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Role of Phe286 in the recognition mechanism of cyclomaltooligosaccharides (cyclodextrins) by *Thermoactinomyces vulgaris* R-47 α-amylase 2 (TVAII). X-ray structures of the mutant TVAIIs, F286A and F286Y, and kinetic analyses of the Phe286-replaced mutant TVAIIs

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Abstract

Phe286 located in the center of the active site of α-amylase 2 from *Thermoactinomyces vulgaris* R-47 (TVAII) plays an important role in the substrate recognition for cyclomaltooligosaccharides (cyclodextrins). The X-ray structures of mutant TVAIIs with the replacement of Phe286 by Ala (F286A) and Tyr (F286Y) were determined at 3.2 Å resolution. Their structures have no significant differences from that of the wild-type enzyme. The kinetic analyses of Phe286-replaced variants showed that the variants with non-aromatic residues, Ala (F286A) and Leu (F286L), have lower enzymatic activities than those with aromatic residues, Tyr (F286Y) and Trp (F286W), and the replacement of Phe286 affects enzymatic activities for CDs more than those for starch.

Keywords: α-Amylase; Crystal structure; Site-directed mutagenesis; Cyclomaltooligosaccharide (cyclodextrin)

1. Introduction

α-Amylase (α-(1→4)-D-glucan-4-glucanohydrolase) catalyzes the hydrolysis of α-D-(1→4)-glucosidic linkages in starch to release α anomer products and this enzyme has been studied from various aspects: structure, function and industrial application.[1,2] *Thermoactinomyces vulgaris* R-47 produces two α-amylases TVAI (637 amino acid residues, $M_W = 71{,}000$ Da)[3,4] and TVAII (585 amino acid residues, $M_W = 67{,}500$ Da),[5] and they can hydrolyze not only starch but also cyclomaltooligosaccharides (cyclodextrins, CDs), which are scarcely hydrolyzed by other α-amylases.[6,7] The X-ray structures of the wild-type TVAII[8] and the inactive mutant TVAII

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E354A (Glu354 → Ala) complexed with β-CD[9] and kinetic analyses of the mutant TVAIIs, F286A (Phe286 → Ala) and F286L (Phe286 → Leu),[9] have been reported by us, showing that Phe286 at the active site plays an important role for TVAII to recognize CDs. Other structure-solved α-amylases have no spatially corresponding residue to Phe286 of TVAII. To elucidate the role of Phe286 and CD-recognizing mechanism of TVAII more precisely, we report the X-ray structures of the mutant TVAIIs, F286A and F286Y (Phe286 → Tyr), and the kinetic parameters of the mutant TVAIIs, F286A, F286L, F286Y and F286W (Phe286 → Trp).

2. Experimental

Site-directed mutagenesis and purification of the mutant TVAIIs.—The gene manipulation methods were based on those of Sambrook et al.[10] All of the mutant TVAIIs were prepared from recombinant *Escherichia coli* MV 1184 cells. Oligonucleotide-directed mutagenesis was carried out using plasmid pTN302-10 as described[3] according to the Kunkel method[11] for the construction of mutant TVAIIs, F286Y and F286W. The oligonucleotides used to produce these mutations were F286Y: 5′-CAC TTG GAC GGC ATA TGT TTC ATA ATT GG-3′, and F286W: 5′-GGC CGG CAC TTG TAC AGC CCA GGT TTC ATA ATT-3′. Those for F286A and F286L have already been reported.[9] Individual mutations were verified by DNA sequencing. The mutant TVAIIs were purified by the same procedure as the wild-type TVAII.[8]

X-ray structure analysis.—F286A and F286Y were crystallized under the same conditions as wild-type TVAII.[8] X-ray diffraction data were collected at 100 K using an ADSC CCD detector system on the BL6A beam line in Photon Factory (Tsukuba, Japan). Data were processed using the program DPS/MOSFLM[12] and the routines from the CCP4 program suite.[13] Initial phases were determined by molecular replacement with the structure of the wild-type TVAII as a probe model, using the program CNS.[14] Refinements of the structure using 30.0–3.2 Å resolution data were carried out by the simulated annealing protocol of the program CNS.[14] Finally, refinement of the structures converged at an R-factor of 0.213 ($R_{free} = 0.275$) for F286A, and an R-factor of 0.204 ($R_{free} = 0.283$) for F286Y.[15] Solvent molecules were not included in the refined structures due to the relatively low-resolution range of the diffraction data.

Table 1
Crystallographic parameters and refinement statistics

	F286A	F286Y
SR facility (beam line)	Photon factory (BL6A)	Photon factory (BL6A)
Resolution range (Å)	30.0–3.2	30.0–3.2
Wavelength (Å)	1.0	1.0
Measured references	130,745	131,344
Unique references	25,420	23,309
Completeness (%)	99.8	91.7
R_{merge}	0.122	0.121
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions (Å)		
a	112.60	112.25
b	118.05	117.94
c	113.13	113.32
R-factor (R_{free})	0.213 (0.275)	0.204 (0.283)
Amino acids	1170 (Mol−1 +Mol−2)	1170 (Mol−1 +Mol−2)
rms deviations		
Bonds (Å)	0.011	0.010
Angles (°)	1.5	1.4

Enzyme assays.—The activities for CDs and starch were assayed as described.[3,5] Enzymatic reactions were carried out in 100 mM phosphate buffer (pH 6.0) at 40 °C. The products were analyzed by the Nelson–Somogyi method by monitoring the reducing power of the hydrolysate. CDs and soluble starch were purchased from Wako Pure Chemical Industries, Ltd, and were used without any purification procedures.

3. Results and discussion

X-ray structures of F286A and F286Y[†].—The structure refinement statistics are listed in

[†] Atomic coordinates of F286A and F286Y have been deposited in the Protein Data Bank with reference ID codes of 1JF5 and 1JF6, respectively.

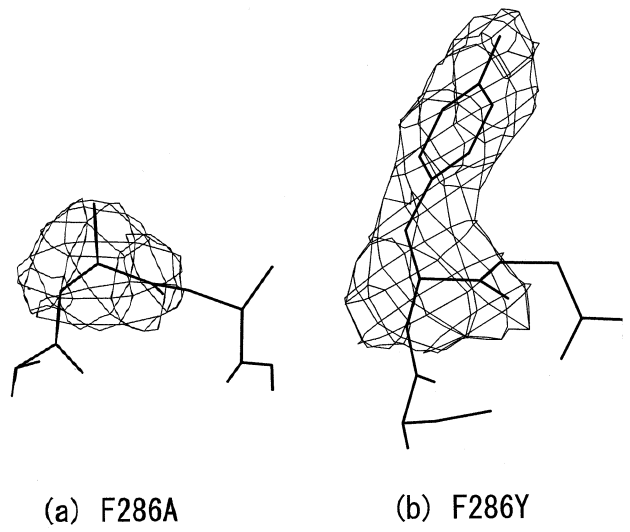

Fig. 1. The annealed omit map electron density for the exchanged residues: (a) Ala286 of F286A; and (b) Tyr286 of F286Y. The omit map was calculated from the coefficients ($F_{obs} - F_{calc}$) and the resultant phase angles after several cycles of annealed refinement of the model excluding the replaced residue at position 286.

Table 1, and annealed omit electron densities of exchanged amino acids at position 286 are illustrated in Fig. 1.[16] In a Ramachandran plot,[17] 85.9% of residues in F286A and 86.3% of residues in F286Y are in the most favored regions as determined by the program PROCHECK.[18] There are two molecules (Mol-1 and Mol-2) in the asymmetric unit related by a non-crystallographic twofold axis. As Mol-1 and Mol-2 have almost identical structures in each case of wild-type TVAII, F286A and F286Y, the structural description concentrates on Mol-1. Due to the relatively low resolution of 3.2 Å, it is difficult to determine the side-chain conformation of the amino acid residues with confidence. However, this resolution range is sufficient to discuss the overall main-chain conformation and the orientations of the side chains of the amino acid residues.

The overall three-dimensional structures of F286A and F286Y are almost equivalent to that of wild-type TVAII. The rms deviations of the Cα atoms compared to wild-type TVAII are 0.41 Å for F286A and 0.51 Å for F286Y. The structures of the active sites are also almost identical, except for the exchanged residues. The superimposed structures of active sites of F286A and wild-type TVAII are shown in Fig. 2. For later discussion, β-CD from the X-ray structure of the E354A–β-CD complex is superimposed in Fig. 2. As the three-dimensional structures of F286A and F286Y are very similar to that of the wild-type TVAII, the replacement of Phe286 is expected not to lead to drastic structural changes in TVAII. Although Trp is larger than Tyr, the computer-graphical replacement procedure of Tyr286 by Trp286 shows that there is no short contact between Trp286 and other amino acid residues. Thus, amino acid residue 286 is expected to predominate the kinetic parameters, as discussed below.

Kinetic analyses on CDs and starch.—The kinetic parameters of the wild-type TVAII and F286A,[9] F286L,[9] F286Y (this work) and F286W (this work) are listed in Table 2.

On CDs as substrate, the replacement of Phe286 by non-aromatic residues (F286A and F286L) cause a more drastic decrease in the k_{cat}/K_m values than the replacement by aromatic residues (F286Y and F286W). For example, the k_{cat}/K_m values for β-CD of F286A and F286 L are 0.44 and 0.091% of that of the wild enzyme, whereas those of the F286Y and F286W enzymes are 19 and 59%, respectively. As previously reported, the X-ray structure of the E354A–β-CD complex shows that Phe286 contacts both C-6 atoms at subsites -1 and $+1$ from the inside of β-CD (see Fig. 2), and that Ala286 and/or Leu286 are not expected to make any contact with β-CDs like Phe286 does. On the other hand, Tyr286 and/or Trp286 can make similar interactions with β-CD to Phe286, as shown in Fig. 3. This may be the reason why F286Y and F286W have comparable k_{cat}/K_m values to that of wild-type TVAII.

With starch as a substrate, the k_{cat}/K_m values of all variants, compared to that of wild-type TVAII, do not change as much as with CDs. The k_{cat}/K_m values of F286A, F286L, F286Y and F286W are 8.6, 3.3, 59 and 151% of that of wild-type TVAII. These results show that the replacement of Phe286 more extensively affects the enzymatic activities for CDs than for starch. This is because the number of glucose units interacting with the enzyme is different for starch binding than for CD binding. In starch binding, a total of eight glucose units interacts with the enzyme (eight subsites),[19] while in CD binding, only four

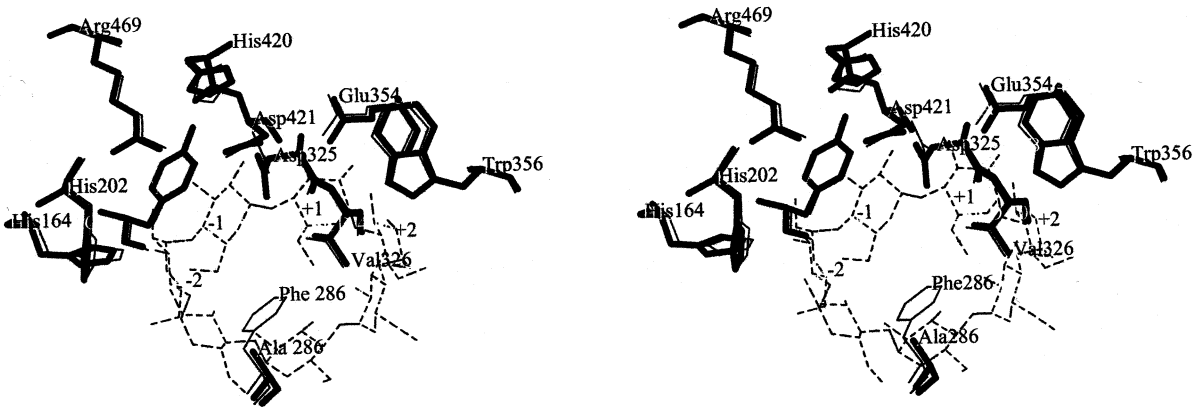

Fig. 2. Stereoview of the active site structure of F286A superimposed on that of the wild-type TVAII. The active site residues of F286A and those of the wild-type TVAII are shown by thick and thin lines, respectively, and β-CD from the X-ray structures of the E354A–β-CD complex is illustrated by broken lines. Subsite numbering for glucose units is indicated as -2, -1, $+1$, $+2$.

Table 2
Kinetic parameters for hydrolysis of starch and CDs with wild-type TVAII and Phe286 mutant enzyme

	F286A	F286L	Wild-type	F286Y	F286W
K_m *(mM)*					
α-CD	5.1	3.2	0.43	0.64	0.61
β-CD	3.6	1	0.99	1.3	1.9
γ-CD	5.4	2.8	0.59	0.61	1.1
Starch	1.3 [a]	1.4 [a]	0.25 [a]	0.3 [a]	0.35 [a]
k_{cat} (s^{-1})					
α-CD	0.55	0.26	22	5.7	18
β-CD	0.35	0.055	22	5.5	25
γ-CD	1.8	0.027	38	32.4	53
Starch	5.5	2.3	12.2	8.8	36
k_{cat}/K_m [b]					
α-CD	0.11 (0.22)	0.081 (0.19)	51 (100)	8.9 (17)	29 (57)
β-CD	0.097 (0.44)	0.020 (0.091)	22 (100)	4.2 (19)	13 (59)
γ-CD	0.33 (0.51)	0.096 (0.15)	64 (100)	53 (82)	48 (75)
Starch	4.2 (8.6)	1.64 (3.3)	49 (100)	29 (59)	74 (151)

[a] K_m values of starch were % (w/v).
[b] The ratio of k_{cat}/K_m values for each variant relative to wild-type enzyme (%) is given in parentheses.

glucose units are involved in the interactions with enzyme (subsites -2, -1, $+1$ and $+2$ in Fig. 2), owing to its cyclic structure. Therefore, the interaction of the 286th residue with the substrate is more important and essential in CD binding than in starch binding.

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19), belonging to the α-amylase family, also has an aromatic residue (Tyr or Phe) to recognize CDs. Sin et al. suggested that the size of the residue in CGTase-recognizing-CDs influences the preferred cavity size of CD, and that the substitution of the aromatic residue by other amino acids affects CD product ratios.[20] From the present work, there is found no correlation of k_{cat}/K_m values between the cavity size of CDs and the side-chain size of the 286th residues, suggesting that the 286th residue interacting with two C-6 atoms of CD cannot recognize the cavity size of the CDs. This observation confirms our previous report that the CD-recognizing mechanism of TVAII is different from that of CGTase.[9]

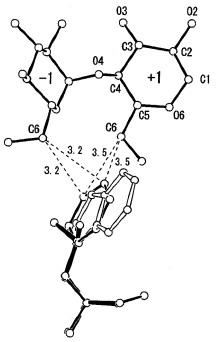

Fig. 3. Interactions between Phe286 and two C-6 atoms of glucose units at subsite −1 and +1 are shown by the broken lines with the distance (Å). Leu and Trp generated by the computer-graphical replacement procedure are superimposed. Leu is shown by thick solid bonds and Trp by open bonds.

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