Crystal Structure of the Covalent Intermediate of Amylosucrase from *Neisseria polysaccharea*†

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ABSTRACT: The α-retaining amylosucrase from the glycoside hydrolase family 13 performs a transfer reaction of a glucosyl moiety from sucrose to an acceptor molecule. Amylosucrase has previously been shown to be able to use α-D-glucopyranosyl fluoride as a substrate, which suggested that it could also be used for trapping the reaction intermediate for crystallographic studies. In this paper, the crystal structure of the acid/base catalyst mutant, E328Q, with a covalently bound glucopyranosyl moiety is presented. Sucrose cocrystallized crystals were soaked with α-D-glucopyranosyl fluoride, which resulted in the trapping of a covalent intermediate in the active site of the enzyme. The structure is refined to a resolution of 2.2 Å and showed that binding of the covalent intermediate resulted in a backbone movement of 1 Å around the location of the nucleophile, Asp286. This structure reveals the first covalent intermediate of an α-retaining glycoside hydrolase where the glucosyl moiety is identical to the expected biologically relevant entity. Comparison to other enzymes with anticipated glucosylic covalent intermediates suggests that this structure is a representative model for such intermediates. Analysis of the active site shows how oligosaccharide binding disrupts the putative nucleophilic water binding site found in the hydrolases of the GH family 13. This reveals important parts of the structural background for the shift in function from hydrolase to transglycosidase seen in amylosucrase.

Amylosucrase (AS)[1] (EC 2.4.1.4) is a transglucosidase with the unique property of producing an amylose-like polymer from sucrose. Hehre et al. first identified AS from *Neisseria perflava* in 1946 (*1*), and it has since been found in several other bacterial strains (*2*−*8*). The reaction, which is catalyzed by AS, is the transfer of an α-D-glucopyranosyl moiety from sucrose onto an acceptor molecule upon release of fructose, but it has also been shown that it can utilize glucosyl moieties from α-D-glucopyranosyl fluoride (*9*) or longer maltooligosaccharides (*10*, *11*). In the case where the acceptor molecule is another saccharide, AS produces only α-1,4 linkages; however, water can also act as an acceptor. In vitro the enzyme has shown the ability to perform both hydrolase and transferase activity in the absence of an acceptor polymer. This results in the synthesis of a mixture of smaller different-sized oligosaccharides and hydrolysis products, such as glucose, maltose, turanose, various maltooligosaccharides, and insoluble polymer (*12*). The ratio between hydrolysis and transfer activity depends mainly on the concentration of sucrose and acceptor molecules. In the presence of glycogen, however, AS primarily performs the elongation reaction (*13*). In vivo the main function of AS is thought to be elongation of glycogen branches.

The full-length recombinant AS from *Neisseria polysaccharea* comprises 636 amino acids, but only 628 amino acids are visible in the crystal structures. AS acts as an α-retaining glycosidase with the catalytic residues Asp286 and Glu328 operating as the nucleophile and general acid/base, respectively (*14*). The double-displacement mechanism, which AS is expected to use, was first described by Koshland in 1953 (*15*) and involves the formation of a covalent intermediate as can be seen in Scheme 1. The first step is the nucleophilic attack by Asp286 at the anomeric carbon (residing in the α-configuration), which is assisted by protonation of the oxygen in the glycosidic linkage (O1) by Glu328. Jointly, this leads to release of fructose and formation of the covalent glucosyl-enzyme intermediate, where the anomeric carbon is now in the *β*-configuration. In the second step, Glu328 acts as a general base and activates the incoming acceptor, which therefore attacks the anomeric carbon, and the covalent bond is cleaved. Subsequently, the anomeric carbon returns to the α-configuration. Both steps are expected to proceed via oxocarbenium ion transition states.

Through the sequence-based classification for glycoside hydrolases established by Henrissat in 1991 (*16*, *17*), AS was found to belong to the glycoside hydrolase family 13 (GH 13), also known as the α-amylase family. The GH13

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[1] Abbreviations: AS, amylosucrase from *Neisseria polysaccharea*; GH, glycoside hydrolase; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OB, oligosaccharide binding sites.

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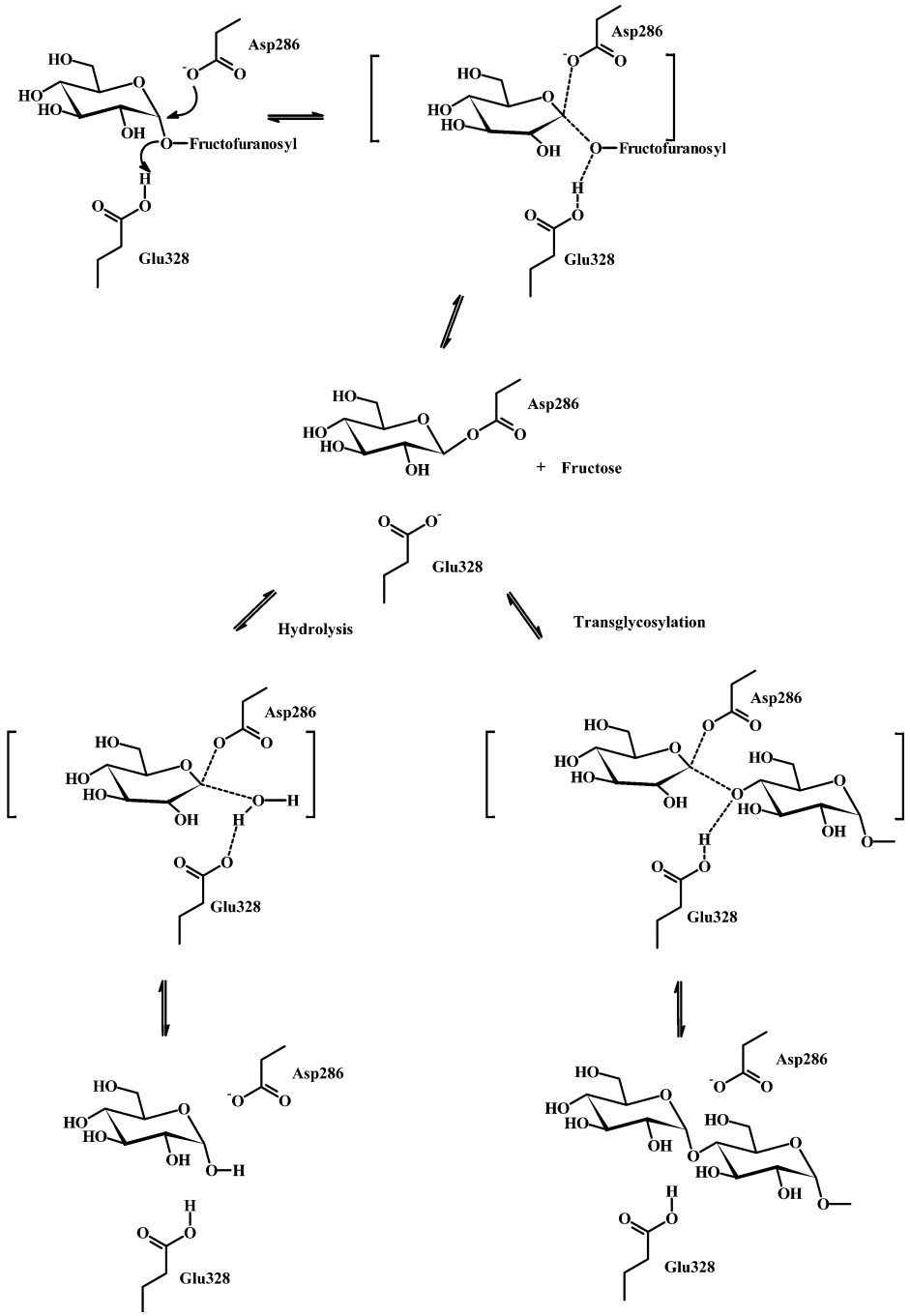
Scheme 1: Schematic Representation of the Reaction Pathway



family consists of enzymes, which all ensure retention of the anomeric carbon configuration, and it falls under the GH-H clan, wherein all of the enzymes share a common fold, the $(\beta/\alpha)_8$ barrel. Several three-dimensional structures of GH13 enzymes have been solved, e.g., cyclodextrin glucosyltransferase (CGTase) (*18*), isomaltulose synthase (PalI) (*19*), oligo-1,6-glucosidase (*20*), and α-amylases from different organisms (e.g., Taka-amylase) (*21*). CGTase was the first α-retaining glycoside hydrolase for which the structure of a covalent intermediate was solved (*22*). Even though it was not the natural intermediate that was formed (since the glycosyl moiety was modified), the structure strongly supported the covalent nature of the intermediate and, thus, the two-step mechanism involving formation and breakdown of the intermediate as proposed by Koshland. Until this date this was the only three-dimensional structure of a covalent intermediate solved for this type of enzyme, in contrast to the β-retaining glycoside hydrolases, where several covalent intermediates have been trapped and the structures solved by X-ray crystallography (*23*−*27*). Although only one structure of a covalent intermediate for the α-retaining enzymes has been solved, a number of covalent intermediates have been trapped and characterized by means of HPLC, mass spectroscopy, and sequencing in order to identify the catalytic nucleophile of the enzymes (*28*−*34*). Recently, Withers and co-workers have reviewed the trapping of covalent intermediates for glycoside hydrolases, both α- and β-retaining enzymes (*35*, *36*).

We have previously solved a number of native and mutant AS structures, alone and in complex with different substrates, which have provided insight into the catalytic mechanism and specificity of AS (*37*−*39*). AS consists of five domains,

A (residues 98−184, 261−395, 461−550), B (residues 185−260), B′ (residues 395−460), C (residues 555−628), and N (residues 1−90). Domain A is the characteristic $(\beta/\alpha)_8$ barrel, where the catalytic pocket is situated. The structure of the active site mutant, E328Q, in complex with sucrose illustrates how the substrate is bound apparently prior to the attack by the nucleophile. The structure of native AS in complex with D-glucose mimics the covalent intermediate formed after the first step in the reaction (*38*). Although most of the enzyme−glucose interactions are expected to be similar to those in the enzyme−intermediate, the bound D-glucose (the β-anomer) induces significant shifts in residues around the active site, and it is therefore of great interest to study the real intermediate. Other structures of AS in complex with either maltoheptaose or sucrose (*39*) have shown a maltoheptaose molecule that spans the active site, imitating the situation of the product immediately after the second step in the reaction. In addition, the presence of another sucrose-binding site of unknown function and two oligosaccharide-binding sites (OB2 and 3) on the surface of the protein was identified. OB2 is in close proximity to the B′ domain, which has been proposed to play an important role in the polymerase activity of the enzyme.

Since AS utilizes a widely accessible and inexpensive compound such as sucrose in glucosyl transfer reactions, it has a great industrial potential in synthesis of various glucans and oligosaccharides. A thorough analysis of the mechanism and specificity of AS is therefore needed in order to understand the catalysis by this rather unique enzyme and to facilitate its optimal utilization.

The E328Q mutant is incapable of activating the substrates by protonation/deprotonation and is hence virtually inactive. In this paper, we have combined this lack of reactivity with a substrate that already possesses a good leaving group. In this system the intermediate can be formed, and the break down is slowed. It was therefore possible to trap the natural glucosyl moiety in the crystalline state and determine the structure.

MATERIALS AND METHODS

Crystallization and Data Collection. The procedure for expression and purification of the AS mutant E328Q was described earlier by Sarçabal et al. (*14*). Apart from the desired mutation in the active site the enzyme contains another mutation, H131Y, which is considered to originate from a PCR error. As noted earlier (*38*), this mutation does not induce any major changes in the structure around the active site.

For crystallization of the AS mutant E328Q, a protein solution (4 mg/mL) containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 1 mM α-dithiothreitol was set up as hanging drops in a ratio of 1:1 against a reservoir solution with 14 mM sucrose, 30% poly(ethylene glycol) 6000, and 0.1 M HEPES, pH 7.0, as described earlier by Skov et al. (*37*, *40*). The crystals were then soaked in a buffer resembling the initial crystallization conditions after equilibrium has occurred, but with sucrose exchanged with 20 mM α-D-glucopyranosyl fluoride for 20 h, after which they were flash cooled in liquid nitrogen. The data collection was carried out at the MAX-LAB synchrotron radiation facility BL711 (λ = 1.011 Å) at cryogenic temperature (110 K). Details concerning the data collection are given in Table 1.

Table 1: Data Collection Statistics[a]

space group	$P2_12_12$
cell dimensions (Å)	$a = 96.3$, $b = 116.6$, $c = 60.5$
resolution (Å)	20−2.2 (2.28−2.20)
total no. of reflections measured	128590
no. of unique reflections	32544
completeness (%)	92 (94.7)
$R(I)^b$sym (%)	8.1 (32.8)
Wilson plot *B*-factor (Å^2)	19.4

[a] Statistics for the highest resolution shell are given in parentheses. [b] $R(I) = \sum_{hkl}(\sum_i(|I_{hkl,i} - \langle I_{hkl}\rangle|))/\sum_{hkl,i}\langle I_{hkl}\rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indicies *h*, *k*, and *l* and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

Table 2: Refinement and Structure Quality Statistics for the Covalent Intermediate

resolution (Å)	20−2.2
no. of protein atoms (residues 1−628)	5072
no. of solvent waters	500
no. of heteroatoms	11
R_{cryst}[a]	18.4
R_{free}[b]	22.1
rms deviation for bonds (Å)	0.012
rms deviation for angles (deg)	1.5
average protein *B*-value (Å^2)	14.8
average solvent *B*-value (Å^2)	20.9

[a] $R_{cryst} = \sum_{hkl}(||F_{o,hkl}| - |F_{c,hkl}||)/|F_{o,hkl}|$, where $|F_{o,hkl}|$ and $|F_{c,hkl}|$ are the observed and calculated structure factor amplitudes. [b] R_{free} is equivalent to the *R*-factor but is calculated with reflections omitted from the refinement process (5% of reflections omitted).

The HKL suite (*41*) was used for processing of the data set. The crystals belong to the orthorhombic space group $P2_12_12$, and the cell dimensions were approximately the same as those of the previous AS crystals.

Structure Determination and Refinement. The initial phases were obtained from the E328Q−sucrose structure, and the first model building was done with ArpWarp (*42*) using the CCP4i interface (*43*). For subsequent model building the program O (*44*) was used, and all refinement was done with CNS (*45*). A test set of 5% was set aside for cross-validation during the refinement process, and the final *R* and R_{free} values were 18.4% and 22.1%, respectively. Information relating to the refinement process can be seen in Table 2.

The density for the covalently bound glucopyranosyl moiety in the active site was very clear in the ArpWarp density map. Water molecules were picked for spherical peaks of 1.5σ in the $2F_o - F_c$ map. Thus the final model comprises 628 residues, one covalently bound D-glucopyranosyl moiety in the active site, and 500 water molecules.

The program Procheck (*46*) was used to analyze the structure with respect to the stereochemistry of the model. In the Ramachandran plot 90.7% non-glycine residues were found to be in the most favored regions, 8.9% in the additionally allowed regions, 0.4% in the generously allowed regions, and none in the disallowed regions. Four residues were found to occupy two conformations; these are Cys138, Met217, Met285, and Gln328. The mutated catalytic residue Gln328 was disordered in the sucrose structure; however, in this structure the Gln328 chain side is better defined and is now confined to two conformations.

The atomic coordinates have been deposited in the Protein Data Bank, Research Collaboration for Structural Bioinformatics, Rutgers, NJ, with the accession code 1S46.

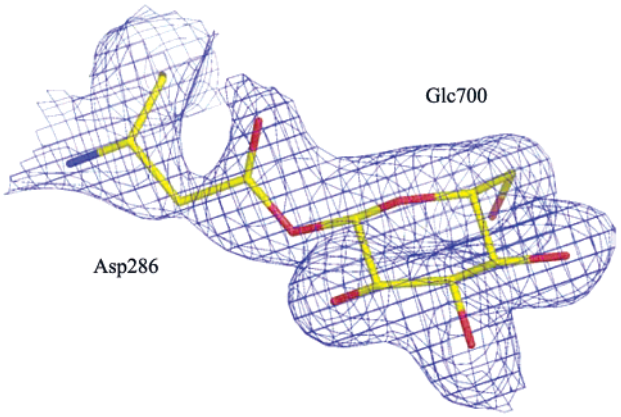


FIGURE 1: Simulated annealing $2F_o - F_c$ omit map of the covalent intermediate in the active site of AS. The map was contoured at 1.0σ around the glucose−AS intermediate (Asp286-Glc700). Atoms within 5 Å of the intermediate were omitted from the map calculation.

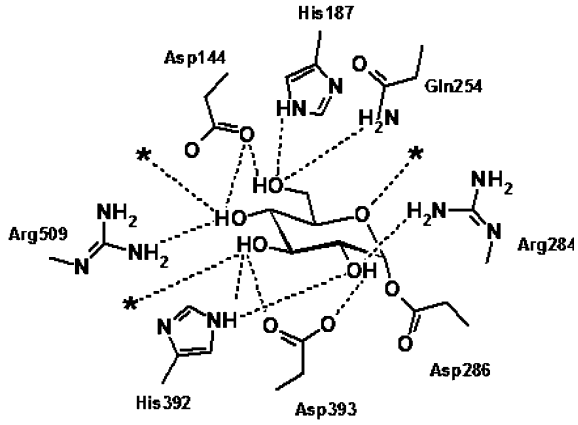


FIGURE 2: Schematic view of interactions between the glucose−AS intermediate and the surrounding enzyme with hydrogen bonds shown as dotted lines. The water molecules are marked as asterisks, where the right asterisk is Wat1294, the upper-left asterisk is Wat1144, and the lower-left asterisk is Wat823.

RESULTS AND DISCUSSION

The Active Site. The electron density associated with the covalent intermediate could already be seen in the electron density map, $2F_o - F_c$, from ArpWarp, and the glucosyl unit was easily modeled into the density at the α-amylase −1 subsite. The density clearly showed the covalent linkage between the glucosyl moiety (adopting the β-configuration) and the nucleophile, Asp286, which is expected for the covalent intermediate of AS. A simulated annealing omit map was calculated, omitting residue Asp286 and the glucosyl ring Glc700, and can be seen in Figure 1. It can be seen that the glucosyl ring lies in an undistorted $^4C_1$ conformation.

The interactions between the covalently linked glucosyl moiety and the surrounding enzyme are dominated by hydrogen bonds, of which some are water-mediated. The active site was investigated for hydrogen bonds with a maximum length of 3.5 Å and a suitable hydrogen bond geometry using the program HBPLUS (*47*). HBPLUS found 13 hydrogen bonds between the intermediate and the surrounding enzyme. The hydrogen-bonding pattern can be seen in Figure 2. The conserved active site residues in the α-amylase family, Asp393, His187, His392, and Arg284, are involved in the majority of hydrogen bonds to the glucosyl moiety and hold the glucosyl ring firmly in the active site pocket; especially His392 and Asp393 are important to the binding, which both participate in two hydrogen bonds to O2 and O3 (of the glucosyl ring). Apart from the hydrogen network, there are also two stacking interactions from Tyr147 and Phe250. The glucosyl ring is resting on Tyr147, while Phe250 interacts with C6.

The entire structure of the AS−glucose complex can be superimposed on the intermediate structure with a rms of 0.24 Å. There are some minor differences in the hydrogen-bonding network around the active site when the two structures are compared. Eleven of the 14 hydrogen bonds in the AS−glucose complex are also found in the intermediate structure. The length of two interactions has decreased significantly; the hydrogen bond between Arg509 and O4 by 0.4 Å and the hydrogen bond between Arg284 and O2 by 0.3 Å. Furthermore, the distance between Wat1294 and O5 has increased by 0.6 Å. The hydrogen bond between Glu328 and O1 no longer exists because of the covalent bond between Asp286 and the glucosyl unit. Lastly, two new hydrogen bonds are observed: between Wat1144 and O4 (3.14 Å) and Gln254 and O6 (3.06 Å).

The most obvious difference between the two structures is the covalent bond between Asp286 and the glucosyl moiety. Formation of this bond results in a significant movement in the backbone involving residues 285−287, which is shifted approximately 1 Å toward the center of the active site. This suggests that this loop is the most flexible part of the active site and that the other active site residues form a rigid entity. Taken as a whole, however, the AS−glucose complex is a good model for how the intermediate is bound in the active site, despite the differences in the positions of Asp286, which is turned away in the AS−glucose structure.

Active Sites of Amylosucrases. Among the amylosucrases recognized to date (*N. polysaccharea*, *Neisseria meningitidis*, *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Caulobacter crescentus*, *Deinococcus radiodurans*, *Magnetococcus* sp., and *Pirellula* sp.), the active site is almost totally conserved. Out of the 14 amino acid residues (Asp144, Tyr147, Ile184, His187, Phe229, Phe250, Gln254, Arg284, Asp286, Ala287, Glu328, His392, Asp393, and Arg509) within a distance of 7 Å from the glucosyl moiety, 11 residues (Asp144, His187, Phe229, Phe250, Gln254, Arg284, Asp286, Glu328, His392, Asp393, and Arg509) are fully conserved in the eight sequences analyzed here. Tyr147 is found in five of the eight sequences, while it is a Phe in the last three. Ile184 is found in two of the eight sequences while Val replaces it in the others. In five out of eight sequences, Ala287 is found while it is a Ser in the remaining three. However, when the structure of the active site of AS is compared with structures of the active sites of other GH family 13 members, both similarities and differences are found (see Table 3). In this structural alignment we have included AS, PalI (*19*), oligo-1,6-glucosidase (*20*), and Taka-amylase (*21*). Here only 7 of the 14 amino acid residues are found at equivalent positions. These are the ones that are all directly involved in binding of the glucosyl moiety and have by mutational studies been shown to be important for catalysis. Among AS, PalI, and oligo-1,6-glucosidase five additional amino acid residues are identical (Asp144, Phe229, Phe250, Gln254, and Arg509). These residues are all

Table 3: Structural Comparison of Amino Acid Residues at Equivalent 3D Positions in the Active Sites of AS and Selected GH Family 13 Members

	AS amino acid no.[a]													
	144	**147**	184	**187**	229	250	254	**284**	**286**	287	**328**	**392**	**393**	509
AS	Asp	**Tyr**	Ile	**His**	Phe	Phe	Gln	**Arg**	**Asp**	Ala	**Glu**	**His**	**Asp**	Arg
PalI[b]	Asp	**Tyr**	Val	**His**	Phe	Phe	Gln	**Arg**	**Asp**	Thr	**Glu**	**His**	**Asp**	Arg
oligo-1,6[c]	Asp	**Tyr**	Val	**His**	Phe	Phe	Gln	**Arg**	**Asp**	Val	**Glu**	**His**	**Asp**	Arg
Taka-amylase[d]		**Tyr**	Val	**His**		Gly	Leu	**Arg**	**Asp**	Thr	**Glu**	**His**	**Asp**	Asp

[a] Numbering of the amino acid residues as in the native AS structure (*37*). Numbers in bold are conserved within these four enzymes. [b] PalI, isomaltulose synthase, PDB code 1M53 (*19*). [c] Oligo-1,6-glucosidase PDB code 1UOK (*20*). [d] Taka-amylase PDB code 7TAA (*21*).

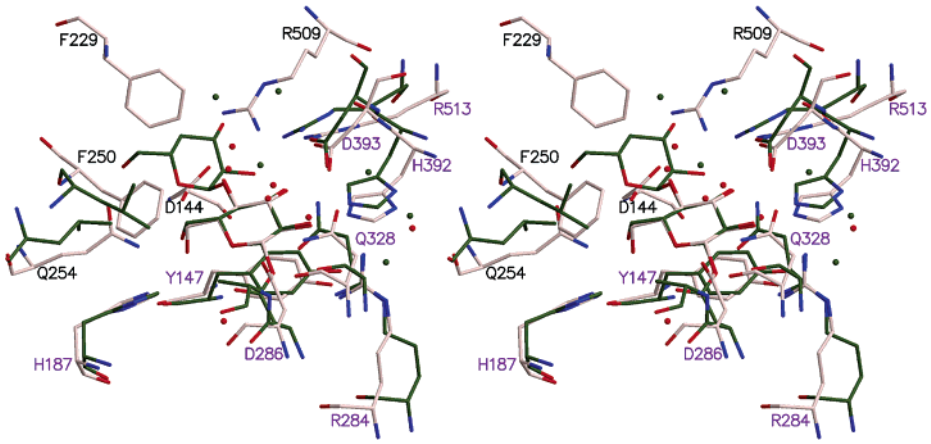


FIGURE 3: Superposition of the covalent intermediate structure of CGTase (green) and AS (light gray). A 4-maltosyl unit is covalently linked to the CGTase nucleophile (only Glc690 and Glc691 are shown), whereas AS has a glucosyl unit attached. The stereo picture shows the residues that lie within a radius of 5 Å of the AS glucosyl moiety.

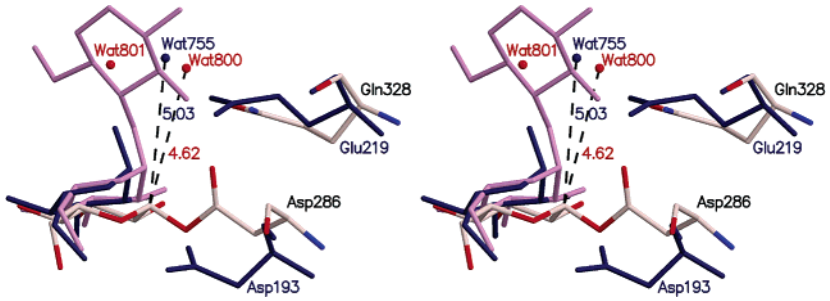


FIGURE 4: Stereoview of the position of the possible hydrolyzing water molecules in the active site of the AS covalent intermediate structure (in light gray, red, and blue) and in the G4-amylase structure (in dark blue) (*49*). The two glucose units [from an AS−maltoheptaose complex (*39*), in lilac] show that when an acceptor is present in the active site, the location of a posible hydrolyzing water molecule (Wat800 or Wat801) is occupied.

involved in formation of the pocket that is a common feature in these three enzymes. The Asp144−Arg509 salt bridge forms the bottom of the pocket, blocking further subsite, while Phe229, Phe250, and Gln254 cover the intermediate on the top. At positions 144 and 229 Taka-amylase has two deletions, followed by a Gly, a Leu, and an Asp at positions 250, 254, and 509, respectively, reflecting the much more open active site in this polymer hydrolase.

Comparison to the CGTase Intermediate. Within the GH family 13 there is only one other structure of a covalent intermediate. This is the structure of CGTase with a covalently bonded 4-deoxymaltotriose molecule (*22*) (PDB entry 1CXL). Figure 3 shows a superposition of the active site residues of the covalent intermediates of AS and CGTase within a radius of 5 Å around the AS glucosyl unit.

The structures are aligned by superposition of the two glucose moieties linked to the enzymes. The conserved general acid/base glutamates (glutamine variants in both structures, Gln328, AS numbering), and obviously the nucleophilic aspartates (Asp286) are seen to adopt very similar positions. Other generally conserved residues also adopt equivalent positions. The two histidines (His187 and His392) involved in subsite −1 hydrogen bonding, the aromatic stacking platform residue (Tyr147), the arginines at AS positions 284 and 513, and the aspartate at AS position 393 are all seen to superimpose very well. These similarities suggest that both enzymes use the same mechanism to form covalent intermediates. Differences reflecting the downstream diversity in function can also be identified. AS has a salt bridge between residues Asp144 and Arg509 that is the primary reason of the pocket topology necessary for this enzyme function as a exo-acting transglycosidase. The CGTase requires an open architecture for the attack exerted by the nonreducing end of the maltoheptaose molecule during the cylization reaction. Both enzymes have a similar open topology above the glucosyl C1 atoms that subsequently will

be attacked by a nucleophile. Only water molecules are seen in the vicinity of the C1 atoms. How hydrolysis is prevented in the working enzymes is not obvious from just looking at the active site and the immediate surroundings. In both cases hydrolysis is controlled somewhat distantly from the active site.

Water Structure in the Active Site. An important aspect of the covalent intermediate structure is the possibility to investigate the reason AS primarily functions as a transglycosidase rather than as a hydrolase. In order to act as a hydrolase, AS must host a water molecule, which is aligned with the general base, Glu328, and the C1 in the glucosyl-enzyme intermediate prior to hydrolysis. The only electron density around this position is very weak and filled as Wat800. The position of Wat800 can be seen in Figure 4. Wat800 lies at a distance of 2.53 Å (NE2) and 3.12 Å (OE1) to Gln328 (the mutated general base), and the distance to the C1 atom of the intermediate is 4.62 Å. The weak electron density of Wat800 could be explained as caused by partial occupancy or by high thermal motion. It should be pointed out that at 2.2 Å resolution it is not possible to discriminate accurately between occupancy and *B*-factors. Wat800 is fitted in the model with an occupancy of 1 and a *B*-factor of 30.7 Å^2. This *B*-factor should be compared to the average of 12.4 Å^2 (range 6−23 Å^2) for the rest of the very well defined water molecules in the active site and the entrance channel. In a covalent intermediate structure of a β-retaining glycoside hydrolase, endoxylanase (Xyl10A) (at 1.65 Å resolution) (*24*), a water molecule poised to attack the intermediate is seen. It forms a 2.6 Å long hydrogen bond to the catalytic acid/base and lies in close distance (3.7 Å) of the intermediate C1. Consequently, if Wat800 in the AS intermediate structure is to function as the hydrolyzing water, it would have to move closer to C1 before it can perform the nucleophilic attack in a manner similar to Xyl10A.

It would be interesting to compare this structure to a covalent intermediate structure of an α-amylase. Such a structure has not been solved; instead, some α-amylase structures exist, which contain water molecules that have been proposed to be responsible for hydrolysis (*48*, *49*).

The presence of a water molecule (Wat755 in the D294Q structure, PDB entry 1QI5) in a maltotetraose-forming α-amylase (G4-amylase) (*49*) has caused speculation about its role in hydrolysis. This water is positioned between Glu219 (the general base, G4-amylase numbering) and the substrate in the active site, more precisely, 5.0 Å from the intermediate C1 and 3.1 Å from OE1 in Glu219. Upon alignment of the structures with an rms of 0.93 Å, it is evident that Wat755 lies in almost the same position as Wat800 in the intermediate, as can be seen in Figure 4. Hence we conclude that Wat800 could be responsible for AS's hydrolysis side reaction. The distant position and weak binding to the enzyme implicate that transglucosylation will be the main reaction.

Finally, a fundamental difference between the hydrolyzing α-amylases and AS is the topology of the active sites and their entrances; the active site of the α-amylases lies in a cleft, whereas the active site of AS is situated in a pocket. This means that the active site of the α-amylases is widely accessible to water molecules for hydrolysis at all times. On the other hand, because of the pocket topology of AS, the active site is closed to incoming water molecules, when the acceptor molecule is present in the channel. This situation can be seen in the AS−maltoheptaose complex, where the maltoheptaose molecule spans the active site. The two glucose moieties in subsites −1 and +1 from the maltoheptaose complex are shown in lilac in Figure 4. It is evident that the glucosyl ring in subsite +1 occupies the position of Wat800 and there is no opportunity for any other water molecules to enter the active site from the channel. This suggests why AS only acts as a pure transglucosidase in the presence of polymer acceptor (e.g., glycogen), whereas in the absence or in a low concentration of the polymer acceptor it can perform both hydrolase and transferase activity.

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