Crystal Structures of 4-α-Glucanotransferase from Thermococcus litoralis and Its Complex with an Inhibitor*

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Thermococcus litoralis 4-α-glucanotransferase (TLGT) belongs to glucoside hydrolase family 57 and catalyzes the disproportionation of amylase and the formation of large cyclic α-1,4-glucan (cycloamylose) from linear amylase. We determined the crystal structure of TLGT with and without an inhibitor, acarbose. TLGT is composed of two domains: an N-terminal domain (domain I), which contains a (βπ)α barrel fold, and a C-terminal domain (domain II), which has a twisted β-sandwich fold. In the structure of TLGT complexed with acarbose, the inhibitor was bound at the cleft within domain I, indicating that domain I is a catalytic domain of TLGT. The acarbose-bound structure also clarified that Glu123 and Asp214 were the catalytic nucleophile and acid/base catalyst, respectively, and revealed the residues involved in substrate binding. It seemed that TLGT produces large cyclic glucans by preventing the production of small cyclic glucans by steric hindrance, which is achieved by three lids protruding into the active site cleft, as well as an extended active site cleft. Interestingly, domain I of TLGT shares some structural features with the catalytic domain of GoTγ α-mannosidase from Drosophila melanogaster, which belongs to glucoside hydrolase family 38. Furthermore, the catalytic residue of the two enzymes is located in the same position. These observations suggest that families 57 and 38 evolved from a common ancestor.

In the maltose metabolism of the hyperthermophilic archaeon, Thermococcus litoralis, 4-α-glucanotransferase (TLGT) (EC 2.4.1.25) plays a key role, producing glucose and a series of maltodextrins through intermolecular transglycosylation of maltose that has been transported into the cells (1). In addition to intermolecular transglycosylation, TLGT also catalyzes intramolecular transectdextrinolysis in vitro, where it cyclizes amylase to produce cyclic α-1,4-glucans (cycloamyloses, CAs) (2) with 16 to several hundred glucose units. The degree of polymerization of known CAs varies, from six to several hundreds of glucose units. α-, β-, and γ-cycloamyloses, which are the smallest CA species, consisting of 6, 7, and 8 glucose units, respectively, are well known doughnut-shaped rigid molecules and are able to accommodate guest molecules in their central cavity, yielding inclusion complexes. Although larger species of CA, which were recently found to be products of potato 4-α-glucoamylase (3), also form inclusion complexes, they have flexible single-helical conformations in an aqueous solution unlike cycloamyloses (4), and they lose their flexibility and fold into a compact structure during complex formation (5). Because of this structural difference from cycloamyloses, large CAs show the advantageous features of the formation of inclusion complexes and higher solubility in water and thus are expected to be valuable for future industrial use (6). Interestingly, an artificial chaperone activity of large CAs has also been reported (7).

According to Henrissat’s classification (8, 9), TLGT belongs to family 57 of the glucosidase hydrolases. Most family 57 enzymes catalyze reactions similar to those of some α-amylase family members (families 13, 70, and 77). However, no sequence similarity has been detected between family 57 and α-amylase family enzymes. The three-dimensional structures of many α-amylase family enzymes, including that of Thermus aquaticus amylomaltase (10), which produces large CAs, have been determined (11–13), and the amino acid residues involved in the catalysis have also been studied extensively (for a review, see Ref. 14). In contrast to α-amylase family enzymes, family 57 enzymes have received less investigation. Although the catalytic nucleophile of TLGT was recently determined (15), its three-dimensional structure, acid/base catalyst, and mechanism for large CA production remain unknown.

Previously, we made a preliminary report of the crystal structure of TLGT (16). In this paper, we describe the detailed structures of TLGT with and without a tetrasaccharide inhibitor, acarbose. The structures revealed the residues involved in

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1 The abbreviations used are: TLGT, 4-α-glucanotransferase from T. litoralis; CA(s), cycloamyloses(s); CGTase, cyclodextrin glucanotransferase.

2 B. S. Jeon and H. Matsuzawa, unpublished results.
catalysis and substrate binding of the enzyme and provided insight to investigate the mechanism for the production of large CAs.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—The nonlabeled enzyme was expressed in *Escherichia coli* strain BL21(DE3) and purified as described previously (15) with some modifications. In the last purification step, the buffer was changed to 5 mM Tris-HCl, pH 7.5, and the enzyme was concentrated to 10–20 mg/ml by centrifugal filtration using a Centriplus 30 (Millipore). The seleno-methionine-labeled enzyme was coexpressed in the methionine auxotrophic *E. coli* strain B834(DE3) (Novagen) with GroELS and tRNA cognates for AGA and AGG in a medium containing seleno-methionine. The purification procedures for the labeled enzyme were the same as those for the nonlabeled enzyme, except that the buffer used in the last step was replaced by 5 mM Tris-HCl, pH 7.5, containing 10 mM dithiothreitol.

Form I crystals were obtained at 25°C by the hanging drop vapor diffusion method by mixing 5 μl of protein solution with 5 μl of reservoir solution comprising 1.9 M ammonium sulfate, 2% (w/v) polyethylene glycol 400, and 0.1 M HEPES-NaOH, pH 7.5. The crystals were harvested in a solution comprising 2.2 M ammonium sulfate, 2% (w/v) polyethylene glycol 400 and 0.1 M HEPES-NaOH, pH 7.5. The concentration of trehalose (a gift from Hayashibara (Okayama, Japan), recrystallized before use) in the harvesting solution was increased stepwise to 25% (w/v), and then the crystals were subjected to flash freezing before data collection. Form II crystals were grown at 25°C by the sitting drop vapor diffusion method. The drops were made by mixing 5 μl of protein solution with 5 μl of a reservoir comprising 35% (w/v) 2,4-dimethylpentanediol (Hampton Research), 20 mM calcium chloride, and 0.1 M Tris-HCl, pH 8.0. The crystals were transferred to a solution comprising 40% (w/v) 2,4-dimethylpentanediol, 20 mM calcium chloride, and 0.1 M Tris-HCl, pH 8.0, followed by flash freezing. Acarbose-bound crystals were obtained by soaking in a 2,4-dimethylpentanediol solution containing 10 μM acarbose.

Catalysis and X-ray diffraction data were collected on a NONius four-circle x-ray diffractometer equipped with an ADSC Quantum 315 detector (Rigaku/MSC), using a graphite monochromator. The crystals were transferred to a cryoprotectant solution comprising 2.4 M 2,4-dimethylpentanediol, 20 mM calcium chloride, 40% (w/v) trehalose, and 0.1 M HEPES-NaOH, pH 7.5, before flash freezing.

Form I crystals were obtained at 25°C by the hanging drop vapor diffusion method by mixing 5 μl of protein solution with 5 μl of reservoir solution comprising 1.9 M ammonium sulfate, 2% (w/v) polyethylene glycol 400, and 0.1 M HEPES-NaOH, pH 7.5. The crystals were harvested in a solution comprising 2.2 M ammonium sulfate, 2% (w/v) polyethylene glycol 400 and 0.1 M HEPES-NaOH, pH 7.5. The concentration of trehalose (a gift from Hayashibara (Okayama, Japan), recrystallized before use) in the harvesting solution was increased stepwise to 25% (w/v), and then the crystals were subjected to flash freezing before data collection. Form II crystals were grown at 25°C by the sitting drop vapor diffusion method. The drops were made by mixing 5 μl of protein solution with 5 μl of a reservoir comprising 35% (w/v) 2,4-dimethylpentanediol (Hampton Research), 20 mM calcium chloride, and 0.1 M Tris-HCl, pH 8.0. The crystals were transferred to a solution comprising 40% (w/v) 2,4-dimethylpentanediol, 20 mM calcium chloride, and 0.1 M Tris-HCl, pH 8.0, followed by flash freezing. Acrasone-bound crystals were obtained by soaking in a 2,4-dimethylpentanediol solution containing 10 μM acarbose.
crystals overnight in a solution comprising 40% (w/v) 2,4-dimethyloctanediol, 20 mM calcium chloride, 10 mM acarbose (a gift from Bayer), and 0.1 M Tris-HCl, pH 8.0, followed by flash freezing.

**Data Collection**

A multiple wavelength anomalous dispersion data set for a seleno-methionine-labeled form I crystal was collected at wavelengths of 1.0400 Å (remote), 0.9793 Å (peak), and 0.9795 Å (edge) at 100 K on beamline BL45PX of SPring-8 (Hyogo, Japan). A native data set for a form II crystal was collected at a wavelength of 1.0200 Å at 100 K on beamline BL45PX of SPring-8. An acarbose-bound data set for a form II crystal was collected at a wavelength of 1.0000 Å at 100 K on beamline BL18B of the Photon Factory (Tsukuba, Japan). The first two data sets were processed with DENZO and Scalepack (17), and the last one was processed with Mosflm (18).

**Phase Calculation and Refinement**

For phase calculation of the multiple wavelength anomalous dispersion data set, the program SOLVE (19) was used. The multiple wavelength anomalous dispersion map was improved by density modification using the program DM (20). The model for “form I-Se” was built into the resultant electron density map using the program O (21) and refined to 2.8 Å resolution, including simulated annealing, bulk solvent correction, and grouped B-factor refinement with the program CNS (22). Water molecules were picked automatically from Fo −Fc electron density maps using the program CNS and checked manually at a graphic station. The phase for form II-free crystal was calculated by molecular replacement using form I-Se as the initial model using the program CNS. Noncrystallographic symmetry was found on the self-rotation function, but it was not used for cross-rotation function nor translation function. Density modification was not applied to the molecular replacement solution. The calculated model was refined to 2.4 Å resolution, including rigid body minimization, simulated annealing, and grouped B-factor refinement with the program CNS. The two TLGT monomers in the asymmetric unit were not restrained during refinement, because there are some variations in the conformation of the two molecules as described later. The final model was found to exhibit good geometry, as determined using the program Procheck (23); 88.7% of the residues have φ/ψ angles in the “most favored region” of a Ramachandran plot. The model for form II complex was also refined using the program CNS. During refinement of form II complex, the ligands were added based on 2Fo −Fc and Fo −Fc electron density maps. The refinement statistics are presented in Table I. A structural data base search was performed using the DALI server (24). Least mean square fitting of the structures was carried out with the program LSQMAN (25). The oligosaccharide model shown in Fig. 7 was constructed using the program XtalView (26).

**Site-directed Mutagenesis and Enzyme Assay**

The D214N variant was generated with a QuikChange site-directed mutagenesis kit (Stratagene). An oligonucleotide with the sequence 5’-GGTTTCTACTGGG-3’ and a complementary oligonucleotide, which replaced the codon for Asp214 (GAC) with TAA and introduced a HphI site for rapid screening of the mutation, were used. The mutation was reconfirmed by sequencing with an ABI PRISM 310 DNA se-

![Fig. 2. Sequence alignment of TLGT and two family 57 enzymes.](http://www.jbc.org/)

**Crystal Structure of Glycoside Hydrolase Family 57**
quencer (Applied Biosystems). Activity toward maltotriose was measured as described previously (27). One unit of activity was defined as the amount of enzyme that liberated 1 μmol of glucose from maltotriose per min at 80 °C.

RESULTS

Structure Determination—TLGT was crystallized in two forms (forms I and II). Form I crystals belonged to hexagonal space group P6_22 and form II crystals to orthorhombic space group P2_1_2_2. First, we determined the structure at 2.8 Å resolution by multiple wavelength anomalous dispersion method using a form I crystal labeled with seleno-methionine. The model was refined to an R-factor of 22.1% (Table I). We called this structural model form I-Se. The form I crystal contained one TLGT monomer/asymmetric unit. Next, we determined the structures of a form II crystal through molecular replacement in the free form, designated as form II-free, and in a complex form with acarbose, designated as the form II-complex. 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Domain II—Domain II of TLGT is composed entirely of β-strands, with the exception of two short α-helices (a19 and a20). This domain is characterized by a β-sandwich fold, in which two layers of anti-parallel β-sheets are arranged in a nearly parallel manner. The layer adjacent to domain II consists of 10 β-strands (β10, β11, β12, β13, β14, β15, β20, β21, β22, and β23), and the other layer consists of seven β-strands (β16, β17, β18, β19, β21, β22, and β24). β21 and β22 bend sharply and lie across the two layers. The same fold is found in chondroitin AC lyase from Flavobacterium heparinum (Protein Data Bank code 1CB8), β-galactosidase from E. coli (1BGL), hyaluronate lyase from Streptococcus pneumoniae (1EGU), methylamine oxidase from Hansenula polymorpha (1AZV), and copper amine oxidases from pea seedlings (1KSI) and E. coli (1OA C). Although the function of domain II is unclear at present, this domain may play a role in transglycosylation reaction of TLGT, because it has been suggested that β-sandwich domain of E. coli β-galactosidase is involved in transglycosylation reaction (28).

Calcium-binding Site—A calcium ion was bound at the loop between β10 and β11 in domain II (Figs. 1a and 2). The calcium ion was coordinated with O61 of Asp292, O62 of Asp394, O61 of Asp396, the main chain carbonyl oxygen of Arg398, and Oε1 and Oε2 of Glu400.

In form II-free, one additional calcium ion was identified between Glu60 of chain B and Asn348 of chain A of an adjacent asymmetric unit (data not shown). Because no form II crystals were observed when calcium chloride was omitted from the crystallization buffer, the latter calcium ion may promote crystallization by tightening the interaction between the two TLGT molecules.

Catalytic Residues—An acarbose-TLGT complex was obtained by soaking a form II crystal in a buffer containing acarbose. The structure of the complex was determined at 2.4 Å resolution and refined to an R-factor of 19.8% (Table I). Electron density corresponding to an intact acarbose molecule was clearly observed for the active site of chain A (Fig. 4a). As expected, the acarbose molecule bound to substrates −1 to +3, where the acarviosine moiety, the inhibitory disaccharide group of acarbose, occupied substrates −1 and +1 and the maltose moiety occupied substrates +2 and +3 (Fig. 5a). The nomenclature for the subsites is according to Davies et al. (29). This is the inhibitory binding mode observed for most of the structures of a-amylase family enzymes in complex with acarbose (30).

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3 H. Imamura and H. Matsuzawa, unpublished results.
Enzymatic hydrolysis of glycosidic linkages can be classified into two major types according to the anomeric configuration of the product, retaining and inverting, and in both cases the catalytic residues are typically two carboxylates (31). In retaining glycoside hydrolases, such as TLGT (2, 15), one residue acts as a nucleophile and the other as an acid/base catalyst. Glu123 and the two catalytic residues.

**FIG. 4. Bound ligands in the form II complex of TLGT.** Stereoviews of the refined models of acarbose in chain A (a) and maltose in chain B (b) are presented, together with $F_o - F_c$ electron density maps contoured at 4σ. Each ligand was omitted from the phase calculation. c, acarbose and the two catalytic residues. a and b were generated using XtalView (26) and Raster3d (45).
is close (3.15 Å) to the C1 atom of the valienamine moiety at subsite -1, allowing nucleophilic attack (Fig. 4c), which is consistent with the results of a cross-linking study that demonstrated Glu123 to be a catalytic nucleophile (15). It is considered most likely that Asp214 is the acid/base catalyst of TLGT, because O62 of Asp214 is only 2.96 Å away from the amide group of the valienamine moiety (this amide group is replaced by a glucosidic oxygen in a native substrate) (Fig. 4c), and there is no other acidic residue nearby. The average distances between all four pairs of O atoms of Glu123 and Asp214 (6.72 and 6.97 Å in the acarbose-free and acarbose complex structures, respectively) are in appropriate range for retaining enzymes (32). We generated the D214N mutant, in which Asp214 was replaced by Asn. The specific activity of the D214N mutant (0.0016 units/mg) was decreased about 10,000-fold as compared with that of the wild-type enzyme (17.7 units/mg). These results indicate that Asp214 is the acid/base catalyst.

Subsite Structure—Fig. 5a shows interactions between acarbose and the protein. The valienamine moiety at subsite -1 is within hydrogen bonding distance of His11 and Asp354 and interacts with His19, Glu216, and Trp357 via water molecules. The 6-deoxyglucose moiety is fixed at subsite +1 with Arg124, Asp213, and Asp214 through hydrogen bonds, with Tyr172 through aromatic stacking, and with Trp271 through a hydrophobic interaction. The glucose moiety at subsite +2 is bound to Arg282 and Asp213 though hydrogen bonds, to Tyr283 through an aromatic stacking interaction, and to Trp271 through a hydrophobic interaction. The glucose moiety at subsite +3 seems to be more flexible, because it only exhibits a stacking interaction with Phe187 and a hydrophobic interaction with Trp271, i.e., no hydrogen bonding interaction with the protein.

In contrast to chain A, acarbose did not bind at the active site of chain B of the form II complex, and only a Tris molecule was identified there (data not shown). Tris was also found at the active site of chain B of the form II-free (Fig. 1a). Although acarbose did not bind to the active site, we found electron density corresponding to disaccharide, which seems to be maltose, at the edge of the active site cleft of chain B (Fig. 4b). The reducing end of maltose is -14.5 Å apart from subsite -1, suggesting that this binding site corresponds to subsites -5 and -6. The glucose moiety at subsite -5 is bound to His368 through a hydrogen bond and to Phe19 through a hydrophobic interaction (Fig. 5b). The glucose moiety at subsite -6 is within hydrogen bonding distance of Arg272 and exhibits hydrophobic interactions with Phe19 and Tyr601 (Fig. 5b). In chain A, maltose did not bind to this site, because this region was involved in the crystal contact. From the complex structure with acarbose, TLGT was revealed to possess at least nine subsites, -6 to +3, although the residues forming subsites -4 to -2 could not be determined in this study.

Unexpectedly, a glucose molecule was found at subsite +1 of form I-Se (data not shown). Glucose interacted with Arg124, Asp214, Asp213, and Tyr272. Interactions between glucose and these residues in form I-Se are the same as those observed between the +1 glucose moiety of acarbose and the corresponding residues in the form II complex. Glucose was probably derived from trehalose reagent, which was used as a cryoprotectant and contained a trace amount of glucose.

The active site cleft of TLGT is tunnel-like in shape, as evidenced by the three lids that cover the cleft (Figs. 6 and 7). The first lid (lid 1, residues 220–224) protrudes from the β(α) barrel (Fig. 2). The second (lid 2, residues 358–363) and third (lid 3, 627–630) lids protrude from the three-helix bundle and domain II, respectively (Fig. 2). Upon binding of acarbose, the conformations of lids 2 and 3 change significantly (Fig. 6). In the absence of acarbose, the side chains of Val360 and Phe361 were directed toward the active site cleft. The bound acarbose collides with the side chains of Val360 and Phe361, leading to movement of lid 2. This movement induces a large movement of lid 3 (Fig. 6). For example, the Cα of Ser257 and Glu258 move 4.2 and 6.2 Å, respectively. In addition to the movements of these two regions, the χ1 axis of Tyr183 and the χ2 axis of Phe187 rotated to interact with the pyranose rings of the maltose moiety via hydrophobic stacking at subsites +2 and +3, respectively (Fig. 6).
the same manner (data not shown). This observation indicates that the two TLGT monomers interact in the same manner as observed in these crystals. Two hydrogen bond networks and a hydrophobic patch form the primary contribution to the interactions between the two subunits (Fig. 8a). One hydrogen bond network is constructed from two water molecules and six residues: Glu166 and Tyr266 from one monomer, and Ala91, Lys314, Asn324, and Lys328 from the other (Fig. 8a). The hydrophobic patch is formed by Leu287, Phe288, Phe291, Leu295, Tyr304, Phe307, and Val308 (Fig. 8b). In particular, Leu295 and Val308 are conserved (Fig. 2) and in contact with their counterparts in the other monomer (Fig. 8b), which suggests that they play a central role in the hydrophobic contact in this region. The proportion of the buried surface area (H110111500 Å2) is large enough for dimerization, compared with the total molecular surface of a monomer (H1101122300 Å2). Oligomerization is known to be one of the strategies by which proteins acquire thermostability (33). Because the dimer interface is located on the opposite side of the active site, dimerization seems to contribute to the thermostability rather than the activity including amylose cyclization.

DISCUSSION

One of the interesting features of TLGT is the (β/α)7 barrel fold, which forms the core of domain I (Fig. 3a). Proteins having the (β/α)7 barrel fold are very rare (34), although there is a large number of β-barrel proteins. Cellobiohydrolase (35) and endoglucanase (36), which both belong to glycoside hydrolase family 6, are two examples of (β/α)7 barrel proteins. The central β-sheet of the barrel of family 6 proteins is not completely closed, because there is only one hydrogen bond between the main chains of the first (β1) and last (β7) strands. The barrel of TLGT has a more open conformation, because there are two such “nonclosures” in the barrel of TLGT: between β3 and β4 and between β7 and β8.

Previously, we found that the nucleophilic residues of TLGT and class II α-mannosidases (glycoside hydrolase family 38) are located at the same position in the amino acid sequences despite their low sequence similarity (15). When the two families were compared at the three-dimensional level, striking structural similarities were observed in the catalytic domains of TLGT (Fig. 3b) and Drosophila melanogaster Golgi α-mannosi-
dase II (37) (Fig. 3c): a β-sheet core surrounded by α-helices, a three-helix bundle, and a catalytic nucleophile at the fourth β-α loop. These findings strongly suggest that the two families have
evolved from a common ancestor. Despite the similarities, however, there are also significant structural differences between the two enzymes. The most important one is that the catalytic domain of α-mannosidase II does not have a barrel structure, because there is no hydrogen bond that connects the two β-strands (7 and 8 in Fig. 3c) corresponding to β7 and β8 of TLGT. The αβ fold in α-mannosidase II may be a result of protein evolution from the (βα)2 barrel fold found in TLGT and vice versa. This difference is interesting from the standpoint of the evolution of protein folding.

In the crystal structures of amylose-α-acarbose complexes, α-acarbose was often found in a modified form, because of the transglycosylation activity of α-amylase (Ref. 30). However, it is clear that α-acarbose bound to the active site of TLGT (chain A in the form II-complex) is an intact one, because the electron density indicates that the sugar ring at the subsite –1 is somewhat flattened and that O-6 is not present at the subsite +1. Why maltose bound to the subsite –5 and –6 of chain B in the form II-complex is uncertain. Because maltose was not detected in the α-acarbose reagent when analyzed on thin layer chromatography (data not shown), acarbose moiety of α-acarbose may have flexible conformation, and only maltose moiety may be observed as a clear electron density. Additional electron density at the nonreducing end seen in Fig. 4b can be explained by this idea. Such a case has been also reported in the crystal structure of Bacillus circulans xylanase complexed with xylotetraose, in which electron density for only two xylose residues was observed (38). It is also unclear why α-acarbose did not bind to the active site chain B in the form II crystal. Although TLGT has a larger K<sub>v</sub> value (0.6 × 10<sup>−5</sup> M) for α-acarbose than α-amylase family enzymes (0.6 × 10<sup>−7</sup> to 0.8 × 10<sup>−4</sup> M) (39), the acarbose concentration (10 mM) in the soaking solution is sufficient. One possibility is that conformational changes in chain B prevent the binding of α-acarbose. When compared with form I and chain A of form II, the slight movements of O-2 – O-3 – O-4 and O-5 are observed (data not shown), probably because of crystal packing. These movements seem to change chain B into an inactive form that cannot bind the substrate.

There are several kinds of 4-α-glucanotransferases, including cyclodextrin glucanotransferase (CGTase), many of which produce CAs (6). The minimum ring size of CAs varies with the enzyme. The smallest products of CGTase are CAs with 6 glucose units. Potato 4-α-glucanotransferase (also a family 57 enzyme) (41). What is the evolution of protein folding.

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