Crystal Structure of a Maltogenic Amylase Provides Insights into a Catalytic Versatility*

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Amylases catalyze the hydrolysis of starch material and play central roles in carbohydrate metabolism. Compared with many different amylases that are able to hydrolyze only α-D-(1,4)-glycosidic bonds, maltogenic amylases exhibit catalytic versatility: hydrolysis of α-D-(1,4)- and α-D-(1,6)-glycosidic bonds and transglycosylation of oligosaccharides to C3-, C4-, or C6-hydroxyl groups of various acceptor mono- or disaccharides. It has been speculated that the catalytic property of the enzymes is linked to the additional ~130 residues at the N terminus that are absent in other typical α-amylases. The crystal structure of a maltogenic amylase from a Thermus strain was determined at 2.8 Å. The structure, an analytical centrifugation, and a size exclusion column chromatography proved that the enzyme is a dimer in solution. The N-terminal segment of the enzyme folds into a distinct domain and comprises the enzyme active site together with the central (αβ)8 barrel of the adjacent subunit. The active site is a narrow and deep cleft suitable for binding cyclodextrins, which are the preferred substrates to other starch materials. At the bottom of the active site cleft, an extra space, absent in the other typical α-amylases, is present whose size is comparable with that of a disaccharide. The space is most likely to host an acceptor molecule for the transglycosylation and to allow binding of a branched oligosaccharide for hydrolysis of α-D-(1,4)-glycosidic or α-D-(1,6)-glycosidic bond. The (αβ)8 barrel of the enzyme is the preserved scaffold in all the known amylases. The structure represents a novel example of how an enzyme acquires a different substrate profile and a catalytic versatility from a common active site and represents a framework for explaining the catalytic activities of transglycosylation and hydrolysis of α-D-(1,6)-glycosidic bond.

Starch is the main source of energy for a wide variety of living organisms. It is the polymer of glucose, linked by either α-D-(1,4)- or α-D-(1,6)-glycosidic bonds. Starch is hydrolyzed by several different types of hydrolytic enzymes widespread in nature, most of which can be grouped in the α-amylase family (1). Amylases are classified according to their enzymatic action pattern. Glucoamylases (EC 3.2.1.3) and β-amylases (EC 3.2.1.2) are exo-type enzymes cleaving glucose from maltose units, respectively, from the nonreducing end of starchy materials by hydrolyzing α-D-(1,4)-glycosidic bonds. α-Amylases (EC 3.2.1.1) are endo-type enzymes catalyzing the cleavage of the internal α-D-(1,4)-glycosidic bonds of starch, glycogen, and various oligosaccharides. Pullulanases (EC 3.2.1.41) cleave the internal α-D-(1,6)-glycosidic bonds of the substrate pullulan, which is the polymer of maltotriose linked by α-D-(1,6)-glycosidic bond.

Several groups of starch-hydrolyzing enzymes are known to harbor more than single enzyme activity. One group of these, maltogenic amylases (MAs; EC 3.2.1.133) exhibit unique characteristics that are different from other α-amylases (2–5) in that they exhibit (i) a dual activity of α-D-(1,4)- and α-D-(1,6)-glycosidic bond cleavages; (ii) an activity of α-D-(1,4)- to α-D-(1,3), α-D-(1,4)-, or α-D-(1,6)-transglycosylation; and (iii) an activity of cleaving acarbose, a pseudo-tetrasaccharide competitive inhibitor of α-amylases. The enzymes prefer cyclodextrins (CDs) to starch or pullulan as substrates in that the hydrolysis of β-CDs (seven glucose units) is ~100 times faster than that of starch or pullulan (5). In contrast, the other α-amylases with the single hydrolysis activity cannot hydrolyze CDs or pullulan. MAs cleave α-D-(1,4)-glycosidic bond much more efficiently than α-D-(1,6)-glycosidic bond. The enzymes hydrolyze CDs and starch mainly to maltose at low concentration of the substrates, thereby they are named “maltogenic amylase”. Different sugar molecules, including glucose, fructose, maltose, and cellobiose, can serve as acceptors for the transglycosylation. Compared with cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19), which catalyze the formation of CDs from starch by transglycosylation, the transglycosylation activity of MAs is much more inefficient because they convert the substrates mainly to hydrolysis products. The property, if not all, is shared by other

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The atomic coordinates and structure factors (code 1SMA) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: MAase, maltogenic amylase; ThMa, Thermus maltogenic amylase; BSMA, B. stearothermophilus maltogenic amylase; CGTase, cyclodextrin glycosyltransferase; NPase, neopullulanase; CDase, cyclomaltodextrinase; CD, cyclodextrin; PTS, pseudo-trisaccharide; MIR, multiple isomorphous replacement; MR, molecular replacement; BES, N,N-[bis[2-hydroxyethyl]amino]ethanesulfonic acid; NCS, noncrystallographic symmetry; TVAII, T. vulgaris R-47-amylase II.
amylolytic enzymes with different names, including neopullulanases (NPases; EC 3.2.1.135) (6, 7) and cyclomaltodextrinases (CDases; EC 3.2.1.54) (8, 9), both of which are homologous to maltogenic amylases with sequence identity of 40–86%. The three groups of amylases are high molecular weight amylases because of a unique addition of about 130 residues at the N terminus compared with the other \( \alpha \)-amylases containing the single activity of hydrolyzing \( \alpha \)-D-(1,4)-glycosidic bonds.

The C-terminal region of the enzymes (about 490 amino acids) exhibits some extent of sequence homology with the smaller \( \alpha \)-amylases and contains invariant catalytic residues (1). Unlike other starch-hydrolyzing enzymes, the three groups of the enzymes are intracellular enzymes. In \textit{Klebsiella oxytoca}, the open reading frames for CDase and an extracellular enzyme CGTase are clustered on the chromosomal DNA together with genes coding for products homologous to the maltose and linear maltodextrin uptake system (10). The finding suggested a starch degradation pathway where CDase is involved in the

![FIG. 1. Sequence alignment of ThMA (GenBank™ accession number O069007), BSMA (Q45490), \textit{B. stearothermophilus} NPase (P39440), \textit{Bacillus} sp. CDase (O82982), and \textit{A. oryzae} TAKA-amylase (P10529). The secondary structure assignment and numbering at the top of the alignment correspond to ThMA. In the secondary structure notations, N\( \beta \)1, B\( \beta \)1, and C\( \beta \)1 represent the first \( \beta \)-strand of the N-, (\( \alpha \)/\( \beta \))8-barrel-, and C-domain, respectively, and so on. Amino acids that are not conserved, compared with the ThMA sequence, are lightly shaded. The black boxes represent the catalytic residues. Glu-332 is indicated by an asterisk whose substitution with histidine severely affects the transglycosylation activity of ThMA.](image-url)
TABLE I
Structure determination and crystallographic statistics

| Source | Native | HcCl$_2$ | PCl$_2$(NH$_3$)$_2$ | Se-Met |
|--------|--------|----------|-------------------|--------|
| Wavelength (Å) | 1.0715 | 1.0715 | 1.0715 | 0.9789 |
| Resolution range | 20.0–2.8 | 20.0–3.0 | 20.0–3.0 | 20.0–3.1 |
| $R_{	ext{cryst}}$ (outer shell) (%) | 3.4 (12.0) | 3.7 (13.6) | 4.0 (12.2) | 4.4 (13.6) |
| Completeness (%) | 94.3 | 87.6 | 85.7 | 84.8 |
| r.m.s. deviation bond lengths/angles | 0.005/1.117 |
| Phasing power (acen/cent) | 0.70/0.78 |
| Number of sites | 1 | 13 | 7 |
| Number of refined atoms, Protein/water/erythritol | 93,128/285/2 |
| $R_{	ext{free}}$ (acentric) | 0.46/0.59/0.47 |
| $R$-factor/ $R_{	ext{free}}$ | 29.2/27.0 |

a $R_{	ext{cryst}} = \frac{\Sigma |F_o| - |F_c|}{\Sigma |F_o|}$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes respectively.

b $R_{	ext{free}}$ was calculated with 5% of the data.

c $R$-factor = $\frac{\Sigma |F_o| - |F_c|}{\Sigma |F_o|}$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes respectively.

d $R_{	ext{free}}$ was calculated with 5% of the data.

Intracellular degradation of CDs that are generated and transported into the cell by CGTase and by a specific uptake system, respectively. The crystal structures of α-amylases from many different sources (Kadziola et al. (11) and Fujimoto et al. (12), and references therein) revealed that this family of proteins has a common folding scaffold, the (αβ)$_8$ barrel, and that the active site is located on top of the barrel. However, structural information of MAases has been lacking. We have determined the structure of maltogenic amylase from a Thermus strain (ThMA). ThMA shows optimum temperature of 60°C, which is higher than that of any other maltogenic or similar enzyme reported so far. The structure reveals how the unique ~130 extra residues at the N terminus modify the common active site preserved throughout the α-amylase family to achieve the distinct property of the enzyme.

MATERIALS AND METHODS

Crystallization—Initial effort was put into the structure determination of Bacillus stearothermophilus maltogenic amylase (BSMA) whose crystallization condition we have reported (13). We suffered because of fragility of the crystals, especially in the presence of a heavy metal compound, and subsequently switched to the crystallization of ThMA which led to the structure determination of the both enzymes. Gene cloning and overproduction of ThMA was described recently (5). The ThMA crystals were obtained by vapor diffusion from droplets containing 2 μl of protein solution (10 mg/ml in 20 mM maleate, pH 6.8) plus 0.5 μl of precipitant solution containing 0.5 M lithium sulfate, 0.3 M ammonium sulfate, 0.1 M sodium citrate (pH 5.6), and 4% ethanol (v/v), which were equilibrated against 1 ml of the same precipitant solution at 22 °C. The crystals belong to the space group P6$_1$, with cell dimensions of $a = b = 118.04$ Å, $c = 266.88$ Å, and contain two molecules in the asymmetric unit.

X-ray Data Collection, Structure Determination, and Refinement—Because of the large unit cell dimensions of ThMA crystals, all x-ray diffraction data were collected from flash-cooled crystals by using synchrotron radiation from TARA and BL18B beamlines at Photon Factory, Japan. The cryoprotectant solution contained 0.5 M lithium sulfate, 0.3 M ammonium sulfate, 0.1 M BES (pH 6.5), 4% ethanol (v/v), and saturated erythritol. Data reduction, merging, and scaling were accomplished with the programs DENOVO and SCALEPACK (14). While searching for heavy atom derivative crystals, the (αβ)$_8$ structures of various amylases were used as search models in the MR (molecular replacement) method. Low sequence homologies of ThMA to other typical concentration distribution of the protein as a function of the square of the radial position is shown. The partial specific volume of ThMA was calculated as 0.7354 cm$^3$/g from the amino acid sequence of the protein. The solid line indicates the calculated curve for dimeric species. $R^2$, the coefficient of determination, indicates an excellent fit to an ideal dimeric species model.
ical α-amylases prevented us from obtaining a correct solution. To our surprise, the \((\alpha/\beta)_8\) structure of \(\beta\)-amylase (Protein Data Bank code 1BYA) as a search model, which bears no meaningful sequence homology to ThMA, yielded correct positions of the two molecules in the asymmetric unit with CCP4 version of AMoRe (15) (for the first solution) and with X-PLOR (16) (for the second solution). The translation functions were calculated with the highest peak with a correlation of 7.3% in a rotation search using AMoRe. The search found the highest peak with a correlation of 10.8% and an \(R\)-factor of 56.5%. The increase in the correlation coefficient suggested that the peak was a correct solution. However, efforts to find the position of the second molecule after fixing the position of the first solution did not yield a promising solution. A self-rotation search with X-PLOR yielded a very strong peak for \(\phi = 7.5^\circ, \psi = 0^\circ, \kappa = 180^\circ\). The first solution was rotated according to the noncrystallographic symmetry (NCS), and a translation search along the \(xy\) plane was performed, which was followed by a translation search along the \(z\)-axis to correlate the relative \(z\)-positions of the two solutions. The calculations generated the final solutions with an \(R\)-factor of 49.9% after a rigid body refinement. When examined on a graphics computer, the two molecules showed no overlap with symmetry-related molecules. Furthermore, the phase derived from MR solutions identified a holmium position in a Fourier difference map which coincides with the position located from 3.0 Å resolution isomorphous Patterson difference map. The successful MR demonstrates that initial phase information can be derived using available structures coupled with a correct prediction of the tertiary folding pattern of an interested protein. However, we do not understand why the \((\alpha/\beta)_8\) barrel of TAKA-amyrase A, which should be more homologous to ThMA than the \(\beta\)-amyrase (see below), failed to give a correct solution.

It was not at all straightforward to solve the structure by using the phase information derived from MR, and additional phase information was obtained from three heavy atom derivative crystals (Table I) by MIR (multiple isomorphous replacement) method. Heavy atom binding sites were identified by Fourier difference analysis using the phases derived from MR. The heavy atom positions were used to calculate MIR phases. The MIR phases with the three derivative data had a mean figure of merit of 0.47 at 3.1 Å resolution and were improved by solvent flattening and by 2-fold NCS density averaging. Some parts of the partial model inconsistent with the MIR map were further truncated. A combination of the partial model and the MIR phases and a 2-fold NCS

### Fig. 3
- **a**: stereoview of Ca tracings of the ThMA monomer. Every other 20th residue positions are numbered. The drawing does not contain the Ca positions for residues 160–163, 259–264, and 277–280.
- **b**: the ribbon diagram of the ThMA monomer. The N-, \((\alpha/\beta)_8\) barrel, and C-domain are in green, cyan, and yellow, respectively.
- **c**: the ribbon diagram of the ThMA dimer in complex with a computationally docked \(\beta\)-CD, which is the most preferred substrate for the maltogenic and related enzymes. The two subunits, related by the molecular 2-fold axis lying on the figure, are labeled with different colors. The docking of the substrate molecule did not require reorientation of the protein amino acid side chains and change in torsion angles of substrate. The model of \(\beta\)-CD bound to soybean \(\beta\)-amylase (27) was used without modification.
Crystal Structure of Maltogenic Amylase

RESULTS

\begin{figure}
\centering
\includegraphics[width=\textwidth]{schematic_products.png}
\caption{Schematic drawings of products from reaction of acarbose with ThMA. Acarbose is hydrolyzed to PTS and glucose. Three different products can be generated by transglycosylation of PTS to glucose. One of those generated by \(\alpha\)-\(\beta\)-transglycosylation is acarbose. The activity of \(\alpha\)-\(\beta\)-\(\gamma\)-transglycosylation can be detected by using an acceptor molecule such as \(\alpha\)-methylglucoside.}
\end{figure}

Averaging at 8-3.1 Å resulted in a significantly improved electron density map in which many regions could be traced. The MR phasing and density modifications were carried out using the CCP4 suite (17). Crystallographic refinements, iterative map calculations, and model buildings were done using X-PLOR, CCP4 suite, respectively. The 2-fold NCS restraints were maintained until the last refinement. From the beginning of the refinement, 5% of total reflections from the native data set were set aside for monitoring \(R_{total}\) value. The final model at 2.8 Å resolution consists of 574 amino acids with an \(R\) value of 20.9% and an \(R_{free}\) of 27.0%. The model does not contain highly disordered residues 161-163, 259-264, and 277-280 for which electron densities are lacking.

The refined dimeric structure of ThMA was used to determine the structure of BSMA by MR, which shares 69% sequence identity to ThMA. Contrary to our earlier prediction that the asymmetric unit of BSMA crystals would contain three to four molecules (13), only one dimer is present with a high solvent content of 70.1%. The BSMA structure is very similar to that of ThMA and is not discussed in detail in this report.

Analytical Ultracentrifugation—Sedimentation equilibrium measurements were performed at 20 °C on a Beckman Optima XL-A analytical ultracentrifuge, using a four-hole rotor with standard double sector cell at a rotor speed of 10,000 rpm. The values of the two variables, absorbances at 280 nm versus radial positions were obtained. The apparent molecular weight of ThMA was calculated by fitting data sets to a single species model using the software BMPD-Nonlinear regression. The partial specific volume of ThMA and solvent density were calculated as described by Zamyatnin (18).

Acarbose is hydrolyzed to PTS and glucose. One of those generated by \(\alpha\)-\(\beta\)-\(\gamma\)-transglycosylation is acarbose. The activity of \(\alpha\)-\(\beta\)-\(\gamma\)-transglycosylation can be detected by using an acceptor molecule such as \(\alpha\)-methylglucoside.

ThMA Is a Homodimer—Fig. 1 shows sequence alignments of MAases, NPase, CDase, and \textit{Aspergillus oryzae} TAKA-amylase A, which is listed as the most homologous smaller \(\alpha\)-amylase in a sequence alignment search using the BLAST algorithm (19). The N-terminal 129 amino acids and the C-terminal \(\sim\)30 amino acids in the former three enzymes are completely absent in TAKA-amylase A. The central region exhibits a low degree of sequence homology, but the three catalytic residues, Asp-328, Glu-357, and Asp-424 (ThMA numbering) are invariant. The structure of ThMA can be divided into three distinct domains, the N-domain (residues 1-124) composed of \(\beta\)-strands exclusively, the central (\(\alpha\)/\(\beta\))\(_8\) barrel domain, and the C-domain composed of eight \(\beta\)-strands (residues 505-588) (Fig. 3). Superposition of the structures of ThMA and TAKA-amylase A reveals that the C-domain as well as the (\(\alpha\)/\(\beta\))\(_8\) barrel of the two enzymes are structurally conserved in contrary to the sequence alignment. However, a structural domain corresponding to the N-domain of ThMA is totally absent in the structure of TAKA-amylase A. It is a \(\beta\)-sandwich-like structure composed of 12 anti-parallel \(\beta\)-strands (Fig. 3). The domain is distinctively separated from the central body of the ThMA structure, but it is involved in extensive interactions with the (\(\alpha\)/\(\beta\))\(_8\) domain of the adjacent molecule in the asymmetric unit. A total of 1390 Å\(^2\) in solvent accessible surface of one molecule is excluded by the interactions, which is an indication of a dimer formation of the enzyme in solution. More than two-thirds of the excluded surface is hydrophobic surface. The observation led us to run ThMA on a size exclusion column to find that the enzyme was eluted as if the apparent molecular weight of the enzyme is \(\sim\)130,000, close to the calculated dimeric molecular weight (136, 414) (Fig. 2a). An ultracentrifugation analysis further evidenced the dimerization of ThMA in solution as shown in Fig. 2b. BSMA exhibits the same chromatographic profile and is also dimeric with tertiary and quaternary structures nearly identical to those of ThMA. Therefore, it is firmly established that MAases are dimeric in solution. Very recently, the crystal structure of a related enzyme, \textit{Thermoactinomyces vulgaris} R-47-amyII (TVAII) hydrolyzing cyclodextrins and pullulan, was reported (20) which shows 44% sequence identity to ThMA. The overall structure of TVAII is very similar to that of ThMA. Two molecules of TVAII were also contained in the asymmetric unit of the crystals of the enzyme (space group \(P_2_1_2_1_2_1\)). The two molecules exhibit the same molecular contacts as the ThMA dimer does. Although the oligomerization state of TVAII was not investigated, the cumulative evidence strongly suggest that TVAII and other homologous enzymes classified as CDases and NPases should be dimeric in solution. Therefore, the substrate profile and catalytic property of these enzymes would be best explained by the active site configuration in the dimeric state.

The N-domain Modifying the Common Active Site Structure—The consequence of the dimer formation of ThMA is striking. The N-terminal domain of one subunit covers a part of the (\(\alpha\)/\(\beta\))\(_8\) barrel of the other subunit, which corresponds to the active site cleft of all the known \(\alpha\)- or \(\beta\)-amylases. As a result, the dimer interface at the top of the barrel forms a narrow and deep groove that is \(\sim\)17 Å in length, \(\sim\)8 Å in width, and \(\sim\)18 Å in depth, distinctively different from the wide and shallow active site clefts of the smaller \(\alpha\)-amylases. The groove must be the active site cleft because the three invariant catalytic residues are located at the bottom of the groove. They are found in the same relative position in space as the corresponding residues in TAKA-amyI A. The shape of the active site cleft of ThMA explains much slower hydrolysis of starch than CDs by the enzyme. The ring structures of CDs should be ring structures of CDs by the enzyme. The ring structures of CDs should be

\begin{align*}
\text{ThMA} & \quad \text{Is a Homodimer} \\
\text{Analytical Ultracentrifugation} & \quad \text{Sedimentation equilibrium measurements were performed at 20 °C on a Beckman Optima XL-A analytical ultracentrifuge, using a four-hole rotor with standard double sector cell at a rotor speed of 10,000 rpm. The values of the two variables, absorbances at 280 nm versus radial positions were obtained. The apparent molecular weight of ThMA was calculated by fitting data sets to a single species model using the software BMPD-Nonlinear regression. The partial specific volume of ThMA and solvent density were calculated as described by Zamyatnin (18).}
\end{align*}
computationally into the groove (Fig. 3c) using the “solid docking” module in QUANTA (Molecular Simulation, Inc.). The program allows a successful docking only when an electrostatic and geometric complementarity is accomplished between a host and a guest molecule. The deep groove is well occupied by the ring structure of the \( \beta \)-CD and many residues from both the \((\alpha/\beta)_8\) barrel and N-terminal domains are involved in favorable interactions with the modeled \( \beta \)-CD as shown in a close-up view of the substrate-binding model (Fig. 5). At the bottom of the cleft, one of the glycosidic bonds of the \( \beta \)-CD is located close to the catalytic residues. The catalytic residues of ThMA would be reached only by the disordered part of starch. Pullulan, a branched molecule by the presence of \( \alpha-D-(1,6) \)-glycosidic bond, would not reach the catalytic residues as easily as CDs either.

**Fig. 5. Extra sugar-binding space at the active site of ThMA.** a, molecular surfaces of ThMA and a model-built maltose at the extra sugar-binding space. In this model, the protein and docked \( \beta \)-CD atom positions were held fixed, and the torsion angle of the maltose glycosidic bond was rotated until the “solid docking” in program QUANTA allowed the favorable docking. The docking did not require reorientation of the protein side chains. b, stereo view of the active site of ThMA with hypothetical binding modes of the substrate \( \beta \)-CD (in coral) and maltose (in light blue). The position of \( \beta \)-CD and maltose is the same as in Fig. 3c and in Fig. 5a, respectively. The active site residues (Asp-328, Glu-357, Asp-424) plus Glu-332, identified as important for transglycosylation, are labeled. All oxygen atoms are in red. Several other residues (His-205, Tyr-207, Phe-289, Trp-359, His-423, Asp-468, Pro-469, and Trp-47 from the adjacent subunit) in close contact with the modeled \( \beta \)-CD are shown without labels. The segments of the \((\alpha/\beta)_8\) barrel (from one subunit) and the N-domain (from the other subunit) comprising the active site cleft are shown in green and magenta. Fig. 3 and Fig. 5, a and b, were prepared using the programs MOLSCRIPT (28), GRASP (29), and QUANTA, respectively.
pseudo-trisaccharide (PTS), glucose, and branched PTSs as transglycosylation products (Fig. 4). PTS can be recovered in pure form from an enzyme reaction mixture. When PTS and glucose, both in large amounts, reacted with ThMA, no transglycosylation product was formed. Therefore, the transglycosylation reaction by ThMA is concomitant with the hydrolysis of glycosidic bond. Catalysis by a-amylases is widely believed to proceed via a double-displacement reaction, in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed (22, 23). Given this mechanism and the fact that the transglycosylation reaction requires a high concentration of an acceptor sugar, it is reasonable to think that the acceptor molecule competes with a water molecule at the active site for attacking the glycosyl-enzyme intermediate. The assumption requires a sugar-binding site adjacent to the main substrate-binding site. Consistently, an extra space, lined by both the N-domain and the (α/β)8 barrel, is found deep at the bottom of the active site cleft (Fig. 5). A corresponding space is not found in the smaller amylases. Restrained substrates, such as CD, cannot reach the space as the bound β-CD model suggests. The size of the space appears to accommodate a disaccharide such as maltose rather easily, as a maltose molecule can be successfully docked into the space (Fig. 5b). In this model, the C4-OH group of a maltose at the nonreducing end is 3.6 Å away from Glu-357. There is some degree of freedom to rotate and translate the model without steric clash. We propose that the space (referred to as “extra sugar-binding space”) is responsible for the transglycosylation activity of ThMA. A mono- or disaccharide occupying the space could serve as an acceptor molecule to compete with a water molecule for attacking enzyme-substrate intermediate catalyzed by the same catalytic armory as shown in Fig. 6. In the extra sugar-binding space, an acceptor sugar molecule may be able to position either the C3-, C4-, or C6-OH group in a proper orientation for nucleophilic attack of the glycosyl-enzyme intermediate. The idea of extra sugar-binding space playing an important role in the transglycosylation explains the effect of Glu-332 mutation. We found that Glu-332 is absolutely conserved among 13 different related enzymes known so far, and the corresponding residue in the smaller α-amylases is conserved as histidine (24, 25) (Fig. 1). While the latter is known to be important for substrate-binding, Glu-332 in ThMA is located at the extra sugar-binding space and does not interact with the bound β-CD model. Substitution of the residue by histidine results in the drastic reduction of α-D-(1,6)-transglycosylation products without affecting the hydrolysis activity. The residue is at hydrogen bonding distance from the modeled maltose molecule. In the context of our proposal, Glu-332 appears to play an important role in the binding of small oligosaccharide acceptors.

ThMA is able to cleave α-D-(1,4)-glycosidic bonds of pullulan, although it does so inefficiently. It can also cleave α-D-(1,6)-glycosidic bonds of pullulan and small oligosaccharides. The cleavage is inefficient, and therefore, branched products resulting from the α-D-(1,6)-transglycosylation can be accumulated. The extra sugar-binding space, we propose, is also responsible for accommodating branched substrate into the active site cleft for cleavage. It was able to place a short pullulan molecule containing two maltotriose units into the active site cleft without a severe steric clash so that an α-D-(1,4)-glycosidic bond after a maltose unit is positioned close to the catalytic residues. Clearly, the extra sugar-binding space was necessary in fitting the molecule.

**DISCUSSION**

MAases, NPases, and CDases are intracellular enzymes, which implicates their biological roles in metabolizing small oligosaccharides, including CDs imported into the cells for energy generation, and storing them in the form of branched oligosaccharides under the condition of high concentration of glucose or maltose. A closely linked enzyme, CGTase, is hypothesized to have evolved from an ancestral hydrolase on the basis of sequence and phylogenetic analysis (26). The conservation of the common (α/β)8 barrel and C-domain in the structure of ThMA also indicates that the three groups of the enzymes are likely to have evolved from a common ancestor. Probably, these enzymes add a significant adaptive value to organisms by enabling efficient metabolism of starch materials.

Most members belonging to the α-amylase family exhibit the single activity of hydrolyzing α-D-(1,4)-glycosidic bond. The unique addition of ~130 residues at the N terminus of these enzymes compared with the other amylases has been speculated to be responsible for a rarely found example of an enzyme acquiring a new catalytic activity. We proved that ThMA is a homodimer in solution. As far as we know, there is no small molecular weight α-amylase whose functional unit is a multimer. The most salient feature of the dimer is that the unique N-terminal domain of one subunit comprises the active site together with the (α/β)8 barrel of the other subunit. The active site is otherwise similar to those of the smaller α-amylases in that it is located at one end of the (α/β)8 barrel and contains the invariant catalytic residues at similar spatial position. The
unique N-terminal domain of ThMA and probably of the related enzymes plays a major role in the modification of the common active site structure through the dimer formation for acquiring the distinct substrate profile and new catalytic property. Both the transglycosylation and the hydrolysis of α-D-(1,6)-glycosidic bond by ThMA are explained on the basis of the structural analysis and modeling with support from the biochemical and the mutagenesis studies. It is surprising that the new catalytic activities are gained not by creating a new catalytic residue, but most likely by creating the extra sugar-binding space at the active site. Although our explanation needs elaboration by structural elucidation of the enzymes in complex with different substrates or analogues, the current model may serve as a paradigm for other members of amylases with the activities of transglycosylation and hydrolysis of α-D-(1,4)- and α-D-(1,6)-glycosidic bonds.

Finally, the natural design of the transglycosylation activity of the enzyme at high concentration of acceptor sugar molecules may now be altered for biotechnological applications to produce branched oligosaccharides with higher efficiency.

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