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Studies on the Hydrolyzing Mechanism for Cyclodextrins of *Thermoactinomyces vulgaris* R-47 α-Amylase 2 (TVAII). X-Ray Structure of the Mutant E354A Complexed with β-Cyclodextrin, and Kinetic Analyses on Cyclodextrins[1]

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Crystals of the mutant E354A of *Thermoactinomyces vulgaris* R-47 α-amylase 2 (TVAII) complexed with β-cyclodextrin were prepared by a soaking method, and the diffraction data were collected at 100 K, using Synchrotron radiation (SPring-8). The crystals belong to an orthorhombic system with the space group $P2_12_12_1$ and cell dimensions a = 111.1 Å, b = 117.7 Å, c = 113.3 Å, which is almost isomorphous with crystals of the wild-type TVAII, and the structure was refined to an R-factor = 0.208 (R_{free} = 0.252) using 3.0 Å resolution data. The refined structure shows that the interactions between Phe286 and two C6 atoms of β-cyclodextrin at the hydrolyzing site are important for TVAII to recognize cyclodextrins as substrates. This observation from the X-ray structure was supported by kinetic analyses of cyclodextrins using the wild-type TVAII, the mutant F286A and F286L. These studies also suggested that the TVAII-hydrolyzing mechanism for cyclodextrins is slightly different from that for starch.

Key words: α-amylase, cyclodextrin, crystal structure, enzymatic glucoside hydrolysis, site-directed mutagenesis.

α-Amylase (1,4-α-D-glucan-4-glucanohydrase; EC 3.2.1.1) hydrolyzes internal 1,4-α-glucosidic linkages in starch to release α-anomer products. This α-retaining hydrolyzing mechanism has been widely studied, and a possible hydrolyzing mechanism of α-amylase has been proposed (*1*, *2*). In the proposed hydrolyzing mechanisms, the Glu residue in an active site plays an important role to protonate the glucosidic oxygen of substrates. Mutant enzymes in which this Glu residue is replaced by another amino acid residues successfully form the stable substrate-enzyme complexes, and several X-ray structures of these complexes have been reported (*3*, *4*). When a substrate binds to α-amylase, the torsion angles of glucosidic bonds at the hydrolyzing site must deviate considerably from those in a regular helical structure of amylose, because the Glu residue needs to protonate the glucosidic oxygen at the first step of the proposed hydrolyzing mechanism. In the X-ray structure of *Bacillus subtilis* mutant α-amylase complexed with maltopentaose (*3*), the torsion angles of O5-C1-O4′-C4′ and C1-O4′-C4′-C3′ at the hydrolyzing site are observed to be 26° and 92°, respectively, while those of typical amylose are 100°, and 109° (*5*).

Thermoactinomyces vulgaris R-47 produces two α-amylases, TVAI (637 amino acid residues, MW = 71,000 Da) (*6*, *7*) and TVAII (585 amino acid residues, MW = 67,500 Da) (*8*), and we recently reported the X-ray structure of TVAII at 2.6 Å resolution (*9*). Interestingly, they can efficiently hydrolyze cyclic oligosaccharides (cyclodextrins (CDs)) with six (α-), seven (β-), or eight (γ-) glucoses, as well as a starch, while most other α-amylases scarcely hydrolyze them (*10*, *11*). Since CDs have a stable round-shaped structure with torsion angles O5-C1-O4′-C4′ and C1-O4′-C4′-C3′ of 109° and 129° (*12*), respectively, it is difficult to change the conformations of their glucosidic bonds, as found in the X-ray structure of *Bacillus subtilis* mutant α-amylase complexed with maltopentaose (*3*). Thus, it is interesting to know how TVAII hydrolyzes CDs, and knowledge of the TVAII-hydrolyzing mechanism for CDs may be useful for understanding the general hydrolyzing mechanisms of α-amylase.

To elucidate TVAII-hydrolyzing mechanism for CDs, we investigated the X-ray structure at 3.0 Å resolution of the inactive TVAII mutant E354A, in which Glu354 protonating the glucosidic oxygen is replaced by Ala, complexed with β-CD as a substrate. Although the X-ray structures of complexes between CDs and cyclodextrin glucanotrans-

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Abbreviations: TVA, *Thermoactinomyces vulgaris* R-47 α-amylase; CD, cyclodextrin.

ferases belonging to an α-amylase family have been reported, those of α-amylase complexed with CDs have not. The present X-ray structure is the first recorded example of a complex between an α-amylase and a CD. It shows that Phe286 located at an active site is the important residue to recognize cyclodextrins as substrates. To confirm this, we also carried out kinetic analyses of TVAII-hydrolyzing activity for CDs, using the wild-type TVAII and two mutant enzymes, in which Phe286 is replaced by other hydrophobic amino acid residues, Ala (F286A) and Leu (F286L).

MATERIALS AND METHODS

Site-Directed Mutagenesis and Purification of Mutant Enzymes—The gene manipulation methods were based on those of Sambrook *et al* (*13*). All of the mutant enzymes were prepared from recombinant *Escherichia coli* MV1184 cells (*6*). Oligonucleotide-directed mutagenesis was carried out using a plasmid pTN302-10 as described (*6*) according to the method of Kunkel (*14*) for the construction of the mutants E354A, F286A, and F286L. Oligonicleotides 5′-GCC AGC CGG ACG CGT CAT GCC AAA TCG CTC CGA CAA T-3′, 5′-GGC CGG CAC TTG TAC AGC AGC GGT TTC ATA ATT GGT-3′, and 5′-GGC CGG CAC TTG TAC AGC CAA GGT TTC ATA ATT-3′ were used for the construction of the mutants E354A, F286A, and F286L, respectively. Individual mutations were verified by DNA sequencing. The mutant enzymes were purified by the same procedure as the wild-type TVAII (*6*).

X-Ray Structure Analysis—The mutant E354A was crystallized by the same procedure as the wild-type TVAII (*9*). The crystals were soaked in a reservoir solution containing 10 mM β-CD for 24 h, and data of 3.3 Å resolution were collected at room temperature using an R-AXISIIc imaging plate system on a Rigaku RU300 rotating anode X-ray generator. To confirm the electron density of the bound β-CD in a crystal, diffraction data were collected at 100 K using a Mar CCD detector system on the BL41XU beam line in SPring-8. Data were processed using the program MOSFLM (*16*) and the routines from the CCP4 program suite (*17*). The measurement conditions, crystal data and observed data statistics are listed in Table I. Initial phases were determined by a molecular replacement method with the structure of the wild-type TVAII as a probe model, using the program X-PLOR (*18*). Refinement of the structure without β-CDs using 20.0–3.0 Å resolution data was carried out by the simulated annealing protocol of the program X-PLOR (*18*, *19*). An electron density map with the coefficients of (F_{obs}–F_{calc}) and the resultant phases was generated to introduce the bound β-CDs, using the program Xfit in XtalView system (*20*). After the refinement of the structure with the bound β-CDs, water molecules were gradually introduced, if the peaks above 3σ contoured level in the (F_{obs}–F_{calc}) map were in the range of a hydrogen bond with the model. Through the refinement, the water molecules with the temperature factor above 40 Å^2 were omitted. Finally, the structure was refined to an *R*-factor of 0.208 (R_{free} = 0.252) (*21*), using 20.0–3.0 Å resolution data. Structure refinement statistics are listed in Table II.

Enzyme Assays—The activities were assayed as described (*6*, *8*). The enzyme reaction was carried out in 100 mM phosphate buffer (pH 6.0) at 40°C. The products were analyzed by the Nelson-Somogyi method for α-, β-, γ-CDs and starch by monitoring the reducing power of the hydrolysate.

RESULTS AND DISCUSSION

Overall Structure of E354A·β-CD Complex—The crystal structure of E354A·β-CD complex is almost isomorphous with that of the wild-type TVAII, with two molecules in an asymmetric unit (Mol-1 and Mol-2), related by non-crystallographic 2-fold symmetry. In a Ramachandran plot (*22*), 89.9% of residues in Mol-1 and 90.7% residues in Mol-2 are shown in the most favored regions as determined by the program PROCHECK (*23*), and no residue is in the disallowed regions or the generously allowed regions. In the final ($2F_{obs}$–F_{calc}) maps with 1σ contoured level, several discontinuous electron densities were found at the residues Asp165, Asp272, Phe273, Val275, Val288, Gln289, Glu305, Glu547, and His563 in Mol-1, and the residues Glu104, Phe167, Glu183, Gln257, Val275, Pro274, Ser276, Val290, Pro291, Asp467, Lys477, and Val545 in Mol-2, indicating partially disordered regions. Since the structures of Mol-1 and Mol-2 are almost identical, with the r.m.s. deviations

TABLE I. **Measurement conditions, crystal data, and observed data statistics.**

SR Facility	SPring-8
Beam Line	BL41XU
Temperature (K)	100
Wavelength (Å)	1.0
Resolution (Å)	3.0
No. of crystals	1
No. of measured refs.	103,006
No. of unique refs.	29,483
Completeness (%)	97.8
R_{merge}[a] (the highest shell)[b]	0.093 (0.173)
Space group	$P2_12_12_1$
Cell dimensions (Å)	
a	111.1
b	117.7
c	113.3

[a]R_{merge} is defined as $\Sigma_{i,hkl}|I(i,hkl)-\langle I(i,hkl)\rangle|/\Sigma_{i,hkl}\ I(i,hkl)$, where *i* runs through the symmetry-related reflections. [b]The highest shell has the 3.11–3.00 Å resolution range.

TABLE II. **Refinement statistics.**

Resolution range (Å)	20.0–3.0
No. of refs.	27,899 (F_o>2.0σF_o)
Completeness (the highest shell)[b](%)	92.2 (87.6)
R-factor[a] (the highest shell)[b]	0.208 (0.327)
R_{free}[a] (10% random subset)	0.252
r.m.s deviations	
Bond (Mol-1, Mol-2)[c] (Å)	0.012 (0.013, 0.012)
Angle (Mol-1, Mol-2)[c] (°)	2.8 (2.8, 2.8)
Dihedral angle (Mol-1, Mol-2)[c] (°)	25.2 (25.1, 25.2)
No. of amino acids	1170 (585 × 2)
No. of non-hydrogen protein atoms	9552 (4776 × 2)
No. of carbohydrate atoms	154 (77 × 2)
No. of solvent molecules	523
Average *B*-factor (Å^2)	
Protein atoms (Mol-1, Mol-2)[c]	23.3 (22.4, 24.1)
Carbohydrate atoms (Mol-1, Mol-2)[c]	48.4 (46.3, 49.4)
Solvent atoms	24.9

[a]*R*-factor is calculated from 100% of reflections. R_{free} is from a random set of 10% of reflections excluded from refinement. [b]The highest shell has the 3.13–3.00 Å resolution range. [c]Mol-1 and Mol-2 are related by non-crystallographic 2-fold symmetry in an asymmetric unit.

between them being 0.62 Å for main chain atoms, the structural description concentrates on Mol-1.

Figure 1 shows the overall structure of E354A·β-CD complex illustrated by the program MOLSCRIPT (*24*). The structure is divided into four domains. They are Domain N (residues 1–121) with a β-structure, Domain A (122–242 and 298–502) with a $(\beta/\alpha)_8$ barrel structure, Domain B (243–297) with a small loop from Domain A, and Domain C (503–585) with a β-sandwich structure. A β-CD binds to the cleft between Domains A and B, and does not interact with Domains N and C. The orientation and position of β-CD relative to the enzyme is very similar to those found in the X-ray structure of the mutant cyclodextrin glucanotransferase from *Bacillus circulans* strain 8 complexed with the β-CD derivative (*4*). Cyclodextrin glucanotransferases belonging to an α-amylase family have five domains, A, B, C, D, E, of which Domains D and E are located at the C-terminal side. In contrast, Domain N of TVAII is at the N-terminal side. In spite of the different locations of domains, the active site structures of both enzymes are very similar to each other (*10*), and the binding modes of β-CD are equivalent.

The partial disordered residues mentioned above are mainly located in Domain B (residues 270–290) close to the bound β-CD, as shown in Fig. 1. The r.m.s deviation of main chain atoms between E354A·β-CD complex and the wild-type TVAII is 0.62 Å, and that of this region (residues 270–290) is 1.69 Å. The binding of β-CD induces significant structural change in this region, and the relatively poor electron density of this region suggests that this region is dynamically moving through the substrate-binding.

Active Site Structure of E354A·β-CD Complex—The omit map for the bound β-CDs in Mol-1 with 2σ contoured level is illustrated in Fig. 2. The quality of electron density is not high enough for each atom to be located, especially the O6 atoms, or for the structure of the bound β-CD to be discussed in detail. This is because the bound β-CD is disordered rotating around its center axis. However, the electron density definitely shows a round-shaped structure of β-CD, and this allowed us to determine the overall orientation and position of β-CDs in Mol-1 and Mol-2.

The active site structure of E354A·β-CD complex (Mol-1) superimposed on that of the wild-type TVAII (Mol-1) is illustrated in Fig. 3. The glucose units of β-CD are labeled as Glc^{I}, Glc^{II}, Glc^{III}, Glc^{IV}, Glc^{V}, Glc^{VI}, and Glc^{VII}, and subsites for glucose units are defined as -2 (Glc^{II}), -1 (Glc^{I}), +1 (Glc^{VII}), and +2 (Glc^{VI}) from the non-reducing end to the reducing end, as shown in Fig. 3. The hydrolyzing site is between subsites −1 and +1. The binding of β-CD induces the significant conformational changes in several amino acid residues to form favorable interactions with β-CD. In the structure of wild-type TVAII without β-CD, Phe286 makes short contacts with His244, but, through the binding of β-CD, Phe286 becomes twisted, breaking the short contacts with His244, and making short contacts with both C6 atoms of Glc^{I} and $GlcV^{II}$ (subsites −1 and +1) from the inside of β-CD, as shown in Fig. 4. As predicted (*9*), these interactions between Phe286 and the two C6 atoms appear to be important for TVAII to recognize CDs as substrates. *Bacillus subtilis* α-amylase has no residue corresponding to Phe286 in TVAII, which interacts with glucose units at subsites −1 and +1. Although Leu144 of *B. subtilis* α-amylase makes short contacts with the substrate, it interacts with two C6 atoms of glucose units of maltopentaose at subsites −1 and −2, not −1 and +1. This is because the structures

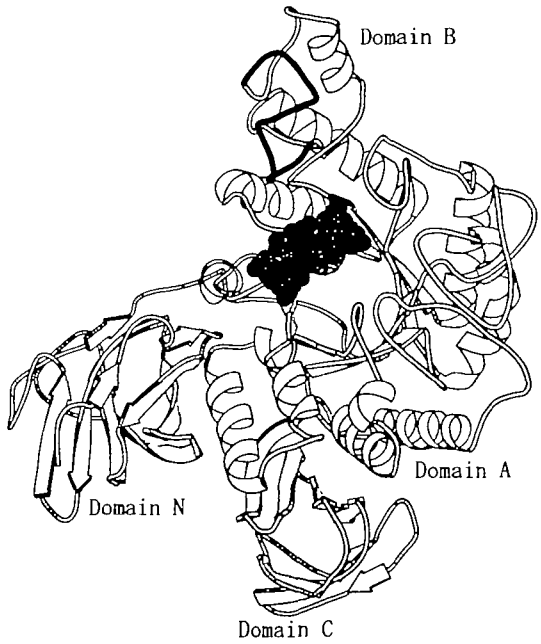

Fig. 1. **Overall structure of E354A·β-CD complex illustrated by the program MOLSCRIPT (*24*).** The bound β-CD is indicated by a space-filling model, and the region of high deviation close to β-CD (residues 270–290) is shaded.

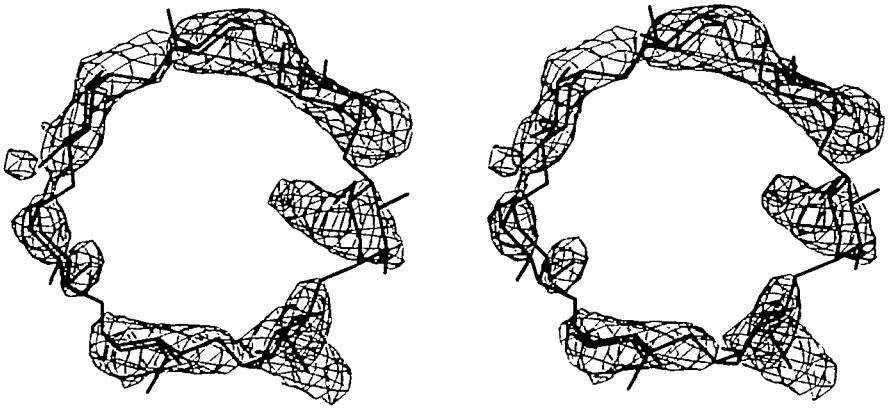

Fig. 2. **Stereoview of the omit map electron density for the bound β-CD with 2.0 σ contoured level.** The omit map was calculated from the coefficients of the $(F_{obs}-F_{calc})$ and the resultant phase angles after several cycles of refinement of the model excluding β-CD.

of domains B are different between TVAII and *B. subtilis* α-amylase.

From the previous modeling study (*9*), *Aspergillus oryzae* α-amylase (TAKA-amylase) also has no residue corresponding to Phe286 in TVAII and it has a relatively narrow and deep active site cleft compared to TVAII, which cannot accommodate β-CD as a substrate. The active site of *B. subtilis* α-amylase is also relatively narrow and deep compared to that of TVAII, and it has some amino acid residues which occupy the positions of Glc^{IV} and Glc^{V} of β-CD in TVAII, suggesting that *B. subtilis* α-amylase also cannot accommodate β-CD. Therefore, as well as Phe286 interacting with glucose units at subsites −1 and +1, the relatively wide and shallow active site cleft of TVAII is also important for recognition of CDs as substrates.

His202, Tyr204, and Trp356 also change their orientations to make van der Waals contacts with β-CD. Although hydrogen bond interactions are difficult to discuss in this X-ray structure due to the poor electron density for β-CD, the positions of His164, His420, Asp421, Asp465, and Arg469 allow the possible formation of hydrogen bonds with β-CD.

Tyr45* from domain N of Mol-2 (non-crystallographic 2-fold symmetry-mate) also changes its orientation to fit the bound β-CD. As reported previously (*9*, *25*), domain N is thought to be the driving force in the formation of dimer structure in a crystal, because Domain N is involved in almost all interactions between Mol-1 and Mol-2. However, it is still not clear whether the interactions between Tyr45* and the bound β-CD are related to the TVAII-hydrolyzing activity for CDs.

The electron density of β-CD indicates that it maintains its round-shaped structure through the binding to an enzyme. Such a conformational change of the glucosidic bonds between subsites −1 to +1 as observed in the X-ray structures of other enzyme-substrate complexes is not found. From the present X-ray structure, it seems to be difficult for Glu354 to protonate the O4 atom between subsites −1 and +1, because the lone pairs of the O4 atom are directed towards the center of β-CD, which is completely opposite to the position of Glu354 (Ala). This suggests that the TVAII-hydrolyzing mechanism for cyclodextrins is slightly different from that for starch. Although Asp421 seems to serve as a hydrogen donor to the O4 atom, Glu354 must be important to the hydrolyzing activity because the hydrolyzing

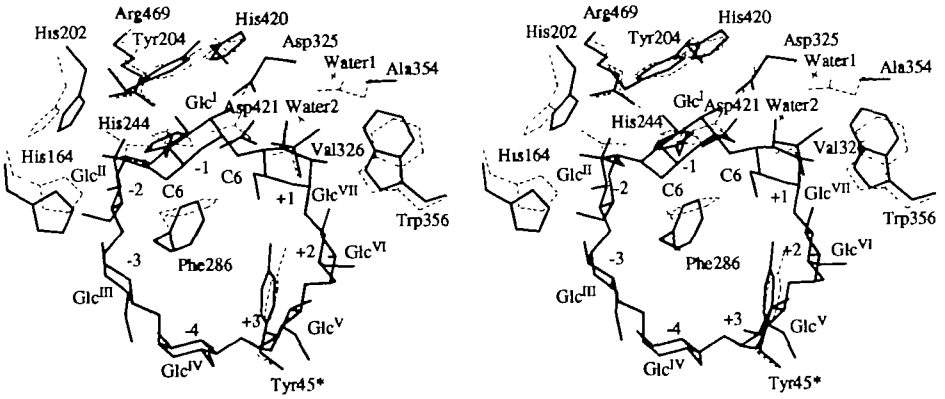

Fig. 3. **Stereoview of the active site structure of E354A·β-CD complex superimposed on that of the wild-type TVAII.** The active site of the wild-type TVAII is illustrated by broken lines.

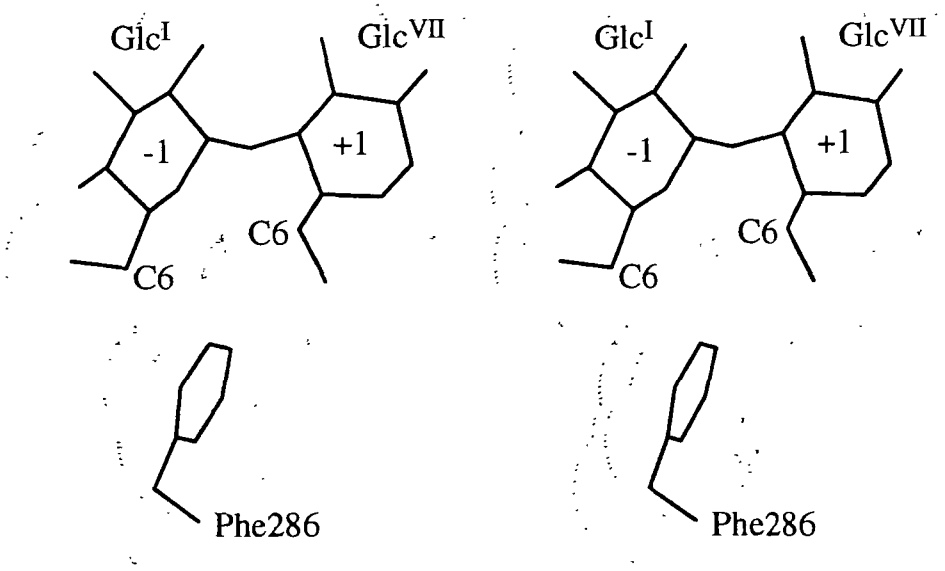

Fig. 4. **Stereoview of the interactions between Phe286 and glucose units at subsite −1 and +1 of β-CD.** All atoms are shown by the van der Waals radii.

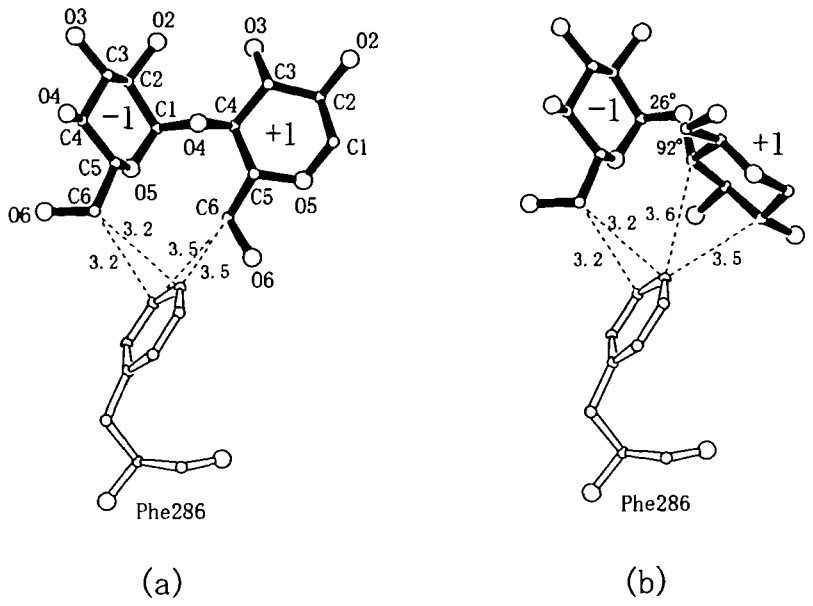

Fig. 5. **Possible van der Waals contacts (broken lines) between Phe286 and glucose units at subsite −1 and +1 are shown in the case of β-CD-binding (X-ray structure) (a) and starch-binding (modeling structure) (b).** Substrates are indicated by solid lines, and carbon atoms are illustrated by small circles. The distances of van der Waals contacts (Å) are labeled. The starch-binding structure is generated from the X-ray structure by rotating the torsion angles of O5-C1-O4′-C4′ and C1-O4′-C4′-C3′ to 26° and 92°, as labeled.

TABLE III. **Kinetic parameters of wild-type TVAII and mutant enzymes.**

	K_m (mM)			k_{cat} (s-1)			k_{cat}/K_m		
	F286A	F286L	Wild-type	F286A	F286L	Wild-type	F286A	F286L	Wild-type
α-CD	5.1	3.2	0.43	0.55	0.26	22	0.11 (1.3)[b]	0.081 (1.0)[b]	51 (629)[b]
β-CD	3.6	2.8	0.99	0.35	0.055	22	0.097 (4.9)[b]	0.020 (1.0)[b]	22 (1100)[b]
γ-CD	5.4	2.8	0.59	1.8	0.27	38	0.33 (3.4)[b]	0.096 (1.0)[b]	64 (666)[b]
Starch	1.3[a]	1.4[a]	0.25[a]	5.5	2.3	12.2	4.2 (2.6)[b]	1.6 (1.0)[b]	49 (31)[b]

[a] K_m values of starch were % (w/v). [b] The ratio of k_{cat}/K_m values for each substrate is given in parentheses.

activities of E354A for CDs and starch completely disappear. Therefore it is proposed that water molecules hydrogen bonded to Glu354 and Asp421 as shown in Fig. 3 (Water-1 and Water-2) and observed in the X-ray structure of wild-type TVAII, may serve as hydrogen donors to the O4 atom of CDs.

Kinetic Studies of Wild-Type TVAII, F286A, and F286L for CDs—Kinetic parameters of the wild-type TVAII and mutant enzymes are listed in Table III. The mutant enzymes have low activities for both CDs and starch compared to the wild-type enzyme. Figure 5a shows the interactions between β-CD and Phe286 based on the presented X-ray structure, and Fig. 5b shows the starch-binding model structure. Since, in the binding of starch, TVAII is thought to be followed by the proposed hydrolyzing mechanism in which Glu354 protonates the glucosidic oxygen of substrates, Fig. 5b is generated by rotating the torsion angles of O5-C1-O4′-C4′ and C1-O4′-C4′-C3′ to 26° and 92°, respectively, as observed in the X-ray structure of *Bacillus subtilis* α-amylase complexed with maltopentaose (*3*). In the binding of CDs, Phe286 makes contact with both C6 atoms at subsites −1 and +1 from the inside of CDs as discussed above, and also, in the binding of starch, Phe286 is expected to make contact with the C6 atom at subsite −1 and the C2 and C4 atoms at subsite +1. Ala286 and/or Leu286 are expected not to make any contacts with a glucose unit at subsite +1 like Phe286 does, in both cases of CD-binding and starch-binding. This is one reason why the mutant enzymes have lower activities. However, activities for different substrates are changed differently by the mutations. The substitution of Phe286 more extensively affects the activities for CDs than for starch. For example, the ratios of k_{cat}/K_m for β-CDs of F286A, F286L, and the wild-type TVAII are 4.9, 1.0, and 1,100, and those for starch are 2.5, 1.0, and 31. This difference is very important, because it shows that Phe286 should more strongly interact with CDs than starch, and that Phe286 must be important for TVAII-hydrolyzing mechanism for CDs, as indicated by the X-ray structure and the fact that other α-amylases have no residue corresponding to Phe286.

There is no correlation between the cavity-size of CDs and the k_{cat}/K_m values, showing that Phe286 cannot recognize the cavity-size of CDs but can recognize their round shape. In the X-ray structure, Phe286 does not enter deeply into the cavity of β-CD as Tyr195 of cyclodextrin glucanotransferase from *Bacillus circulans* strain 8 does (*4*). Independent of the cavity-size of CDs, Phe286 seems to interact with both C6 atoms at subsites −1 and +1 and to recognize the glucosidic bond-conformation between subsites −1 and +1.

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