Role of Phe283 in enzymatic reaction of cyclodextrin glycosyltransferase from alkalophilic *Bacillus* sp.1011: Substrate binding and arrangement of the catalytic site

RYUTA KANAI,^{1,2} KEIKO HAGA,¹ TOSHIHIKO AKIBA,² KUNIO YAMANE,¹ AND KAZUAKI HARATA²

¹Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

²Biological Information Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

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Abstract

Cyclodextrin glycosyltransferase (CGTase) belonging to the α -amylase family mainly catalyzes transglycosylation and produces cyclodextrins from starch and related α -1,4-glucans. The catalytic site of CGTase specifically conserves four aromatic residues, Phe183, Tyr195, Phe259, and Phe283, which are not found in α -amylase. To elucidate the structural role of Phe283, we determined the crystal structures of native and acarbose-complexed mutant CGTases in which Phe283 was replaced with leucine (F283L) or tyrosine (F283Y). The temperature factors of the region 259–269 in native F283L increased $>10 \text{ \AA}^2$ compared with the wild type. The complex formation with acarbose not only increased the temperature factors ($>10 \text{ \AA}^2$) but also changed the structure of the region 257–267. This region is stabilized by interactions of Phe283 with Phe259 and Leu260 and plays an important role in the cyclodextrin binding. The conformation of the side-chains of Glu257, Phe259, His327, and Asp328 in the catalytic site was altered by the mutation of Phe283 with leucine, and this indicates that Phe283 partly arranges the structure of the catalytic site through contacts with Glu257 and Phe259. The replacement of Phe283 with tyrosine decreased the enzymatic activity in the basic pH range. The hydroxyl group of Tyr283 forms hydrogen bonds with the carboxyl group of Glu257, and the pK_a of Glu257 in F283Y may be lower than that in the wild type.

Keywords: CGTase; crystal structure; substrate binding; catalytic activity

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19), which belongs to the α -amylase family (glycosyl hydrolase family 13; Henrissat and Davies 1997), catalyzes the conversion of starch and related α -1,4-glucans to cyclodextrins. The enzyme also catalyzes a coupling reaction and a disproportionating reaction through intermolecular transglycosylation, but its hydrolyzing activity is weak. Most CGTases from bacteria (e.g., *Bacillus macerans*, *B. stearothermophilus*, *B. circulans*) show a bell-shaped pH profile (pH 5.5 to

7.5; Kobayashi et al. 1978; Nitschke et al. 1990; Tanaka et al. 1991), and some CGTases from alkalophilic bacteria (*Bacillus* sp.1011, *B. ohbensis*, *Bacillus* sp.37–2) can function over a wide pH range (pH 4.5 to 10; Kaneko et al. 1990; Sin et al. 1991; Nakamura et al. 1993). The mechanism of the alkalophilic catalysis is not clearly understood, but the pK_a value of the proton donor/acceptor residue in alkalophilic CGTase must be higher than that in other CGTases.

The cyclization reaction that is not observed in α -amylase is the most attractive property of CGTase. The catalytic residues of CGTase and α -amylase are one aspartic acid (Asp229) as the nucleophile, and one glutamic acid (Glu257) as the proton donor/acceptor (Kubota et al. 1991; Nakamura et al. 1992; Uitdehaag et al. 1999b), and both enzymes have been suggested to fundamentally have the

Reprint requests to: Kazuaki Harata, Biological Information Research Center, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan; e-mail: k-harata@aist.go.jp; fax: 81-29-861-6194.

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same catalytic mechanism (Uitdehaag et al. 1999b). The key to the cyclization reaction is how the nonreducing end of the donor molecule, instead of the other sugar and water molecules, is transferred at subsites +1 and +2 of the acceptor-binding site in CGTase. Nakamura et al. (1994) have reported the biochemical analysis of the four mutant CGTases in which the specifically conserved aromatic amino acid residues (Phe183, Tyr195, Phe259, and Phe283) of the catalytic active site are replaced with leucine. Stochastic reaction path calculations of the process to form γ -CD from maltooctaose showed that Phe183 transports the nonreducing end of a donor molecule to subsite +1 at the acceptor binding site through the stacking interaction between the aromatic ring of phenylalanine and the pyranose ring of the sugar molecule, and Tyr195 cooperatively acts with Phe183 as a hydrophobic center to guide linear substrates to the acceptor-binding site where they are cyclized compactly (Uitdehaag et al. 2001). Phe259 plays an important role in binding the substrate, especially an acceptor residue, to cooperatively cyclize it on Phe183 (Nakamura et al. 1994; Schmidt et al. 1998; Uitdehaag et al. 2001). Furthermore, Nakamura et al. (1994) have shown that the K_m values of F283L CGTase for some linear substrates are similar to those of the wild-type enzyme, whereas those for CDs are higher. However, the relationship between the enzymatic property and the structural change caused by the mutation of Phe283 has remained unknown despite the significance of this residue that is specifically conserved in CGTase.

Here, we discuss the structural role of Phe283 on substrate binding and the catalytic reaction based on four crystal structures of mutant CGTases replaced with leucine (F283L) or tyrosine (F283Y) and their complexes with acarbose (F283LACA and F283YACA), which is a powerful inhibitor for most glucoside hydrolases.

Results

Enzyme assays of F283L and F283Y mutants

Nakamura et al. (1994) have reported that F283L CGTase has about half of the starch degrading activity of the wild type within a neutral pH range. The activity was considerably decreased at both acidic and basic pH ranges (Fig. 1). In contrast, the starch degrading activity of F283Y CGTase was similar to that of wild type between the acidic and neutral pH ranges but decreased to 10% at pH 10.0. The pH value of half activity at basic pH side was shifted to 8.6 from 10.0 of the wild type.

Replacement of Phe283 with leucine resulted in 23% to 67% decreases in the K_m values for 3-ketobutylidene- β -2-chloro-4-nitrophenylmalto-pentaoside (3KB-GSCNP), which is a maltopentaose with its nonreducing end blocked and with aglycon at its reducing end, in the disproportionation reaction, and no significant correlation to pH was detected.

Figure 1. Absolute starch degrading activity. pH Profiles for F283L (Nakamura et al. 1994 [solid circles]), F283Y mutant (solid squares), and wild-type CGTase (solid diamonds).

On the other hand, the k_{cat} values for the disproportionating reaction at various pH conditions decreased to 1.6% to 4.4% compared with those of wild type.

Crystal structures of two mutants, F283L and F283Y, and their acarbose complexes (F283LACA and F283YACA)

The crystallographic properties of these mutant enzymes (Table 1) are almost identical to those of wild-type CGTase (Harata et al. 1996; Haga et al. 2003), and the asymmetric unit contains two independent molecules (MOL1, MOL2). F283LACA and F283YACA bind a pseudo-tetrose derived from acarbose at subsites from -2 to +2 in the active site pocket (Figs. 2, 3). Acarbose is composed of an unsaturated cyclitol (A) at the nonreducing end, a 4,6-dideoxy-4-amino-D-glucose (B), and two D-glucose residues (C, D) in that order. However, it was converted to a pseudo-tetrose consisting of A-B-C and one glucose residue (G) at the nonreducing end (G-A-B-C). This conversion has been also confirmed in other CGTase complexes (Mosi et al. 1998; Haga et al. 2003).

Structural change in the catalytic active site of F283L

The backbone structure of F283L is almost identical to that of the wild-type CGTase. However, the isotropic temperature factors of C α atoms of the residues 259–269 of F283L, which construct subsites +1 to +3 (van der Veen et al. 2001; Haga et al. 2003), increased by $>10 \text{ \AA}^2$ (Fig. 4A). Therefore, the structure of this region is more flexible in F283L than in the wild type.

In F283L, the phenyl group of Phe259 is moved toward the carboxyl group of Glu257, the proton donor/acceptor group, for $\sim 1 \text{ \AA}$ with a +30-degree change in the χ^1 angle of Phe259 (Fig. 5A). The side-chain of Glu257 is also shifted

Table 1. Data processing and structure refinement of the residue 283 mutant CGTases

	(F283L)	(F283Y)	(F283LACA)	(F283YACA)
Data processing				
Cell axis				
a (Å)	63.9	64.9	65.0	64.1
b	73.7	74.4	73.7	73.6
c	78.2	79.0	80.1	78.3
α (°)	85.1	85.2	85.5	84.9
β	105.0	105.1	105.5	105.1
γ	101.0	101.0	101.5	101.0
Resolution range (Å)	29.1–1.83	29.1–1.83	48.4–1.80	18.9–1.82
No. of unique reflections	102,927	92,133	83,161	103,110
R_{merge} (%)	7.3	10.1	8.6	9.5
Structure refinement				
Resolution range (Å)	10.0–2.0	10.0–2.0	10.0–2.1	10.0–2.0
No. of unique reflections ($F > 2\sigma$)	78,142	84,562	67,131	83,723
Completeness (%)	86.2	90.1	82.8	92.2
Final R_{factor} (%)	17.0	15.5	16.9	16.3
Final R_{free} factor (%)	24.2	20.0	23.2	21.7
Root mean square deviation				
Bond length (Å)	0.015	0.013	0.013	0.013
Bond angle (°)	2.94	2.64	2.90	2.76

by ~ 0.5 Å toward Leu283. This indicates that Phe283 sterically regulates the conformation of Glu257 because the C ζ atom of Phe283 is within 4.0 Å to the carboxyl group of Glu257, and the replacement of Phe283 with leucine creates a space into which the side-chain of Glu257 can move. The side-chains of Asp328 and His327 are moved by ~ 0.4 Å, but they still form hydrogen bonds with the carboxyl group of Glu257 as observed in the wild type (Fig. 5A).

Structural change in the catalytic active site of F283LACA

When wild-type CGTase forms the complex with acarbose (Mosi et al. 1998; Haga et al. 2003), the structural change was minimal compared with the native enzyme. In contrast, several significant changes were found between native F283L and its complex. The isotropic temperature factors of the regions 257–278 and 310–317 of F283L are increased >10 Å² by the complex formation with acarbose (Fig. 4B). In addition, region 257–267 undergoes the remarkable structural changes, as shown by the positional difference (>0.5 Å) of the equivalent C α atoms between F283L and F283LACA (Fig. 4C). Therefore, these regions in F283L are more flexible in the complexed state than those in the native state. Furthermore, because the regions 269–280 and 314–320 are connected to each other by four and five hydrogen bonds in MOL1 and MOL2, respectively, and these hydrogen bonds are conserved in the native and acarbose-complexed structures of F283L and wild type, the structure of these regions may alter cooperatively through hydrogen bonding interactions in between the native and complexed

states. The electron density of the aromatic ring of Phe259 in F283LACA is much weaker than that observed in F283L. This indicates the high flexibility of the side-chain of Phe259. Contacts between the glucose residue and Phe259 may be flexible but not weak because the K_m for 3KB-G5CNP in the disproportionation is rather decreased in F283L compared with the wild-type enzyme.

The C α atoms of Phe259 are shifted by 1.3 Å (MOL1) and 1.7 Å (MOL2) toward substrates (Fig. 5B) compared with the wild type complexed with acarbose (Haga et al. 2003). Glu257 is shifted 1.5 Å toward Leu283 in MOL1 and 0.89 Å in MOL2, but the carboxyl group of Glu257 forms a hydrogen bond with the carboxyl group of Asp328, as observed in the wild type (Fig. 5B). The carboxyl group of Asp229 is shifted toward the bottom of the active cleft by 0.9 Å in MOL1 and 0.6 Å in MOL2 (Fig. 2).

Binding of pseudo-tetrose derived from acarbose in F283LACA

The torsion angles of the cleavage sites [ϕ , C7(A)-C1(A)-N4(B)-C4(B); ψ , C1(A)-N4(B)-C4(B)-C5(B)] of the pseudo-tetrose molecule of F283LACA differ from those of acarbose complexed with the wild type ($\Delta\phi = -37^\circ$ [MOL1], -23° [MOL2] and $\Delta\psi = 26^\circ$ [MOL1], -48° [MOL2]; Fig. 2). The torsion angles of the glycosidic linkage that connects sugar residues at subsite +1 and +2 in MOL2 were also changed by -17° ($\Delta\phi$) and 58° ($\Delta\psi$). In MOL2 of F283LACA, Phe259 was shifted 1.6 Å toward the 4-amino-4,6-dideoxyglucose (GLD) residue at subsite +1 from its position in the wild type complex. This move-

Figure 2. Stereo views of the structures of pseudo-tetrose and catalytic active site of F283LACA and wild-type CGTase complexed with acarbose (A, MOL1; B, MOL2). Thick green lines and thin blue lines indicate F283L and wild-type CGTases, respectively. Red lines denote the replace residue Leu283. Omit $|F_o - F_c|$ electron density of pseudo-tetrose in F283LACA is drawn at 1.5 σ (blue) and 3.0 σ level (red).

ment of Phe259 disrupted the hydrogen bonds between GLD and Glu257, and Phe259 was in close contacts within 4 Å distance with the GLD residue at subsite +1 (Fig. 5B).

The distance between the N4 atom of the pseudo-glycosidic bond and the carboxyl group of Glu257 was not significantly changed by the mutation of F283L, whereas the distance between the C1 atom of the cyclitol residue at subsite -1 and the carboxyl group of Asp229 was changed >0.3 (MOL1) and 0.1 Å (MOL2; Fig. 2). This indicates that the efficiency of nucleophilic attack by Asp229 is decreased in F283L.

As observed in the wild-type complex (Mosi et al. 1998; Haga et al. 2003), the unsaturated cyclitol ring of the sugar

residue in F283LACA is parallel to the aromatic ring of Tyr100, and the pyranose ring located at the subsite +2 is also parallel to the aromatic ring of Phe259 or Phe183 (Fig. 2). As a result, their intermolecular contacts are mostly conserved. This may be the reason for the relatively low K_m values of F283L for linear substrates (Nakamura et al. 1994).

Structural change in the catalytic active site of F283Y and F283YACA

The hydroxyl group of Tyr283 in F283Y forms de novo hydrogen bonds with the carboxyl groups of Glu257 (2.6 Å in MOL1 and MOL2) and the side-chain of Asn326 (2.9 Å

Figure 3. Stereo views of the structures of pseudo-tetrose and catalytic active site of the acarbose complexes of F283Y and wild-type CGTases (A, MOL1; B, MOL2). Thick green lines and thin blue lines indicate F283Y and wild-type CGTases, respectively. Red lines show the replaced residue Tyr283. Omit $|F_o - F_c|$ electron density of pseudo-tetrose in F283LACA is drawn at 1.5 σ (blue) and 3.0 σ level (red).

in MOL1 and 3.1 Å in MOL2; Fig. 5C). Compared with the wild type (Harata et al. 1996), the χ^2 angle of Glu257 in F283Y was changed by 7.9° in MOL1 and 4.5° in MOL2, and the carboxyl oxygen atoms of Glu257 are shifted 0.3 to 0.4 Å, although they still retained the hydrogen bonds with Asp328 and His327 (Fig. 5C).

Compared with the wild-type CGTase-acarbose complex (Mosi et al. 1998; Haga et al. 2003), the carboxyl oxygen atoms of Glu257 in F283Y_ACA were shifted 0.3 to 0.8 Å by the rotation of χ^2 or χ^3 and formed hydrogen bonds with the hydroxyl group of Tyr283. The imidazolyl group of His327 forms a de novo hydrogen bond with the O2H hydroxyl group of the cyclitol residue with the distance of 2.7 Å in MOL1 and 2.6 Å in MOL2 (Fig. 3). The other contacts

between the protein and sugar molecules in F283Y_ACA are mostly conserved in the wild type complexed with acarbose. These findings indicate that the change in pseudo-tetrose binding was caused by structural changes of Glu257, Phe259, His327, and Asp328 induced by the mutation.

Discussion

Phe283 represses the flexibility of region 259–269 neighboring the catalytic site

The crystal structure of F283L revealed the increased flexibility of the region 259–269, which constructs subsite +2 and +3 (Fig. 4). In the wild type, the aromatic ring of Phe283 is situated ~3.3 Å far from the H α -C α of Phe259

Figure 4. Comparison of structures and temperature factors. (A) Difference of isotropic temperature factors for equivalent Cα atoms between F283L and wild-type CGTase. (B) Difference of isotropic temperature factors for equivalent Cα atoms between F283L_ACA and F283L. (C) Positional difference for equivalent Cα atoms in domain A (residues 1–138 and 204–406) between F283L_ACA and F283L.

and perpendicular to the aromatic ring of Phe323 with the H ζ atom of Phe323 2.8 Å away (Harata et al. 1996). This geometry indicates that the aromatic ring of Phe283 is in the CH– π interaction with Phe259 and Phe323. In F283L, the replacement of Phe283 with leucine breaks the CH– π interaction, and the side-chain of Leu283 causes steric hindrance with the phenyl groups of Phe259 and Phe323 (Fig. 5B). This is supported by the fact that such changes are not evident in the structure of F283Y (Fig. 5C). In addition, the C β , C γ , and C δ 1 atoms of Phe283 are 4.0 Å far from Leu260 (Fig. 5B), indicating that Phe283 suppresses the

structural flexibility of Leu260 through close contact. Except for such interactions, the region 259–269 that is exposed to solvent has few interactions. Therefore, the increased flexibility of this region may result from the disruption of the CH– π interaction of Phe283 with Phe259 and close contacts with Leu260.

Phe283 maintains the structure of the catalytic site

We compared the conformational changes of residues Phe259, Glu257, His327, and Asp328 of the native and acarbose-complex of F283L with the wild type (Fig. 5; Harata et al. 1996; Haga et al. 2003). The replacement of Phe283 by leucine induces movement of the side-chain of Glu257 by disrupting the close contact of the carboxyl group with the phenyl group. This conformational change of Glu257 is associated with movement of the side-chains of Asp328 and His327 to retain hydrogen bonds with the carboxyl group of Glu257. Moreover, the conformation of the side-chain of Phe259 alters to move toward the side-chain of Glu257. These findings indicate that the phenyl group of residue 283 plays an important role in maintaining the structure of the “active” catalytic site by regulating the conformation of the side-chains of Phe259 and Glu257 via CH– π interaction and close contact, and indirectly arranges the structure of the side-chains of His327 and Asp328. Because the k_{cat} value of F283L CGTase for β -CD formation was ~40% of the wild type (Nakamura et al. 1994), these structural changes may slightly affect the catalytic reaction. In fact, the distance between the carboxyl group of Asp229 and the C1 atom of the cyclitol residue at subsite –1 in F283L_ACA was increased by 0.3 and 0.1 Å in MOL1 and MOL2, respectively (Fig. 2), and the efficiency of Asp229 as a nucleophile would be lower than that of the wild-type enzyme.

Phe283 may play important role in substrate binding by repressing the flexibility of region 259–269

The K_m values of F283L for various linear substrates in coupling, disproportionation, and hydrolysis were similar to or less than those of the wild type, whereas the K_m values for α -, β -, and γ -CD were increased 1.3-fold to threefold in cyclization, coupling, and hydrolysis (Nakamura et al. 1994). These results indicate that the region 259–269 involved in the binding of linear sugars has flexibility that slightly affects the catalytic reaction but does not reduce binding affinity. However, the increased structural flexibility of Phe259 in F283L causes the increase of K_m values for α -, β -, γ -CD. In fact, although the glucose residue at subsite +2 of maltonaose is sandwiched between Phe183 and Phe259 (Uitdehaag et al. 1999b), the glucose residue of γ -CD at subsite +2 is in the stack interaction with only Phe259 (Uitdehaag et al. 1999a). This indicates that Phe259 is more important for cyclodextrin binding than is Phe183. Therefore, we suggest that Phe283 plays an important role in the

Figure 5. Comparison of the catalytic active site. Broken lines denote hydrogen bonds (≤ 3.3 Å). The replaced residue 283 is shown by red lines. (A) MOL1 of F283L (thick lines) and wild type (thin blue lines). (B) MOL2 of F283L_ACA (thick lines) and wild-type complex (thin blue lines). (C) MOL2 of F283Y (thick lines) and wild type (thin blue lines).

binding of substrates, especially CD, by repressing the flexibility of the region 259–269.

New hydrogen bond with the carboxyl group of Glu257 in F283Y may reduce enzymatic activity in the basic pH region

F283Y CGTase is less active at the basic pH region than wild-type CGTase in the starch degradation (Fig. 1). The

crystal structure of F283Y shows that a new hydrogen bond is formed between the hydroxyl group of Tyr283 and the carboxyl group of Glu257 (Fig. 5C). Therefore, pH profile of F283Y CGTase may be changed because the proton of the carboxyl group of Glu257 will be easily released by the hydrogen bond formation with the hydroxyl group of Tyr283, which decreased the pK_a value. Although the experimental pK_a value of Glu257 was not measurable, the present results indicate that the pK_a value of wild-type

Glu257 is relatively high to maintain its protonation state in the basic pH range.

Material and methods

Chemicals and enzymes

All restriction and modification enzymes used for recombinant DNA manipulations were purchased from Takara Shuzo or Toyobo. Tetracycline and ampicillin were from Wako Pure Chemical Industry. Soluble starch was from E. Merck. Acarbose was a gift from Drs. A. Mullen and K. Hornberg (Bayer AG). All other chemicals were of reagent grade.

Bacterial strains and plasmids

Recombinant DNA was manipulated in *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ[lac proAB] / F': traD36 proAB lacI^r ZΔM15*). Mutant CGTases were produced in the protease-deficient mutant *E. coli* ME8417 (*lon::Tn10[ter^r] thr leu lacY*), provided by Dr. H. Takahashi (University of Tokyo). Plasmid pTUE254 contains the CGTase gene region of an alkalophilic *Bacillus* sp.1011 (Kimura et al. 1987).

Construction of mutant CGTase genes

The F283L mutant CGTase gene was constructed as described (Nakamura et al. 1994). The F283Y mutant CGTase gene was prepared in the same manner as that of F283L (Nakamura et al. 1994). The oligonucleotide, 5'-GCTCGATTACCGCTTTG-3', was used in site-directed mutation of the F283Y mutant CGTase gene.

Expression and purification of mutant CGTases

Mutant CGTases were expressed and purified as described (Nakamura et al. 1992; Haga et al. 1994). ME8417 containing the plasmid pTUE254 encoding mutant CGTase was incubated in Luria-Bertani medium containing ampicillin (50 μg/mL) and tetracycline (20 μg/mL) for 14 h at 37°C. Target proteins were abstracted by osmotic shock (Chan et al. 1981). The supernatant obtained after ammonium sulfate (28%) precipitation was loaded onto TOYOPEARL HW55-F, and then the target proteins were eluted with 10 mM sodium phosphate buffer (pH 6.0). The proteins were desalted over a Bio-Gel column (BioRad) and concentrated to 25 mg/mL by using an Amicon concentration kit. The proteins were >99% pure according to SDS-PAGE.

Enzyme assay

All reactions proceeded at 37°C in Britton-Robinson buffer (50 mM phosphoric, acetic, and boric acids and adjusted to suitable pH by NaOH) with various pH values. Starch degrading activity was measured by the blue value method with slight modification (Nakamura et al. 1994). The enzyme digest was composed of 600 μL of 0.5% (w/w) soluble starch and 300 μL of enzyme (finally ~3 to 5 U/mL). The reaction mixture was incubated for 5, 10, and 15 min. The reaction was halted, and starch was colored by adding stop solution (0.17 mM I₂, 1.7 mM KI, 1.7 mM HCl). One unit of

starch degrading activity was defined as the amount that generated a 1% decrease per minute of absorbance at 660 nm.

Disproportionating activity between 3KB-G5CNP as a donor and glucose as an acceptor in various pH conditions (5.0 to 9.0) were determined as described (Nakamura et al. 1994), and the kinetic parameters k_{cat} and K_m were determined by the nonlinear least-squares methods with Taylor expansion (Sakoda and Hiromi 1976).

Crystallization of F283L and F283Y and their acarbose complexes

All crystals were prepared according to Haga et al. (1994), and the acarbose complexes were obtained by cocrystallization with acarbose. Crystallization was performed by hanging drop vapor diffusion using 2.5% protein solution and a reservoir solution containing 20% (w/v) PEG3000, 20% (v/v) 2-propanol, 100 mM sodium citrate, 1 mM calcium chloride, and additive 1 mM acarbose for the cocrystallization. Rod-like crystals of 1.0 × 0.3 × 0.2 mm were grown within 2 weeks at room temperature.

Data collection

X-ray diffraction data of F283L crystals were collected to 1.8 Å resolution at 286 K by using an Enraf-Nonius FAST diffractometer (40 kV, 50 mA) at three goniometer settings as described (Harata et al. 1996). X-ray diffraction data of the crystals of F283Y, F283L_ACA, and F283Y_ACA were collected at 290K by using a Bruker SMART 6000 diffractometer (50 kV, 90 mA). The $R_{\text{merge}}(I)$ values for these data were <11%. Table 1 summarizes the statistics of data collection.

Structure determination and refinement

All of the four structures were determined by molecular replacement using the coordinates of the wild-type structure (Harata et al. 1996) and refined by using the program X-PLOR (Brünger et al. 1987). In the starting model, the residue 283 was glycine, which was replaced with leucine or tyrosine after $|3F_o - 2F_c|$ electron density maps ($>1 \sigma$) confirmed their shape. The pseudo-tetrose molecules derived from acarbose were introduced in the structures of F283L_ACA and F283Y_ACA by reference to their $|3F_o - 2F_c|$ and $|F_o - F_c|$ electron density maps ($>1.5 \sigma$). Water molecules were introduced in the structures as the same manner as previously described (Harata et al. 1996). The stereo-chemical qualities of these structures were confirmed by the program PROCHECK (Laskowski et al. 1993). The program TURBO-FRODO examined the graphics and drew the structures. The results are summarized in Table 1. Atomic coordinates have been deposited with Protein Data Bank (F283L: 1V3J, F283Y: 1V3K, F283L_ACA: 1V3L, F283Y_ACA: 1V3M).

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