400 and the 6-hydroxyl of 1-deoxynojirimycin and is at an approximate distance of 3.3 Å from the "anomeric" carbon of the inhibitor. The structural arrangement of functional groups near the inhibitor molecule suggests that Glu 179 is a catalytic acid, Glu 400 a catalytic base, and water 500 the attacking nucleophile in the hydrolysis of maltooligosaccharides. The relevance of the X-ray work to proposed mechanisms of enzymatic hydrolysis of oligosacchrides is discussed.

Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3) is an exohydrolase which catalyzes the removal of β-D-glucose from the nonreducing ends of starch and other related poly- and oligosaccharides (Weill et al., 1954; Manjunath et al., 1983). 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, in the production of ethanol by fermentation, and in the brewing of light beer (Saha & Zeikus, 1989). Unfortunately, glucoamylase is not suited ideally as a catalyst in its industrial applications. The enzyme has low thermostability, and its pH of optimum catalysis is well below those of other enzymes (α-amylases and xylose isomerases) employed in the processing of raw starch or the products of starch degradation. In order to engineer a better industrial catalyst, a thorough understanding of the relationship of the structure of glucoamylase to its function is essential.

Glucoamylase cleaves the α-1,4-glucosidic bond most rapidly (Abdullah et al., 1963). However, the enzyme also hydrolyses α-1,6-glucosidic bonds at an appreciable rate (Abdullah et al., 1963), thus allowing it to completely hydrolyze starch. The enyzme also shows a preference for maltoologosaccharides of at least six residues. The rate of hydrolysis rises and the K_m decreases as the chain length of maltooligosaccharides increases to six (Abdullah et al., 1963; Sierks et al., 1989).

Several species of *Aspergillus* produce at least two major forms of glucoamylase (Svensson et al., 1982; Hayashida, 1975). These forms result from the limited proteolysis of a single parent protein (called glucoamylase I) of 615 or 616 amino acid residues (Svensson et al., 1986a; Hayashida et al., 1989a,b). 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 α-helices, 12 of which are arranged in a polypeptide fold that is a variation on the α/β-barrel (Aleshin et al., 1992). This fold has been dubbed an α/α-barrel (Aleshin et al., 1992) or a twisted α-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,b) 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, 1990; Dalmia & Nikolov, 1991; Williamson et al., 1992; Belshaw & Williamson, 1990, 1991).

Previous crystallographic work has focused on a proteolytic fragment of glucoamylase I from *Aspergillus awamori* var. X100, which contains residues corresponding to residues 1–471 of glucoamylase I from *Aspergillus niger*. This fragment has been called glucoamylase II(471). In the above appellation, we use the numeral "II" to designate a form of glucoamylase that lacks the ability to digest raw starch granules. The number in parentheses indicates our best estimate of the length of the polypeptide chain in the numbering scheme of glucoamylase from *A. niger*. Throughout the paper the term glucoamylase I designates the form of the enzyme that has the ability to

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‡ Iowa State University.

§ Leningrad Nuclear Physics Institute.

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degrade raw starch granules, whereas the term glucoamylase II represents an enzyme of unspecified chain length that has the ability to hydrolyze only soluble maltooligosaccharides.

1-Deoxynojirimycin is a strong inhibitor of glucoamylase with K_i = 96 μM (Truscheit et al., 1981). We describe here the three-dimensional model of the complex of 1-deoxynojirimycin with glucoamylase II(471) from *A. awamori* var. X100. Two molecules of 1-deoxynojirimycin bind in close proximity to each other. However, one of the sites is occupied partially and is probably a secondary site of low affinity for the inhibitor. The X-ray model clearly presents a subset of residues directly involved in binding the inhibitor or in a position to assist catalytically in the hydrolysis of a true substrate. We discuss the relevance of the crystallographic results to proposed mechanisms for the enzymic hydrolysis of maltooligosaccharides the subject of a recent review by Sinnott (1990).

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 = 85 000 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) poly(ethylene glycol) 6000 (Fluka) was equilibrated against a solution of 50 mM phosphate, pH 5.95, and 20% (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) poly(ethylene glycol) 6000. The 1-deoxynojirimycin complex was formed by soaking crystals for 2–3 days in a solution containing 1 mM 1-deoxynojirimycin (Sigma), 33% (w/v) poly(ethylene glycol) 6000, and 50 mM potassium phosphate, pH 6.0.

Data collection for the 1-deoxynojirimycin 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 52 047 reflections were recorded from a single crystal. Of the 26 797 possible unique reflections to 2.3-Å resolution, 20 987 were actually collected with an R merge of 0.033. Of those, 20 472 had $|F| > 2\sigma(|F|)$. The data were 90% complete to 2.5-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. The refined unit cell parameters were a = 116.6 Å, b = 103.6 Å, and c = 48.3 Å, which are comparable to those of the native crystals at pH 6.0, namely, a = 116.7 Å, b = 104.3 Å, c = 48.49 Å (Aleshin et al., 1992). 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).

Initial phases for the complex of 1-deoxynojirimycin with glucoamylase II(471) were calculated from the model for the native enzyme at pH 6.0 (Aleshin et al., 1992). The model for the free enzyme at pH 6.0 also represented the initial conformation of glucoamylaseII(471) in the inhibitor complex.

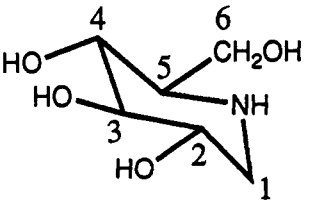

FIGURE 1: 1-Deoxynojirimycin in the C1-chair conformation.

Two models of 1-deoxynojirimycin, differing primarily in the conformation of the ring, were generated using software from the POLYGEN package. One model (Figure 1) was developed directly from the structure of D-glucose in the C1 chair conformation, which was then energy minimized using CHARMm. Similarly, the half-chair conformation of the inhibitor was developed by introducing a double bond between the endocyclic nitrogen (N5) and the C1 atom of 1-deoxynojirimycin, followed by energy minimization.

Each of these models of 1-deoxynojirimycin was built into the density for the inhibitor and refined independently by restrained least-squares (Hendrickson & Konnert, 1980) in an attempt to ascertain whether the X-ray data were of sufficient resolution to determine the pucker of the ring. A Silicon Graphics 4D25 and the program TOM (Cambillau & Horjales, 1987) were used for building 1-deoxynojirimycin into the model. All two-, three-, and four-center distances were restrained in the two models for 1-deoxynojirimycin, except for the four-center distances involving the O6 atom. Restraints on chiral volumes associated with atoms C2, C3, C4, and C5 were also applied during refinement. Intramolecular nonbonded contacts were only considered between the O6 atom and atoms N5 and C4 of the ring. The combination of distance and chiral restraints essentially fixed the conformation of the ring in each of the two models for 1-deoxynojirimycin.

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 α-D-mannose, in accordance with the findings of Gunnarsson et al. (1984). Standard groups for α-D-mannose and *N*-acetyl-D-glucosamine were based on the coordinates of monosaccharides obtained by X-ray diffraction studies (Chu & Jeffrey, 1968; Longchambon et al., 1976). The glycosidic bond angles were set initally 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.

Refinement proceeded over the course of 207 cycles and involved the interpretation and building of the solvent structure, the refinement of 1-deoxynojirimycin in the C1-chair and

half-chair conformations, the refinement of 1-deoxynojirimycin in the C1 chair conformation at a secondary binding site, 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. Sites for water molecules were added to the model provided that (1) electron density was present at a level of at least 3σ in maps based on the Fourier coefficients $(|F_o| - |F_c|)e^{i\alpha_{calc}}$ and $(2|F_o| - |F_c|)e^{i\alpha_{calc}}$, (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 90 Å^2 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 90 Å^2 probably represent water molecules with occupancy parameters below 1.0 and true thermal parameters substantially 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 3σ in maps based on the Fourier coefficients $(|F_o| - |F_c|)e^{i\alpha_{calc}}$ and $(2|F_o| - |F_c|)E^{i\alpha_{calc}}$, (2) alternative conformers must have reasonable contacts with nearby atoms, and (3) each conformer must adopt or be close to a conformation with staggered χ angles.

Our best interpretation of electron density at the secondary binding site for 1-deoxynojirimycin superimposes the inhibitor molecule on three water molecules. The occupancy factors for the atoms of the inhibitor and the three overlapping sites for water were fixed arbitrarily at 0.5. The water sites were chosen by reference to the solvent structure of the native enzyme at pH 6.0 (Aleshin et al., unpublished results) as well as to the highest levels of electron density at the secondary binding site for the inhibitor. The high levels of electron density represent regions of overlap between the inhibitor molecule and water molecules present only when the inhibitor is absent. The water molecules were defined as atoms of "disorder", thereby automatically eliminating the influence of nonbonded contacts between these and other atoms of the surrounding environment. The three water molecules, however, were tethered by the specification of special distance restraints to each other and to other atoms which could accommodate hydrogen bonds.

RESULTS AND DISCUSSION

Quality of the Refined Model. The model for the complex of glucoamylase II(471) with 1-deoxynojirimycin has been deposited with the Protein Data Bank, Brookhaven National Laboratory. In keeping with prior work (Aleshin et al., 1992), 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 sequence used in refinement appears in Figure 2 and is identical to that used by Aleshin et al. (1992). Relevant statistics for the model of the 1-deoxynojirimycin complex of glucoamylase II(471) appear in Table I. A Luzzati analysis (1952) infers an overall root-mean-square uncertainty in atomic coordinates of between 0.1 and 0.2 Å.

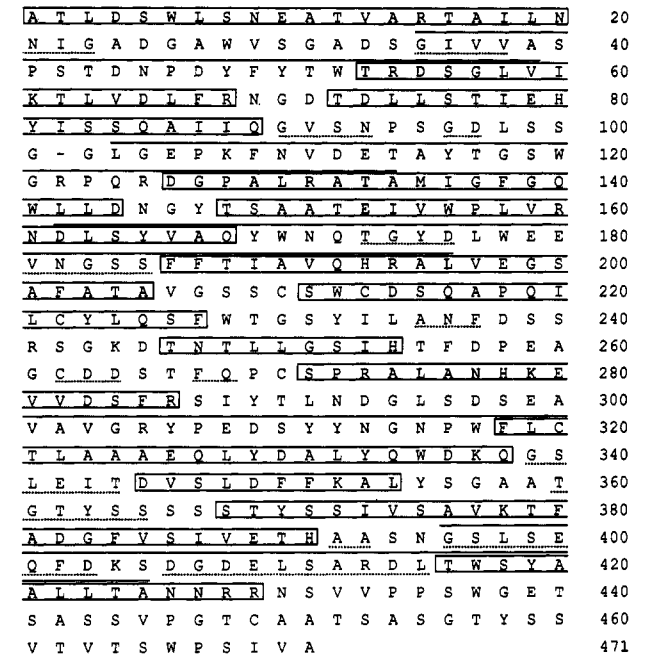

FIGURE 2: Amino acid sequence used in the refinement of the complex of 1-deoxynojirimycin with glucoamylase II(471). Single letters correspond to amino acids as established by the IUPAC-IUB Commission on Biochemical Nomenclature [(1970) *Biochemistry 9*, 3471–3479]. Residues that belong to α-helices are enclosed in boxes, residues that belong to β-strands are underlined, and regions of conserved sequence among glucoamylases, as defined by Itoh et al. (1987), are overlined. Details regarding the assignment of secondary structure are provided by Aleshin et al. (1992).

Table I: Agreement between Target and Observed Stereochemistry for the Complex of 1-Deoxynojirimycin with Glucoamylase II(471)[a]

total number of atoms	4472
total number of solvent sites	605
number of structure factors used in refinement[b]	20387
R factor[c]	0.119
mean B (Å^2) for protein	10.02
RMS[d] deviation (Å) for distances	
two center	0.012 (0.02)
three center	0.030 (0.30)
four center	0.040 (0.05)
RMS[d] deviation (Å) for planar groups	0.012 (0.02)
RMS[d] deviation (Å^3) for chiral volumes	0.137 (0.15)
RMS[d] deviation (Å) for nonbonded contacts	
determined by single torsion angles	0.225 (0.25)
determined by multiple torsion angles	0.159 (0.25)
hydrogen bond	0.201 (0.25)
RMS[d] deviation (deg) for torsion angles	
planar peptides (ω = 180°)	2.2 (3)
staggered side chains	14.2 (15)
orthonormal	28.5 (20)
RMS[d] ΔB (Å^2)	
main chain bond	0.605 (1.0)
main chain angle	1.037 (1.5)
side chain bond	1.865 (2.0)
side chain angle	2.817 (3.0)

[a] Target root-mean-square deviations used in refinement are in parentheses. [b] Comprises all observed moduli from 10- to 2.3-Å resolution, with $|F| \geq \sigma(|F|)$ and $|F| \geq 3.0$. [c] R factor = $\Sigma||F_o| - |F_c||/\Sigma|F_o|$. [d] Root-mean-square.

Atoms of Ala 1 and Thr 2 have high thermal parameters (well in excess of 30 Å^2). As noted previously by Aleshin et al. (1992), the high thermal parameters may be due to proteolytic degradation at the N-terminus. Svensson et al. (1982) discovered that 50% of the glucoamylase molecules from *A. niger* are missing the first three residues from the N-terminus. Atoms of the last six C-terminal residues also show high thermal parameters, perhaps as a consequence of

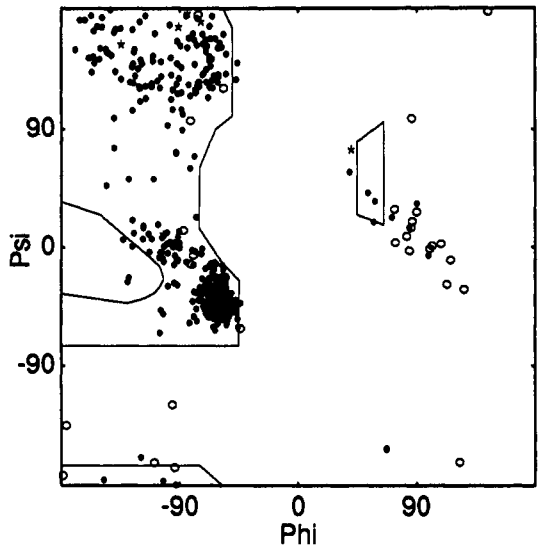

FIGURE 3: Distribution of ϕ–ψ angles for the amino acid residues of the complex of 1-deoxynojirimycin with glucoamylase-II(471). (*) Residue flanking a cis petide; (O) glycine; (●) all other residues.

nonuniform proteolysis. Glucoamylase II from *A. niger*, for instance, is a mixture of polypeptide chains of 512 and 514 residues (Svensson et al., 1986a).

The distribution of ϕ–ψ angles (Ramachandran et al., 1963) for all amino acid residues of the inhibitor complex is given in Figure 3. Residues Asn 313 and Ser 411 have ϕ–ψ angles which lie significantly outside the allowed regions. As noted previously Aleshin et al. (1992), the local hydrogen-bonding environments of Asn 313 and Ser 411 determine the conformations of these residues.

Modeling indicates alternative conformations for the side chains of Asp 25, Asp 44, Thr 246, Asp 293, and Lys 352. Asp 25, Asp 44, Asp 293, and Lys 352 lie on the surface of the protein near a lattice contact of the crystal. In one conformer of Asp 25, the OD1 atom makes hydrogen bonds to three water molecules, one of which (water 565) hydrogen bonds to carbonyl 360 of glucoamylase in a symmetry equivalent position. In the second conformer, atom OD1 interacts weakly with OG1 of Thr 360. In one of its conformations, Asp 44 OD2 interacts with water 1153, which in turn forms a hydrogen bond with carbonyl 468 of glucoamylase in a symmetry equivalent postion. In the second conformer, atom OD1 makes a strong hydrogen bond to atom OG1 of Thr 43. The disorder at Asp 44 may be related to its hydrogen bond with carbonyl 468 and the nonuniform proteolysis of residues lying close to the C-terminus (Aleshin et al., unpublished results). In the case of Asp 293, the side chain is stabilized by a hydrogen bond between atom OD1 and water 685. The second conformer makes no hydrogen bonds but does have a staggered χ^1 angle. Finally, atom NZ for Lys 352 has two positions, each of which permits the formation of a hydrogen bond to a nearby water molecule. In contrast to the other disordered residues, Thr 246 lies close to the active site "behind" Arg 305. In one conformer, its OG1 atom interacts with amide 246, while in the other conformer it is involved in hydrogen bonds with amide 246 and carbonyl 305.

Comparison of the Complex and Native Proteins. The overall conformation of the protein in the inhibitor complex is essentially the same as the native structure at pH 6.0. The statistics in Table I are almost identical to those published by Aleshin et al. (1992) for the native structure at pH 6.0 without solvent. The root-mean-squared differences in atomic coordinates and the differences in thermal parameters between the native and the inhibitor complex generally are small. Significant differences (greater than 1.0 Å) exist, however, in the positions of Ala 1, Thr 2, Ser 5, Asp 44, Ser 298, Lys 337, Lys 352, and Thr 464. As mentioned above, the density is weak for the first three residues possibly due to limited proteolytic degradation. Differences here may reflect uncertainty in the interpretation of weak electron density and/or real structural changes as a consequence of different levels of proteolytic degradation. The enzyme used in the study of the native crystals (Aleshin et al., 1992 and unpublished results) and the inhibitor complex comes from separate prepartions. Thr–mannose 464 has weak density in both the structure of the native enzyme and its inhibitor complex. Differences in the conformation of the side chain truly reflect the uncertainty in the atomic coordinates of residue 464. Other glycosyl chains are unchanged between the structures of the inhibitor complex and the native enzyme. (A complete description of the glycosyl chains and their interactions with the protein will be presented elsewhere.) Asp 44 and Lys 352 are disordered in the inhibitor complex but not in the native structure at pH 6.0 (Aleshin et al., unpublished results) and vice versa for Lys 337. Furthermore, Ser 5 and Ser 298 have significant differences in their χ^1 angles in the two structures. Significant differences in thermal parameters exist for the side chains of Asp 97, Asp 293, and Lys 337, all of which are disordered in only one of the two structures. Some of the differences above may be due in fact to the slight dehydration of the crystals exposed to 1-deoxynojirimycin. Native crystals were soaked in a buffer containing the inhibitor and an elevated concentration of poly(ethylene glycol) 6000. The dessicating effect of the elevated content of poly(ethylene glycol) may be responsible for the observed reduction in the unit cell parameters for the crystals of the complex relative to the native enzyme. Generally, however, the differences in atomic coordinates and thermal parameters are within the uncertainty levels for these parameters (0.1–0.2 Å for atomic positions and 2–3 Å^2 for thermal parameters). Thus, glucoamylase II(471) does not undergo a large and global conformational change upon binding 1-deoxynojirimycin.

The close similarity in the structures of the native enzyme and its inhibitor complex extends beyond the protein to the solvent region. A total of 605 sites for water are present in the inhibitor complex, including three disordered water molecules described above. The solvent structure for the inhibitor complex is essentially identical to that for the native structure of pH 6.0 (Aleshin et al., unpublished results). The root-mean-square difference in position for water sites of the two structures, excluding the waters displaced from the active site by the inhibitor, is 0.22 Å. Figure 4 shows the distribution of the distances of closest approach of water sites to the enzyme in the inhibitor complex. The peak of the distribution (between 2.75 and 3.0 Å) corresponds to the expected hydrogen-bond contact distance between a water molecule and protein atoms. The tail of the distribution reflects water molecules, which interact with the protein by way of intermediate waters. All contact distances are greater than 2.4 Å.

Interpretation of Inhibitor Binding Sites. Thermal parameters for atoms of 1-deoxynojirimycin at its primary site (NOJ1) range from 7–10 Å^2. Strong density (above 10σ) is present for the whole molecule (Figure 5). Although placement of 1-deoxynojirimycin in the density of its primary site is unambiguous, the local environment does not permit assignment of the state of protonation of the endocyclic nitrogen (N5). The endocyclic nitrogen of 1-deoxynojirimycin has a pK_a of 6.6 in water (Inouye et al., 1966, 1968). At pH

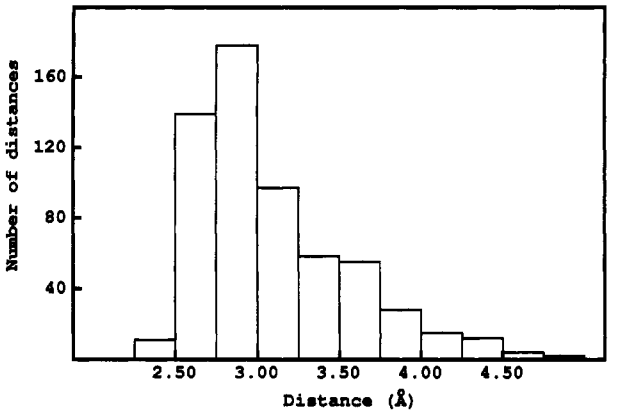

FIGURE 4: Distribution of distances of closest approach between water molecules and the protein.

6.0 approximately 80% of the inhibitor should be in the protonated form. However, of the two hydrogen-bonding interactions involving the N5 atom, only that with water 500 (see below), requires that N5 serves as a proton donor.

The current study reveals the major interactions between the inhibitor at its primary binding site and functional groups of the active site (Figures 5, 6, and 7). The major binding site is in the deepest recess of the packing void of the innermost set of helices of the α/α-barrel. Aleshin et al. (1992) designated the packing void as the active site on the basis of published studies of sequence homology, chemical modification, and directed mutation (Svensson, 1988; Clarke & Svensson, 1984a,b; Svensson et al., 1986b, 1990; Sierks et al., 1989, 1990; Itoh et al., 1987, 1989). Table II is a list of the major contacts between the inhibitor at its primary binding site and nearby atoms. The interactions are similar whether 1-deoxynojirimycin is restrained in the C1 chair conformation or the half-chair conformation, believed typical of a glucopyranosyl cation. However, the protein contacts of atoms N5 and C1 of the inhibitor differ significantly for the C1 chair and half-chair conformers. In the C1 chair conformation, N5 of 1-deoxynojirimycin has a tight contact (2.82 Å) with water 500, indicative of a hydrogen bond, whereas C1 has an acceptable nonbonded contact (3.28 Å) with the same water molecule. In contrast, the half-chair conformation puts N5 at a distance (3.02 Å) from water 500, which is not optimal for hydrogen bonding, and C1 at a distance relatively close (3.06 Å) to water 500. The interactions with atoms in the active site for 1-deoxynojirimycin in the C1 chair conformation are more favorable than in the half-chair conformation. The relevance of the above to the proposed catalytic mechanism of glucoamylase is discussed below.

Comparison of the active sites of the inhibitor complex with the free enzyme revealed the presence of seven well-ordered water molecules in the absence of the inhibitor and only one water molecule when the inhibitor is bound. The single water molecule (water 500) that is not displaced by the binding of 1-deoxynojirimycin has a well-defined orientation with respect to its local environment. Water 500 is hydrogen bonded to OE1 of Glu 400 and to the 6-hydroxyl of the inhibitor. The 6-hydroxyl of 1-deoxynojirimycin must be a proton donor to OD1 of Asp 55 and a proton acceptor to water 500. In addition, water 500 must act as a proton donor to OE1 of Glu 400 and as a proton acceptor to N5 of 1-deoxynojirimycin. Thus a lone pair orbital of water 500 is oriented toward the C1 atom of the inhibitor. The above relationships suggest that water 500 is the nucleophile of a general base-catalyzed mechanism and that Glu 400 is an important catalytic residue of the enzyme.

The water molecules in native glucoamylase occupy the approximate positions of the 2-, 3-, 4-, and 6-hydroxyls, C6, and the absent 1-hydroxyl of the inhibitor. The mechanism by which substrate and inhibitor molecules purge water from the active site is unclear. Little or no "free room" is available to allow water to slide by the inhibitor as it enters this innermost recess of the active pocket. Presumably, transient conformations of the active site must exist that facilitate the flow of water out of the active site in response to inhibitor binding.

The available electron density for 1-deoxynojirimycin at its secondary site (NOJ2) is relatively weak (3σ). Assignment of 0.5 to occupancy parameters for inhibitor atoms at this site resulted in refined thermal parameters ranging from 26 to 29 Å^2. These refined values are high relative to those of nearby side chains. Thus, the arbitrarily assigned occupancy factor of 0.5 may be a modest overestimate of the binding of 1-deoxynojirimycin at its secondary site. Although the inhibitor makes reasonable contacts with nearby protein atoms (Table III), we cannot exclude alternative fits of 1-deoxynojirimycin to the density of its secondary site. In our current interpretation, the inhibitor molecule is in a nonbonded contact with a symmetry related glucoamylase molecule. Thus, the secondary binding site for 1-deoxynojirimycin should be regarded with caution and may not reflect the true binding of substrate at the second subsite.

As the inhibitor at its secondary site is present less than 50% of the time under the experimental conditions used in the present work, the electron density here represents a composite of two structures. We observe diffuse density at a low level in the general shape of the inhibitor, superimposed on peaks of electron density corresponding to water molecules. At sufficiently high contour levels in the electron density map, we observe only three discrete peaks of density, presumably positions of water molecules which overlap and reinforce the density of the inhibitor molecule. Water molecules were inserted at the three locations and each assigned an occupancy of 50%. Two of the three water molecules correspond closely to waters in the native structure. The third water molecule (water 659) hydrogen bonds to the 1-deoxynojirimycin at the primary site and, therefore, is not present in the native structure.

Svensson et al. (1990) found that the inhibitor acarbose, when bound to glucoamylase from *A. niger*, protects Asp 176, Glu 179, and Glu 180 from modification with 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide (EAC). Mutation of Asp 176 to Asn in glucoamylase from *A. niger* led to a 3–4–fold increase in K_m and a 10–20–fold decrease in k_{cat} (Sierks et al., 1990). Sierks et al., 1990) suggested that Asp 176 occupies a position near subsite 1, acting as a general catalytic base. The present study shows that although Asp 176 is close to subsite 1, it is not near the inhibitor molecule at the primary site (Figure 8). Asp 176 cannot serve therefore as a catalytic base; we favor instead Glu 400 in that role (see below). The effects on catalysis by mutation of Asp 176 to Asn may be due instead to the disruption of a hydrogen-bonding network. Atom OD2 of Asp 176 hydrogen bonds to amide 179 and amide 180, while OD1 of Asp 176 interacts with water 627 and water 620 (Figure 8). The hydrogen bonds of water 627 and water 620 are unambiguous as to whether the water molecules act as proton acceptors or donors to nearby atoms. The side chain of the mutant Asn 176 either remains hydrogen bonded to amide 179 and amide 180 (through atom OD1) and disrupts its hydrogen bonds with the two water molecules or the side chain hydrogen bonds to the two water molecules, thereby disrupting its hydrogen bonds

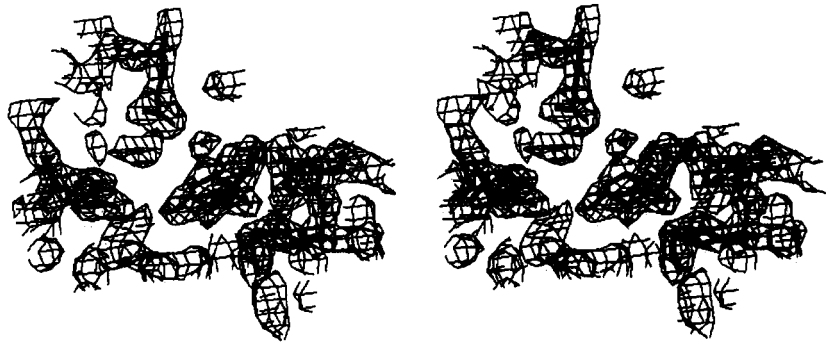

FIGURE 5: Stereoview of the 1-deoxynojirimycin in its primary binding site with associated electron density. Only selected side chains and water molecules are shown. Starting from the top and moving clockwise are Glu 400, water 500, Asp 55, Arg 54, and Arg 305.

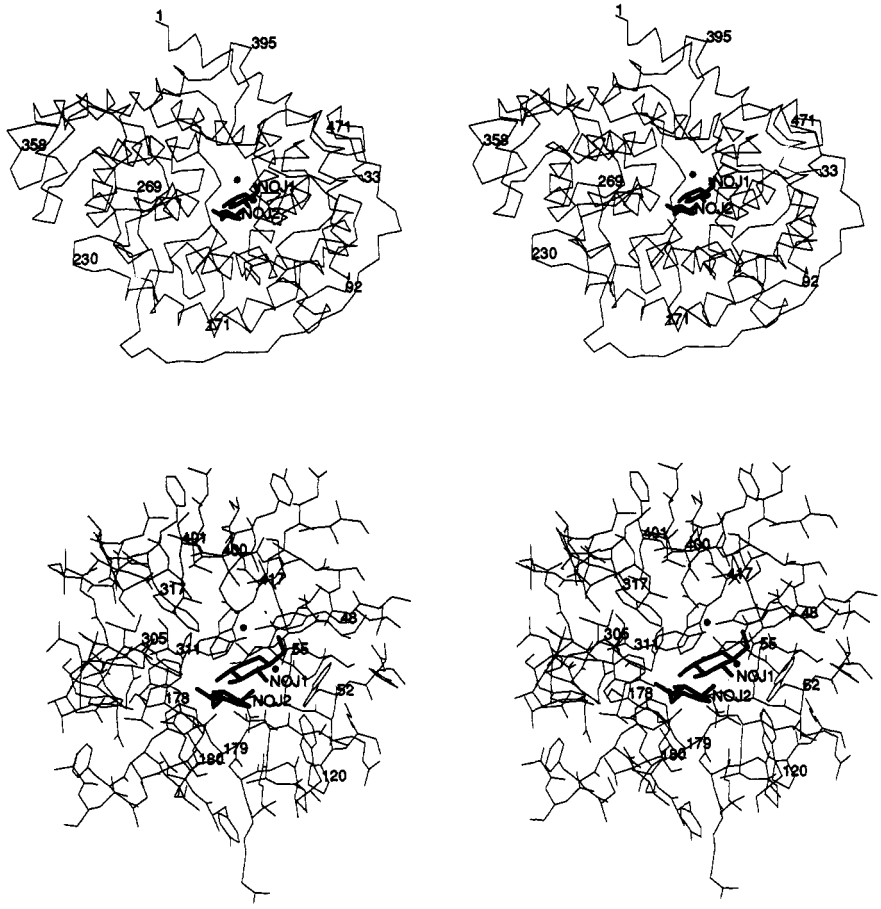

FIGURE 6: Stereoview of an α-carbon plot of glucoamylase II(471) (top) and a more detailed view of the active site (bottom). The 1-deoxynojirimycin molecules are highlighted in the active site. (●) Water molecule; NOJ1 and NOJ2 are 1-deoxynojirimycin at the primary and secondary site, respectively.

with amide 179 and amide 180. In addition, Asp 176 does not interact directly with Trp 120 as suggested by Sierks et al. (1990). Instead, OD2 of Glu 179 hydrogen bonds to NE1 of both Trp 120 and Trp 52 (Aleshin et al., 1992).

The inhibitor complex also confirms the importance of residues and functional groups in the vicinity of the nonreducing end of the substrate. Mutation of Arg 54 to Lys or Thr, and of Asp 55 to Asn or Tyr leads to a complete loss of activity (Itoh et al., 1989). Both of these residues are strongly hydrogen bonded to the 4- and 6-hydroxyls of the inhibitor at its primary site, suggesting that they are required for substrate binding at subsite 1. These interactions also confirm the predictions made by Bock and Pedersen (1987) that the 4- and 6-hydroxyls of the nonreducing end of the substrate are essential.

Relevance of the Structure to the Mechanism of Enzyme Action. Although the spatial relationships between key functional groups in the 1-deoxynojirimycin complex are now clear, the precise mechanism of hydrolysis is still obscure. The catalytic roles of specific side chains of the enzyme and the catalytic mechanism depend on the lifetime of the glucopyranosyl cation in the active site of glucoamylase. The environment at the active site may promote the formation of the glucopyranosyl cation and stabilize it for a significant duration ($>10^{-10}$ s). On the other hand, if the lifetime of the glucopyranosyl cation is short ($<10^{-12}$ s), then the most probable pathway is a preassociation S_N2 mechanism (Banait & Jencks, 1991a,b). The strength and orientation of the attacking nucleophile, the involvement of protein functional groups as acid or base catalysts, and the proximity and distribution of electrostatic charges in the active site are factors which determine the lifetime of the glucopyranosyl cation.

Model studies of Bennet and Sinnott (1986) of the acid-catalyzed hydrolysis of methyl glucopyranosides suggest that,

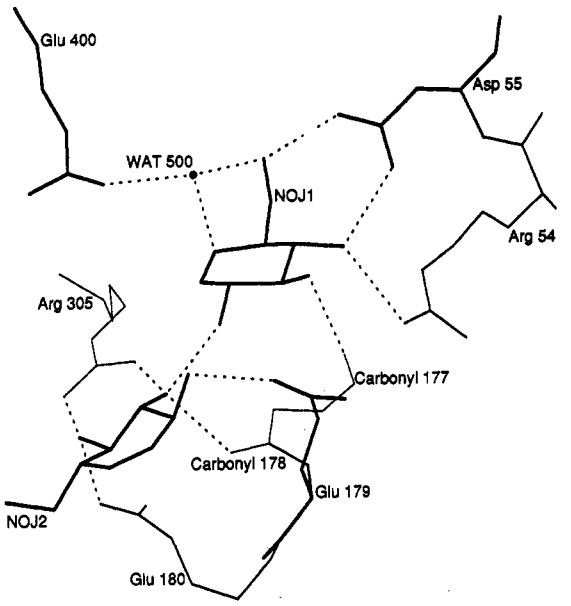

FIGURE 7: Schematic of the active site of glucoamylase II(471). Dashed lines represent donor–acceptor distances less than 3.2 Å. NOJ1 and NOJ2 are 1-deoxynojirimycin at the primary and secondary site, respectively.

Table II: Nonbonded Contacts between Glucoamylase II(471) and 1-Deoxynojirimycin at Its Primary Binding Site[a]

atom of the inhibitor	atom of the protein	contact distance (Å)[b]
O6	water 500	2.97 (2.97)
	Asp 55 OD1	2.57 (2.51)
N5	water 500	2.82 (3.02)
	water 587	2.82 (2.58)
O2	Arg 305 NH1	3.23 (3.25)
	Trp 178 CA	3.43 (3.44)
	O3 NOJ2[c]	2.57 (2.64)
O3	carbonyl 177	2.65 (2.67)
	Arg 54 NE	3.27 (3.23)
	Trp 417 CZ3	3.41 (3.39)
O4	Asp 55 OD2	2.73 (2.66)
	Arg 54 NE	3.32 (3.31)
	Arg 54 NH2	2.91 (2.95)
C1	water 500	3.28 (3.06)
	water 587	3.06 (3.14)
C2	water 500	3.39 (3.34)
C3	carbonyl 177	3.46 (3.49)
C5	water 500	3.49 (3.47)
	water 587	3.44 (3.44)
C6	Asp 55 OD1	3.41 (3.41)

[a] Only distances less than 3.5 Å are listed. [b] Distances in parentheses are for the half-chair conformation of the inhibitor. [c] NOJ2 is the 1-deoxynojirimycin molecule at its secondary binding site.

Table III: Nonbonded Contacts between Glucoamylase II(471) and 1-Deoxynojirimycin at Its Secondary Binding Site[a].

atom of the inhibitor	atom of the protein	contact distance (Å)
O2	Glu 179 OE1	2.46
	water 587	2.48
	NOJ1 C1[b]	2.92
O3	carbonyl 178	2.61
	NOJ1 O2[b]	2.57
	Arg 305 NH1	2.73
O4	Glu 180 OE2	2.43
	Glu 180 CD	3.39
	Arg 305 NH2	3.01
	Arg 305 NH1	3.49
	Tyr 311 CG	3.37
O6	Tyr 311 CD1	3.31
	carbonyl 143[c]	3.27
	water 718[c]	2.77
C2	Glu 179 OE1	3.20
C3	carbonyl 178	3.44
C4	Glu 180 OE2	2.95
C6	water 718[c]	3.15
	water 1087[c]	3.19

[a] Only distances less than 3.5 Å are listed. [b] NOJ1 is the 1-deoxynojirimycin molecule at its primary binding site. [c] The distance listed for this contact uses this atom in a symmetry equivalent position.

for poor and neutral nucleophiles, the preferred pathway is a limiting S_N1 mechanism, with a completely formed glucopyranosyl cation as an intermediate. On the other hand, model studies by Banait and Jencks (1991a) indicate that for good and anionic nucleophiles the preferred reaction pathway for the cleavage of α-D-glucopyranosyl fluoride is an enforced S_N2 mechanism. For the S_N2 mechanism, the nucleophile must be associated with and properly oriented with respect to the anomeric carbon before a successful reaction occurs. Studies by Banait and Jencks (1991a,b) indicate that the glucopyranosyl cation has a lifetime too short to exist as a discrete intermediate in the presence of a good nucleophile. For glucoamylase, we clearly see a neutral and presumably poor nucleophile (water 500) preassociated with the "anomeric" carbon of 1-deoxynojirimycin. The favorable alignment of the nucleophile with the inhibitor and its association with the anomeric carbon are elements of a preassociation S_N2 pathway. On the other hand, work of Firsov (1978) and Matsui et al. (1989) on glucoamylase reveals an α-secondary kinetic isotope effect, a result consistent with the formation of a glucopyranosyl cation. The observation of a kinetic isotope effect along with a preassociated nucleophile suggests the formation of an "exploded" transition state in which the incoming nucleophile and the leaving group are well separated from a glucopyranosyl group that has significant carbocation character.

Substitution of α-D-glucose for 1-deoxynojirimycin in the active site reveals a set of interactions that may favor a half-chair conformation for the true bound substrate. The endocyclic oxygen (O5) of the substrate cannot function as a hydrogen-bond donor to water 500. Consequently, for substrates the favorable distance of 2.82 Å between N5 of the inhibitor and water 500 would become an unfavorable nonbonded contact. This unfavorable contact could be relieved by a transition to a half-chair conformation, which should increase the distance of O5 to water 500 to approximately 3.0 Å (see N5 interactions of Table II) and bring the anomeric carbon C1 into close contact with water 500 (approximately 3.0 Å).

Glu 179 and Glu 400 presumably adopt the roles of catalytic acid and catalytic base, respectively. Sierks et al. (1990) demonstrated the importance of Glu 179, but the identification of Glu 400 as the catalytic base comes solely from the crystallographic work and requires corroboration by directed mutation. The extent to which Glu 400 acts as a catalytic base depends on the degree to which the transition state resembles a glucopyranosyl cation. According to Banait and Jencks (1991b), the reaction of a glucopyranosyl cation with water is so fast that a catalytic base would have no effect on the rate of reaction. However, if the glucopyranosyl cation is not formed completely or if it is stabilized sufficiently in the active site of the enzyme, then Glu 400 could play a catalytic role. Furthermore, Glu 400 may stabilize a completely formed glucopyranosyl cation through electrostatic interactions.

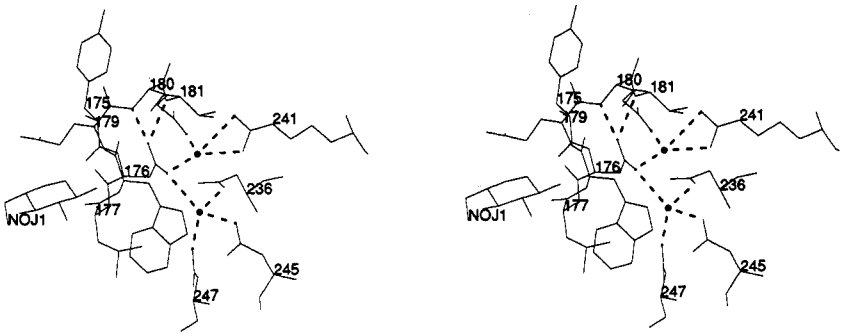

FIGURE 8: Stereoview of the immediate environment of Asp 176 in the inhibitor complex of glucoamylase II(471). (●) Water molecule, with the upper position corresponding to water 627 and the lower portion corresponding to water 620. Dashed lines represent donor–acceptor distances less than 3.2 Å.

Similarly, the putative catalytic role of Glu 179 also depends on the reaction pathway. For a pathway that leads to the complete formation of a glucopyranosyl cation, Glu 179 could serve as a general acid catalyst, protonating the oxygen of the glucosidic linkage early in the reaction. Once ionized, Glu 179 would stabilize the glucopyranosyl cation through electrostatic interactions. On the other hand, for a preassociation S_N2 mechanism, Glu 179 could catalyze the reaction by initially hydrogen bonding to the oxygen atom of the glucosidic linkage and transferring its proton late in the transition state.

On the basis of the current crystallographic study, we cannot distinguish between the formation of a stable glucopyranosyl cation and a situation in which water 500 attacks the anomeric carbon prior to the complete formation of the cation. However, we can argue against the possibility of other mechanistic pathways that involve the formation of covalent substrate–enzyme intermediates. The OE1 atom of Glu 400 and Glu 179 are 4.0 and 3.9 Å, respectively, from carbon C1 of 1-deoxynojirimycin. Furthermore, the approach of the side chain of Glu 400 to the "anomeric" carbon of the inhibitor is blocked by water 500. Glu 179 would also be blocked from the anomeric carbon by the oxygen atom of the glycosidic linkage. Thus, a covalent linkage between either Glu 179 or Glu 400 and the substrate is unlikely.

The present study offers a detailed explanation of the observed difference in the rate of hydrolysis of the α- and β-anomers of D-glucopyranosyl fluoride (Kitahata et al., 1981). The α-anomer of D-glucopyranosyl fluoride undergoes hydrolysis by glucoamylase from *Rhizopus niveus* approximately 2700-fold faster than does the β-anomer. In order for the β-anomer of glucose or glucose analogues to bind to the active site of glucoamylase, the catalytic water, water 500, must be displaced from the active site. However, no steric conflict would exist between water 500 and the α-anomer of glucose or glucose analogues. Thus, the slow rate of hydrolysis of the β-anomer of D-glucopyranosyl fluoride may be due, as suggested by Kitahata et al. (1981), to the displacement of the catalytic water by the β-anomer and the reversal of the catalytic roles of the two carboxylate side chains. In the case of the β-anomer, Glu 400 would be the catalytic acid, protonating the leaving group (fluoride anion), and Glu 179 would be a general base catalyst, coordinating the nucleophilic attack of a nearby water molecule in the active site.

The crystal structure for cellulase D has been reported recently (Juy et al., 1992) and like glucoamylase the catalytic domain of this enzyme is an α/α-barrel or twisted α-barrel. The spatial relationships of the putative catalytic acid and base in cellulase D with respect to the framework of the α/α-barrel are approximately the reverse of those found in glucoamylase. In other words, residue Asp 201 of cellulase D is the putative catalytic base, corresponding to Glu 179, the catalytic acid of glucoamylase, and residue Glu 555 of cellulase D is the catalytic acid, corresponding to Glu 400, the catalytic base of glucoamylase. The spatial reversal of functional groups is consistent with the difference in the functions of the two enzymes; cellulase D cleaves β1→4 linkages, leading to the formation of a new anomeric carbon in the α-configuration, whereas glucoamylase cleaves α1→4 linkages, creating a new anomeric carbon in the β-configuration.

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