Four Aromatic Residues in the Active Center of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. 1011: Effects of Replacements on Substrate Binding and Cyclization Characteristics†

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ABSTRACT: Three-dimensional structures of cyclodextrin glucanotransferases (CGTases) have revealed that four aromatic residues, which are highly conserved among CGTases but not found in α-amylases, are located in the active center. To analyze the roles of these aromatic residues, Phe-183, Tyr-195, Phe-259, and Phe-283 of *Bacillus* sp. 1011 CGTase were replaced by site-directed mutagenesis, and the effects of this procedure were examined. Y195L-CGTase, in which Tyr-195 was replaced by a leucine residue, underwent a drastic change in its cyclization characteristics: it produced considerably more γ-cyclodextrin than the wild-type enzyme and virtually no α-cyclodextrin. Y195L-CGTase had increased K_m values for cyclodextrins, whereas the values for a linear maltooligosaccharide donor were insignificantly changed. Taken together with the structural information of CGTase crystals soaked with substrates, we propose that Tyr-195 plays an important role in the spiral binding of substrate. Replacing either Phe-183 or Phe-259 with leucine induced increased K_m values for acceptors. Furthermore, the double mutant F183L/F259L-CGTase had considerably decreased cyclization efficiency, but the intermolecular transglycosylation activity remained normal. These results indicated that Phe-183 and Phe-259 are cooperatively involved in acceptor binding, and that they play a critical role in cyclization when the nonreducing end of amylose binds to the active center of CGTase. Replacing Phe-283 with a leucine residue induced a decrease in k_{cat} and in affinity for acarbose, suggesting that Phe-283 is involved in transition-state stabilization.

Cyclodextrin glucanotransferase (CGTase,[1] EC 2.4.1.19) catalyzes the conversion of starch and related α-1,4-glucans to cyclodextrins through an intramolecular transglycosylation reaction. Besides this cyclization, the enzyme also catalyzes a coupling reaction (opening of the rings of cyclodextrins and transfer of the linear maltooligosaccharides formed to acceptors) and a disproportionation reaction (transfer of linear maltooligosaccharides to acceptors) through intermolecular transglycosylation reactions. Furthermore, CGTase has weak hydrolyzing activity. Since cyclodextrins can form inclusion complexes with many molecules thereby changing the chemical and physical properties of the included molecules, CGTase is an important enzyme in the food and pharmaceutical industries.

CGTases from bacilli consist of 680–690 amino acids and have over 60% identity within their amino acid sequences. Although sequence similarities between CGTases and α-amylases, which hydrolyze the α-1,4-glucosidic bonds of starch, are usually below 25%, four highly conserved regions have been found and designated as regions I, II, III, and IV, respectively (formally called A-, B-, B′-, and C-regions, respectively; Binder et al., 1986; Kimura et al., 1987; Nakamura et al., 1992). Furthermore, the catalytic domains of both enzymes are folded into $(\beta/\alpha)_8$-barrel structures, and the four conserved regions within this domain constitute the active center (Matsuura et al., 1984; Buisson et al., 1987; Hofmann et al., 1989; Boel et al., 1990; Klein & Schulz, 1991; Kubota et al., 1991). The four conserved regions have also been found in other amylolytic enzymes such as isoamylase (Amemura et al., 1986), pullulanase (Katsuragi et al., 1987), and branching enzyme (Baba et al., 1991). Predictions of the three-dimensional structure have suggested that the catalytic domains of these enzymes are also folded into barrel structures (Jespersen et al., 1991). Therefore, it is proposed that the reaction mechanism of the enzymes possessing the conserved regions are similar, and they have been classified as the α-amylase family with several characteristics (Takata et al., 1992): (i) they act on α-glucosidic bonds; (ii) they split α-glucosidic bonds to produce α-anomeric saccharides or to form α-glucosidic linkages; (iii) they have the four conserved regions in their primary sequences; and (iv) they have aspartate, glutamate, and aspartate residues in the regions II, III, and IV, respectively, as the essential amino acid residues for catalysis (Holm et al., 1990; Kuriki et al., 1991; Nagashima et al., 1992; Nakamura et al., 1992; Takase et al., 1992; Podkovyrov et al., 1993; Søgaard et al., 1993). This family includes α-1,4-hydrolases, α-1,6-hydrolases, α-1,4-transglycosylases, and α-1,6-transglycosylases. Among these, CGTase typically catalyzes α-1,4-transglycosylation, and it is a key enzyme for analyzing the reaction mechanism of the α-amylase family. It has been proposed that the variations in substrate specificity and products of the α-amylase family can be ascribed to the relationships between their similar catalytic centers and different subsite structures (Nakamura et al., 1992).

It has been proposed that the active center of CGTase is composed of tandem subsites as are those of α-amylases and lysozymes (Blake et al., 1967; Matsuura et al., 1984; Sakai et al., 1987) and that it contains seven subsites designated, from the nonreducing to the reducing end, as S_5 through S_2'

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[1] Abbreviations: CGTase, cyclodextrin glucanotransferase; kb, kilobase pairs; HPLC, high-performance liquid chromatography; 3KB-G5CNP, 3-ketobutylidene-β-2-chloro-4-nitrophenylmaltopentaoside.

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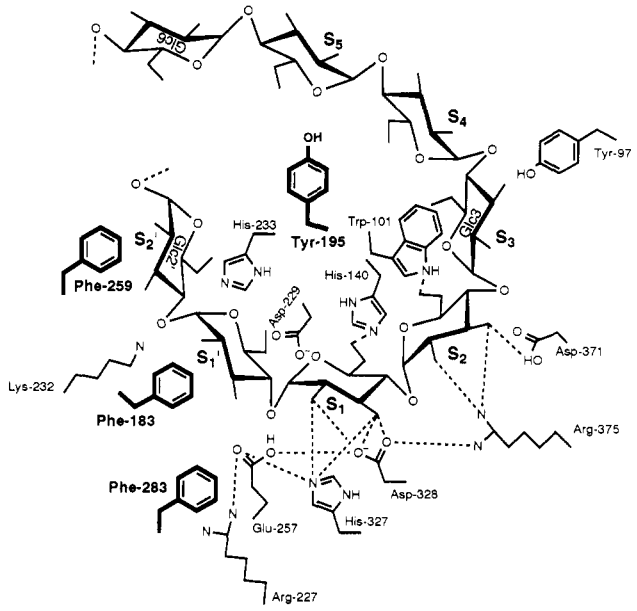

FIGURE 1: Schematic structure of the substrate binding sites of *Bacillus* sp. 1011 CGTase based upon X-ray crystallographic study of *B. circulans* CGTase (Klein et al., 1992). The active center of CGTase has been proposed to contain seven subsites. Each subsite is designated, from the nonreducing to the reducing end, as S_5 through S_2', and the cleavage point is located between subsites S_1 and S_1'. Hydrogen bonds are indicated by dashed lines. Functional roles of His-140, Asp-229, His-233, Glu-257, His-327, and Asp-328 have been examined (Nakamura et al., 1992, 1993), but those of other residues remained unclear. The positions of Tyr-97 and Lys-232 are proposed from the data for α-amylases (Matsuura et al., 1984; Matsui et al., 1992). The four aromatic residues replaced in this study are indicated in boldface.

(Klein et al., 1992; Figure 1). X-ray crystallographic studies of CGTases from *Bacillus circulans* and *Bacillus stearothermophilus* have indicated that many residues at the active center are conserved between CGTases and α-amylases (Klein & Schulz, 1991; Kubota et al., 1991; Klein et al., 1992; Kubota, 1993). Most of these residues are proposed to play similar roles in catalysis between the two enzymes. Indeed, the three conserved histidine residues in the regions I, II, and IV which are located near the catalytic triad (Matsuura et al., 1984; Buisson et al., 1987; Boel et al., 1990; Klein et al., 1992; Kubota et al., 1991) are involved in transition-state stabilization in α-amylase as well as CGTase (Nakamura et al., 1993; Søgaard et al., 1993). Therefore, the difference in reaction products between CGTases and α-amylases should be due to nonconserved residues between the two enzymes.

Comparison of the tertiary structures have revealed that the four aromatic residues are found in the active center of CGTases, but not in that of α-amylases, although their functional roles remain unclear (Klein & Schulz, 1991; Kubota et al., 1991; Klein et al., 1992; Kubota, 1993). These residues, which correspond to Phe-183, Tyr-195, Phe-259, and Phe-283 in *Bacillus* sp. 1011 CGTase, are conserved among all known CGTases including *Klebsiella pneumoniae* CGTase (Binder et al., 1986), which has only 25% amino acid identity with CGTases from bacilli (Figure 2). Although the planar aromatic rings of phenylalanine and tyrosine do not have hydrogen-bonding capability, X-ray crystallography of protein–ligand complexes has shown that an aromatic residue contributes to substrate binding by making partial stacking and face-to-face contact with a sugar residue through van der Waals interactions (Quiocho, 1989; Rouvinen et al., 1990; Spurlino et al., 1991; Muraki et al., 1992; Mikami et al., 1993; Sharon, 1993). Therefore, the four conserved aromatic residues may contribute to specific characteristics of CGTases that are not found in α-amylases, namely, the relatively high transglycosylation activity and the ability to catalyze intramolecular transglycosylation. In order to clarify the functional roles of these four conserved aromatic residues, they were replaced with other amino acid residues by site-directed mutagenesis. In this report, we describe the effects of replacements on enzymatic properties such as cyclization characteristics and kinetic parameters, and discuss their contribution to CGTase catalysis.

MATERIALS AND METHODS

Chemicals and Enzymes. All restriction and modification enzymes used for recombinant DNA manipulations were purchased from Takara Shuzo Co., Ltd., or Toyobo Co., Ltd. Tetracycline and ampicillin were from Wako Pure Chemical Industry Ltd. Soluble starch was from E. Merck. Amylose (average degree of polymerization of 17) was a gift from Hayashibara Biochemical Laboratories, Inc. Acarbose (Bay-g-5421) was a gift from Drs. E. Möller and M. Mardin (Bayer AG). All other chemicals used 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*^{q} *Z*ΔM15]. Wild-type and mutant CGTases were produced in the protease-deficient mutant *E. coli* ME8417 [*lon*::Tn*10*(*tet*^{r}) *thr leu lacY*], provided by Dr. H. Takahashi (The University of Tokyo). Plasmid pTUE254 was constructed by inserting the 4.5 kb *Hin*dIII fragment of pTUE217 (Kimura et al., 1987), which contains the CGTase gene region of an alkalophilic *Bacillus* sp. 1011, into the *Hin*dIII site of pUC13.

Construction of Mutant CGTase Genes. Synthetic oligonucleotides were prepared on an Applied Biosystems 392 DNA synthesizer. Site-directed mutagenesis was performed by the method of Kunkel et al. (1987) with slight modifications as described previously (Nakamura et al., 1992). The mutations were verified by DNA sequencing (Sanger et al., 1977). The following oligonucleotides were used:

F183L	5′-GCACGGATCTCTCCACC-3′
Y195A	5′-TATAAAAACCTGGCCGATCTGGCT-3′
Y195F	5′-AAAACCTGTTCGATCTGG-3′
Y195L	5′-TATAAAAACCTGCTCGATCTGGCT-3′
Y195T	5′-TATAAAAACCTGACCGATCTGGCT-3′
Y195V	5′-TATAAAAACCTGGTCGATCTGGCT-3′
Y195W	5′-ATAAAAACCTGTGGGATCTGGCTG-3′
F259L	5′-GCGAATGGCTCCTAGGC-3′
F283L	5′-TGCTCGATCTCCGCTTT-3′

Expression and Purification of Wild-Type and Mutant CGTases. The *E. coli* ME8417 strains carrying the constructed plasmids were grown in 1 L of L-broth [1% bacto tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% sodium chloride], containing 250 μg/mL ampicillin and 20 μg/mL tetracycline at 37 °C for 12 h. Wild-type and mutant CGTases in the periplasm of the cells were extracted by osmotic shock (Chan et al., 1981) and purified as described previously (Nakamura et al., 1992). To avoid mutual contamination with wild-type and mutant CGTases during purification, all buffer and chromatography gel were freshly prepared each time and used only once. Protein concentrations were determined by means of the BCA protein assay reagent (Pierce), using bovine serum albumin as the standard.

Analysis of the Reaction Products. To analyze the reaction products of wild-type and mutant CGTases, each enzyme (10

Enzymes	183	195	259	283	Reference
Bacillus sp. 1011	...HHYGG-T---D**F**STIEN-GIY--K--NL**Y**DLADLNHNN...	TFGEW**F**LGVNEISPEYH	-----QFANESGMS-LLD**F**RFAQKA...		Kimura et al., 1987
Bacillus sp. 38-2	...HHYGG-T---D**F**STIEN-GIY--K--NL**Y**DLADLNHNN...	NFGEW**F**LGVNEISPEYH	-----QFANESGMS-LLD**F**PFAQKA...		Kaneko et al., 1988
Bacillus sp. 17-1	...HHNGG-T---D**F**STTEN-GIY--K--NL**Y**DLADLNHNN...	TFGEW**F**LGVNEVSAENH	-----KFANVSGMS-LLD**F**RFAQKV...		Kaneko et al., 1989
Bacillus sp. B1018	...HHNGG-T---D**F**STIEN-GIY--K--NL**Y**DLADLNHNN...	TFGEW**F**LGVNEVGPENH	-----KFANESGMS-LLD**F**RFAQKV...		Itkor et al., 1990
B. stearothermophilus	...HHNGG-T---T**F**SSLED-GIY--R--NL**F**DLADLNHQN...	TFGEW**F**LSENEVDANNH	-----YFANESGMS-LLD**F**RFAQKL...		Sakai et al., 1987
B. macerans	...HHNGG-T---D**F**STTES-GIY--K--NL**Y**DLADINQNN...	TFGEW**F**LGANQTDGDNL	-----KFANESGMS-LLD**F**RFAQKA...		Sakai et al., 1987
B. ohbensis	...HHYGG-T---D**F**SSYEN-SIY--R--NL**Y**DLADYDLNN...	TFGEW**F**LGSGEVDPQNH	-----HFANESGMS-LLD**F**QFGQTI...		Sin et al., 1991
B. circulans	...HHYGG-S---D**F**SSLGN-GIY--K--NL**Y**DLADFNHNN...	TFGEW**F**LGSAASDADNT	-----DFANKSGMS-LLD**F**RFNSAV...		Nitschke et al., 1990
B. licheniformis	...HHNGG-S---D**F**STLEN-GIY--K--NL**Y**DLADLNHNN...	TFGEW**F**LGSAAPDADNT	-----DFANESGMS-LLD**F**RFNSAV...		Hill et al., 1990
K. pneumoniae	...HHNGGVTNWNDE**F**NVKNHNLFNVKNHNLE**N**LSDLNQSN...	FFGEW**F**-GASANTTTGVDGNAIDYANTSG-SALLD**F**GFRDTL...			Binder et al., 1986

FIGURE 2: Comparison of the amino acid sequences of various CGTases. The conserved residues are shadowed. The four aromatic residues are highlighted in black.

units/mL) was incubated at 37 °C for appropriate periods with amylose (10 g/L) in 10 mM sodium phosphate, pH 6.5, and the reaction was terminated by boiling the solution for 10 min. The cyclodextrins in the reaction mixtures were identified and quantified by high-performance liquid chromatography (HPLC) with a refractive index detector as described (Nakamura et al., 1993).

Enzyme Assay. All reactions were performed at 37 °C in 10 mM sodium phosphate, pH 6.5. Starch-degrading activity was measured by the blue value method of Fuwa (1954) with a slight modification using 0.3% soluble starch as the substrate. One unit of starch-degrading activity was defined as the amount of enzyme that gave a 1% decrease in absorbance at 660 nm per min. The β-cyclodextrin-forming activity was determined as described (Nakamura et al., 1993). Coupling activity was assayed based upon the method of Thoma et al. (1965) with modifications as described (Nakamura et al., 1993). Disproportionation activity between a maltopentaose with its nonreducing-end blocked and with aglycon at its reducing-end, 3-ketobutylidene-β-2-chloro-4-nitrophenylmaltopentaoside (3KB-G5CNP, Teshima et al., 1991), and suitable acceptors were determined as described (Nakamura et al., 1994). The amounts of reducing sugar liberated by the hydrolyzing activity of the enzymes were measured by the methods of Somogyi (1952) and Nelson (1944) with slight modification. The kinetic parameters k_{cat} and K_m were determined by the nonlinear least-squares method with the Taylor expansion (Sakoda & Hiromi, 1976).

RESULTS AND DISCUSSION

To analyze the roles of the four aromatic residues, each residue was initially replaced individually with the aliphatic residue leucine, because even though it has similar hydrophobicity to that of phenylalanine and tyrosine (Nozaki & Tanford, 1972), leucine does not have to the same extent the ability of aromatic residue to form face-to-face contacts with the glucosyl rings of the substrates. The genes for wild-type and mutant CGTases were expressed in the protease-deficient mutant *E. coli* ME8417. All mutant enzymes produced in the periplasm of the cells were extracted and purified by the same procedure as the wild-type enzyme. The purity of the enzymes exceeded 90%, based upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (data not shown). The molecular masses of all mutant CGTases were estimated to be 70 kDa and were equal to that of wild-type enzyme. The specific activities for the starch-degrading activity of wild-type and mutant enzymes are summarized in Table 1. All mutant enzymes retained sufficient starch-degrading specific activity to analyze the roles of these residues.

Effect of Amino Acid Replacements on Cyclization. The amounts of α-, β-, and γ-cyclodextrin, liberated from amylose by the cyclization activity of wild-type and mutant CGTases, were analyzed by HPLC (Figure 3). Wild-type CGTase produced mostly β-cyclodextrin (over 70% at all incubation periods tested), and the production of α-cyclodextrin gradually increased with the reaction time (Figure 3A). All mutant CGTases retained the ability to produce cyclodextrins, and the production ratios of α-, β-, and γ-cyclodextrin were little changed in Y195F- and F283L-CGTases with that of wild-type (Figure 3C,F). In contrast, F183L-, F259L-, and especially Y195L-CGTases had altered production ratios of α-, β-, and γ-cyclodextrin; in all these cases little α-cyclodextrin was produced (Figure 3B,D,E). The main initial product for Y195L-CGTase changed to γ-cyclodextrin, the absolute production of γ-cyclodextrin being much larger than that of the wild-type CGTase (Figure 3D). Similar results were achieved when soluble starch was the substrate instead of amylose (data not shown). These results suggest that the phenyl groups of Phe-183, Tyr-195, and Phe-259 contribute to the cyclization reaction of CGTase.

Table 1: Starch-Degrading and β-Cyclodextrin-Forming Activities of Wild-Type and Mutant CGTases[a]

enzyme	starch-degrading activity (units/mg)	β-cyclodextrin-forming activity[b] k_{cat} (s^{-1})	K_m (μM)
wild-type	2170	30.5 ± 0.6	57.4 ± 3.8
F183L	338	7.3 ± 0.3	81.5 ± 8.6
Y195F	1920	28.9 ± 0.9	55.6 ± 4.3
Y195L	1160	8.9 ± 0.4	61.8 ± 6.2
F259L	869	8.2 ± 0.2	63.5 ± 5.1
F283L	774	12.0 ± 0.2	72.4 ± 4.0
F183L/F259L	79.0	0.16 ± 0.1	263 ± 42

[a] Assays were performed in 10 mM sodium phosphate, pH 6.5, at 37 °C. [b] Amylose (average degree of polymerization of 17) was used as the substrate.

As the replacement of Tyr-195 by leucine residue induced the most effects on the cyclization characteristics, Tyr-195 was replaced with several amino acid residues, and their effects were analyzed (Figure 4). All mutant CGTases retained more than 50% of starch-degrading specific activities (data not shown). The productivity of γ-cyclodextrin was the largest for Y195L-CGTase, and Y195V-CGTase also produced much higher levels of γ-cyclodextrin than wild-type. Although replacement of Tyr-195 by more hydrophobic (tryptophan) or more hydrophilic (alanine and threonine) residues somewhat affected the cyclization characteristics, no correlation was observed between the productivity of γ-cyclodextrin and the hydrophobicity at the 195th amino acid. Since γ-cyclodextrin has the largest hydrophobic cavity of the three main cyclodextrin products, γ-cyclodextrin has the greatest potential for practical applications. Therefore, Y195L- and Y195V-CGTases acquired better characteristics for industrial use.

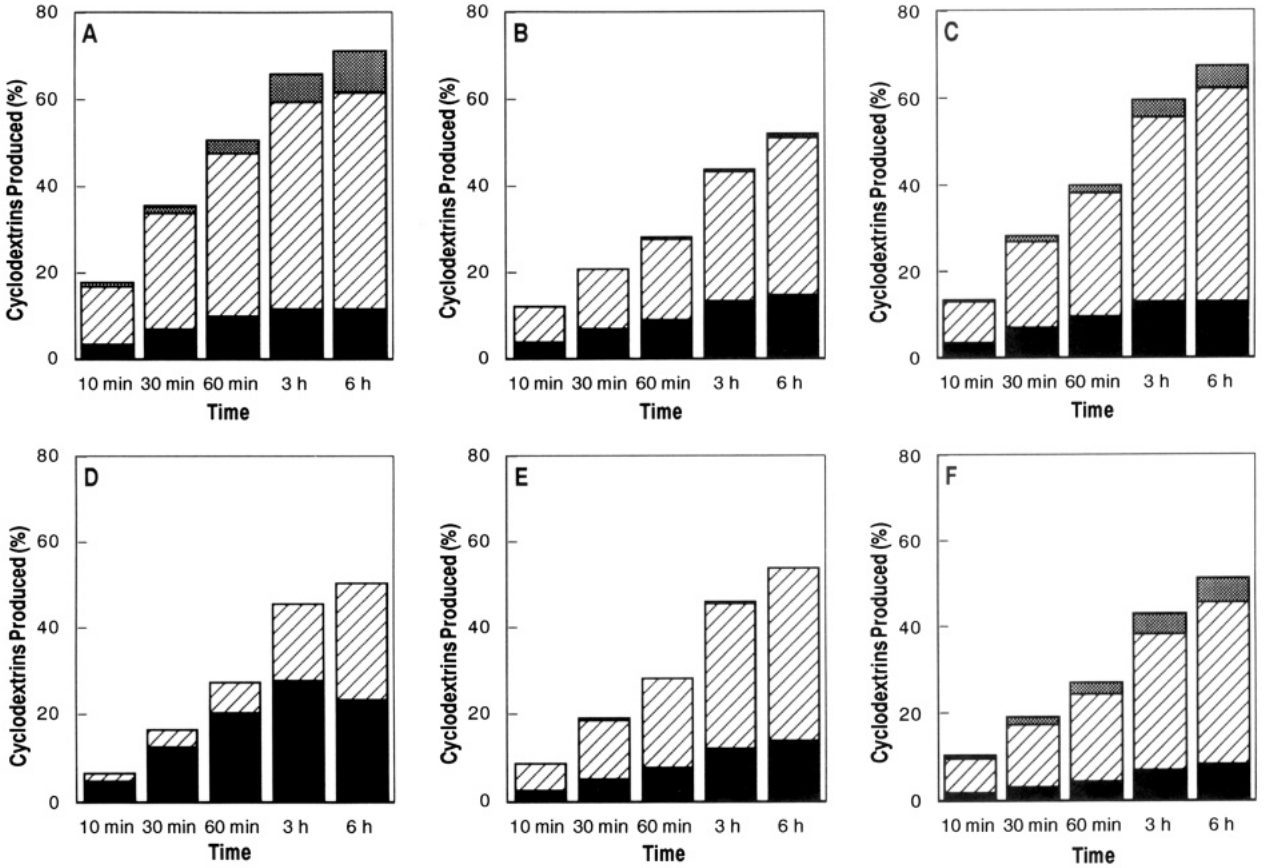

FIGURE 3: Time course of the production of α-, β-, and γ-cyclodextrin from amylose by wild-type (A), F183L- (B), Y195F- (C), Y195L- (D), F259L- (E), and F283L-CGTase (F). The amount of each cyclodextrin was quantified by HPLC and is shown as a percent of the initial amount of substrate. The average degree of polymerization of amylose used as the substrate was 17. Stippling, α-cyclodextrin; diagonal lines, β-cyclodextrin; solid shading, γ-cyclodextrin.

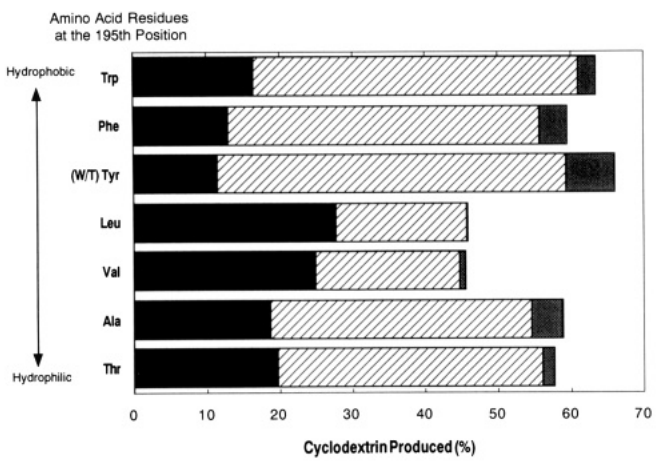

FIGURE 4: Effects of replacing Tyr-195 with several amino acid residues upon the cyclization characteristics. The cyclodextrin levels relative to initial amount of substrate were analyzed by HPLC after each enzyme was incubated with amylose at 37 °C for 3 h. Stippling, α-cyclodextrin; diagonal lines, β-cyclodextrin; solid shading, γ-cyclodextrin.

Since wild-type CGTase produces β-cyclodextrin from amylose as the main product (Figure 3A), the kinetic parameters for cyclization activity were measured as β-cyclodextrin-forming activity using amylose as the substrate (Table 1). Y195F-CGTase had a level of β-cyclodextrin-forming activity similar to that of wild-type CGTase, whereas other mutant CGTases had decreased k_{cat} values. The values of K_m for the β-cyclodextrin-forming activity were not significantly changed by any of the single mutations. Cyclization, however, is a very complicated reaction. Therefore, the roles of each residue should not be evaluated only from the kinetic perspective when the cyclization activity of the enzyme is studied.

Effects of Amino Acid Replacements on Hydrolysis and Intermolecular Transglycosylation. To analyze the roles of the four aromatic residues in more detail, the kinetic parameters for two types of reactions for cyclodextrin degradation (cyclodextrin hydrolysis and coupling; Table 2) and the disproportionation reaction, which is an intermolecular transglycosylation between a linear maltooligosaccharide and an acceptor (Table 3), were determined. To assay the disproportionation, either glucose or maltose were the acceptors, and 3KB-G5CNP was the donor.

The K_m values of γ-cyclodextrin for coupling activity were not significantly changed by replacing Tyr-195 with phenylalanine (Table 2). This value, however, increased 4.3-fold when Tyr-195 was replaced with the aliphatic residue leucine. The K_m values for α- and β-cyclodextrin also increased 4.5- and 4.0-fold, respectively, in Y195L-CGTase. In contrast, the K_m values for the linear maltooligosaccharide, 3KB-G5CNP, in the disproportionation reaction were increased not more than 2-fold by replacing Tyr-195 with leucine (Table 3). The values of K_m for acceptors in both coupling and disproportionation activities were not significantly changed by replacing Tyr-195. Therefore, we suggest that Tyr-195 participates to a very limited extent in the binding of linear maltooligosaccharides.

X-ray crystallography of *B. stearothermophilus* CGTase crystals soaked with maltose has suggested that Phe-191 (the equivalent residue of Tyr-195 in *Bacillus* sp. 1011 CGTase) is located at the active center where its phenyl group could

Table 2: Coupling and Cyclodextrin-Hydrolyzing Activities of Wild-Type and Mutant CGTase[a]

	coupling activity			hydrolyzing activity: α-cyclodextrin		hydrolyzing activity: β-cyclodextrin	
enzyme	k_{cat} (s^{-1})	$K_m^{\gamma\text{-CD}}$ (mM)	$K_m^{\text{methyl-}\alpha\text{-Glc.}}$ (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
wild-type	67.8 ± 1.6	0.12 ± 0.01	16.9 ± 1.4	2.65 ± 0.08	0.57 ± 0.05	0.57 ± 0.05	0.11 ± 0.03
F183L	49.1 ± 4.4	0.21 ± 0.05	44.0 ± 8.9	1.10 ± 0.10	1.15 ± 0.26	ND[b]	ND[b]
Y195F	29.2 ± 1.0	0.10 ± 0.01	17.4 ± 1.8	1.53 ± 0.04	0.55 ± 0.05	ND[b]	ND[b]
Y195L	18.9 ± 0.6	0.51 ± 0.05	12.0 ± 0.9	0.11 ± 0.01	2.56 ± 0.06	0.84 ± 0.02	0.44 ± 0.03
F259L	10.7 ± 0.3	0.35 ± 0.04	11.7 ± 0.8	0.72 ± 0.01	1.25 ± 0.06	ND[b]	ND[b]
F283L	2.1 ± 0.1	0.10 ± 0.02	39.9 ± 5.6	0.23 ± 0.01	0.94 ± 0.14	ND[b]	ND[b]

[a] Assays were performed in 10 mM sodium phosphate, pH 6.5, at 37 °C. [b] Not determined.

Table 3: Disproportionation Activities between 3KB-G5CNP and Acceptors of Wild-Type and Mutant CGTases[a]

	acceptor: glucose			acceptor: maltose		
enzyme	k_{cat} (s^{-1})	$K_m^{\text{3KB-G5CNP}}$ (mM)	K_m^{glucose} (mM)	k_{cat} (s^{-1})	$K_m^{\text{3KB-G5CNP}}$ (mM)	K_m^{maltose} (mM)
wild-type	244 ± 6	0.20 ± 0.02	11.3 ± 0.4	203 ± 3	0.16 ± 0.01	0.49 ± 0.02
F183L	928 ± 19	0.23 ± 0.01	57.8 ± 2.5	333 ± 8	0.08 ± 0.01	1.42 ± 0.09
Y195F	139 ± 5	0.20 ± 0.02	15.9 ± 1.0	192 ± 8	0.22 ± 0.03	0.59 ± 0.05
Y195L	118 ± 6	0.34 ± 0.05	9.8 ± 0.7	132 ± 4	0.31 ± 0.03	0.38 ± 0.04
F259L	20.7 ± 0.6	0.56 ± 0.04	12.0 ± 0.5	25.4 ± 0.8	0.48 ± 0.04	1.30 ± 0.08
F283L	7.3 ± 0.1	0.15 ± 0.01	7.3 ± 0.2	8.2 ± 0.2	0.12 ± 0.01	0.17 ± 0.01
F183L/F259L	228 ± 8	0.34 ± 0.04	160 ± 11	158 ± 4	0.24 ± 0.02	18.2 ● 0.4

[a] Assays were performed in 10 mM sodium phosphate, pH 6.5, at 37 °C.

make an inclusion complex with spiral amylose and cyclodextrin (Kubota, 1993). Tyr-195 of *B. circulans* CGTase is also located at a similar position in the active center (Klein et al., 1992). Indeed, an inclusion complex between cyclodextrin and an enzyme has been observed in soybean β-amylase: the side chain of Leu-383, which is located in the active center, makes an inclusion complex with α-cyclodextrin, a competitive inhibitor for β-amylases (Mikami et al., 1993). The increased K_m values for cyclodextrins in Y195L-CGTase suggest that Tyr-195 can indeed make an inclusion complex with spiral amylose and that replacement of Tyr-195 by leucine affects its formation during substrate-binding thereby resulting in the altered production ratio of α-, β-, and γ-cyclodextrin.

Replacement of Phe-183 with a leucine residue resulted in a 2.9–5.1-fold increase in K_m values for acceptors in both coupling (Table 2) and disproportionation (Table 3), whereas the K_m values for the donors were insignificantly changed in any of reactions tested, indicating that Phe-183 is part of the acceptor binding site. Although the starch-degrading specific activity and the k_{cat} value for the β-cyclodextrin-forming activity in F183L-CGTase were decreased to 16% and 24%, respectively, of those in wild-type CGTase (Table 1), the k_{cat} value for the coupling activity was retained 72% (Table 2). Furthermore, the values for the disproportionation activity were increased to 1.6–3.8-fold those of wild-type CGTase (Table 3). These data indicate that the replacement of Phe-183 with a leucine residue specifically induced the decrease in cyclization efficiency without affecting transglycosylation.

Unusual effects were observed when Phe-259 was replaced with a leucine residue. In all intermolecular transglycosylation reactions tested, F259L-CGTase had increased K_m values for donors: the K_m values of γ-cyclodextrin for coupling activity and of 3KB-G5CNP for disproportionation activity were about 3 times larger than that of wild-type CGTase (Tables 2 and 3). This indicates that replacing Phe-259 with a leucine residue affects donor-binding in the ground-state. The K_m values for acceptors were not significantly changed when the monosaccharides methyl α-glucoside and glucose were used for coupling and disproportionation activities, respectively (Tables 2 and 3). However, the K_m value for the disaccharide acceptor, maltose, was increased 2.7-fold (Table 3). As described previously, the acceptor binding site of CGTase can recognize at least two glucopyranosyl rings (Nakamura et al., 1994). These results suggest that Phe-259 is involved in the binding of both donors and acceptors.

Previously, we have shown that the intermolecular transglycosylation reaction of CGTase is operated by a ping-pong bi-bi mechanism (Nakamura et al., 1994). This model states that before an acceptor binds to its binding site, the reducing side of the cleaved donor is released from the enzyme. Therefore, an acceptor will bind to the same subsites to which the reducing side of the cleaved donor is bound. Phe-259 would be part of the subsite S_2' (Figure 1), because F259L-CGTase had an increased K_m value for the disaccharide maltose, and the values for monosaccharides were not affected by the replacement (Tables 2 and 3). Phe-183 would be part of the subsite S_1', because replacing Phe-183 by leucine affected the K_m values for all the acceptors tested (Tables 2 and 3).

In order to analyze the roles of Phe-183 and Phe-259 in more detail, we constructed the double mutant F183L/F259L-CGTase. The K_m values of glucose and maltose for the disproportionation were increased 14- and 37-fold, respectively, by the double mutation (Table 3). The increasing rates of the K_m values were much greater than those of the single mutants, indicating that Phe-183 and Phe-259 are cooperatively involved in acceptor binding. The K_m value of amylose for the β-cyclodextrin-forming activity also increased 4.6-fold (Table 1). The increased K_m value for the β-cyclodextrin-forming activity would be mainly due to the decreased affinity toward acceptors, that is, the nonreducing end of amylose bound to the enzyme. The k_{cat} values for disproportionation activity were remained largely unchanged at 78–93% of wild-type (Table 3), indicating that the effect of the double mutation on the intermolecular transglycosylation activity is very slight. In contrast, the starch-degrading activity of F183L/F259L-CGTase was decreased 28-fold (Table 1). Furthermore, the k_{cat} value for the β-cyclodextrin-forming activity decreased 190-fold. These results indicate that the subsites S_1' and S_2',

Table 4: Acarbose Concentrations Required for 50% Inhibition of Starch-Degrading Activity (ID_{50})[a]

enzyme	ID^{50} (μM)	enzyme	ID^{50} (μM)
wild-type	0.33	Y195L	1.3
F183L	21	F259L	23
Y195F	0.13	F283L	270

[a] Assays were performed in 10 mM sodium phosphate, pH 6.5, at 37 °C.

of which Phe-183 and Phe-259 are part, play a critical role in cyclization when the nonreducing end of amylose binds to the active center of the enzyme.

It has been reported that cyclodextrins are undetectable when Phe-255 of *B. stearothermophilus* CGTase (equivalent to Phe-259 in *Bacillus* sp. 1011 CGTase) is replaced with an isoleucine residue (Fujiwara et al., 1992). Therefore, the role of this phenylalanine residue appears to be conserved among CGTases.

Although F283L-CGTase had a 2.3-fold higher K_m value for methyl α-glucoside, an acceptor used in coupling (Table 2), the values for acceptors for the disproportionation activities, on the contrary, decreased (Table 3). Replacing Phe-283 with leucine affected the values of k_{cat} rather than the K_m values, indicating that Phe-283 participates very little in ground-state substrate binding.

Effects of Acarbose. The pseudo-tetrasaccharide acarbose is a competitive inhibitor of the cyclization reaction of CGTase (Nakamura et al., 1993). Since the K_i value of acarbose is much smaller than the K_m values for substrate, acarbose has been proposed to act as a transition-state analog toward CGTase (Nakamura et al., 1993). The acarbose concentrations required for 50% inhibition for starch-degrading activity (ID_{50}) are shown in Table 4. The ID_{50} of wild-type CGTase was 0.33 μM. Changes in the values were quite small when Tyr-195 was replaced. In contrast, the values increased 64-, 70-, and 820-fold in F183L-, F259L-, and F283L-CGTase, respectively. Since the values of ID_{50} are proportional to the K_i for acarbose (Nakamura et al., 1993), the changes in the ID_{50} by the replacement probably correlate with changes in the affinities of acarbose for the enzymes. Therefore, replacing Phe-183, Phe-259, or Phe-283 with leucine induce a decrease in the affinity of acarbose, especially in the Phe-283 replacement. Together with the effects of replacing Phe-283 on k_{cat} values (Tables 2 and 3), these results suggest that Phe-283 is involved in transition-state stabilization.

Effects of pH on Stability and Starch-Degrading Activity. The pH stability of wild-type and mutant CGTases is shown in Figure 5A. The residual activities were measured by starch-degrading activity after each enzyme was kept at the indicated pH for 30 min at 50 °C. All enzymes retained more than 80% activity over the pH range of 6.0–9.0 under our experimental conditions. When the enzymes were kept at 37 °C for 30 min, they were all stable over the pH range 5.5–11.0 (data not shown).

The pH profiles of the starch-degrading activity of wild-type and mutant CGTases are shown in Figure 5B. Since *Bacillus* sp. 1011 is an alkalophilic microorganism, *Bacillus* sp. 1011 CGTase retained high activity over an alkaline pH range. Wild-type CGTase had more than 80% activity over the pH range of 4.0–9.0, the optimum being at pH 5.0. The activity at pH 8.0 was 90% of that at pH 5.0. Similar pH profiles were observed in mutant CGTases in which Phe-183, Tyr-195, or Phe-259 were replaced. In contrast, the pH dependence of F283L-CGTase had decreased activity at both acidic and alkaline pH ranges, and the optimum pH for the

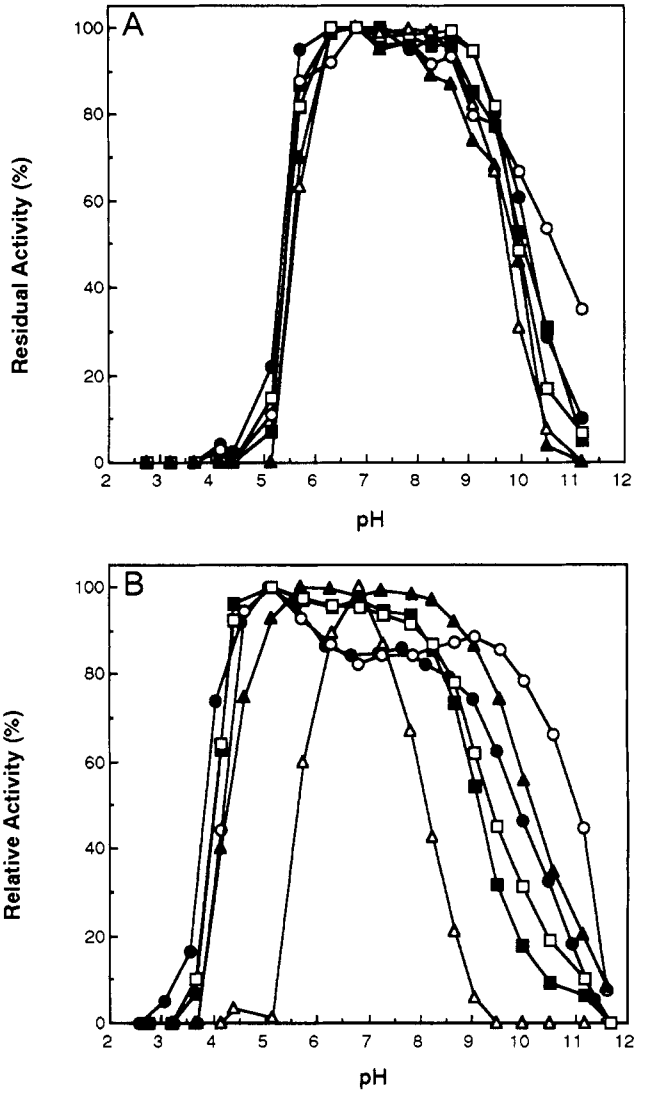

FIGURE 5: Effects of pH on the stability (A) and starch-degrading activity (B) of wild-type and mutant CGTases. To measure the pH stability, each enzyme was incubated at 50 °C for 30 min at the indicated pH and neutralized by sodium phosphate, pH 6.0 at a final concentration of 0.25 M. Residual activity was measured by the starch-degrading activity at pH 6.0. In both analyses, modified Britton-Robinson buffer (containing 10 mM sodium phosphate, 10 mM sodium acetate, and 10 mM sodium borate) was used. Wild-type CGTase, ●; F183L-CGTase, ○; Y195F-CGTase, ■; Y195L-CGTase, □; F259L-CGTase, ▲; and F283L-CGTase, △.

starch-degrading activity was shifted to pH 7.0. Since the pH stability was insignificantly changed (Figure 5A), the drastic changes in pH dependence for F283L-CGTase probably resulted from a direct effect of the replacement, indicating that Phe-283 is important for starch degradation over acidic and alkaline pH ranges.

Changes in the pH dependence have also been found in H327N-CGTases (Nakamura et al., 1993). His-327 is involved in transition-state stabilization, since H327N-CGTase had decreased k_{cat} values for all activities tested and decreased affinity for acarbose without affecting K_m values (Nakamura et al., 1993). All these characteristics of H327N-CGTase are quite similar to those of F283L-CGTase. On the basis of the three-dimensional structure of CGTases, His-327 hydrogen bonds with the carboxylate of Glu-257, which is proposed to be a general acid catalyst, and Phe-283 is located near Glu-257 (Klein et al., 1992; Kubota, 1993). Furthermore, replacement of Arg-227, which is the other residue that hydrogen-bonds with the carboxylate of Glu-257 (Klein et

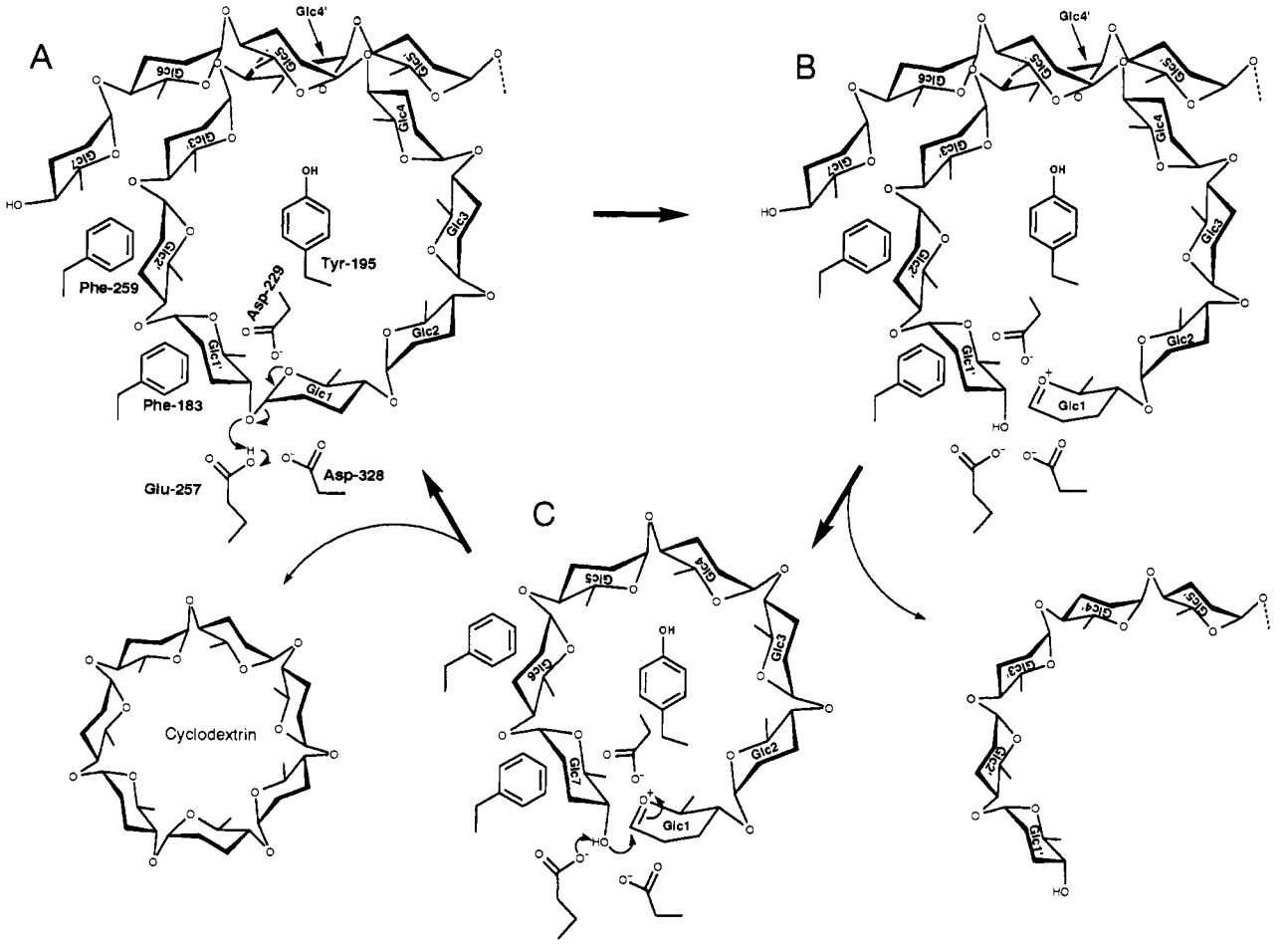

FIGURE 6: Proposed model for the cyclization reaction catalyzed by CGTase. Note that it remains unclear whether the reaction proceeds through an oxocarbonium ion-like intermediate (as shown here; Blake et al., 1992; Cogoli & Semenza, 1975; Matsuura et al., 1994, 1991) or a covalent intermediate (Tao et al., 1989; Isoda et al., 1992). See the text for details.

al., 1992), also induced decreased activity over an alkaline pH range (unpublished result). Thus, we suggest that the environment around Glu-257 is important for the stabilization of the transition-state and for the starch-degrading activity over pH ranges far from its optimum.

Our updated model for the reaction mechanism of the cyclization is shown in Figure 6. Amylose will binds to CGTase in the spiral state due to the ability of Tyr-195 to form an inclusion complex with amylose (Figure 6A). Since the catalytic residues of CGTase has been proposed to be equivalent to those of α-amylases (Nakamura et al., 1992), CGTase will cleave the α-1,4-glucosidic bond of amylose between Glc1′ and Glc1 in the same way as α-amylases (Figure 6B). The transglycosylation reaction of CGTase is operated by a ping-pong mechanism (Nakamura et al., 1994), which states that the transglycosylation occurs after the reducing side of the cleaved amylose has been released from the enzyme. Cyclodextrin will be formed, when the nonreducing end of the cleaved amylose fills subsite S_1' and S_2', of which Phe-183 and Phe-259 are part, respectively (Figure 6C), and the intermolecular transglycosylation will occur when another acceptor binds subsite S_1'. As the k_{cat} values of the disproportionation are larger than the value of the cyclization (Tables 1 and 3), we propose that the main reaction catalyzed by CGTase is the disproportionation. This model is consistent with that based upon the tertiary structure of *B. circulans* CGTase (Klein et al., 1992).

Recently, we succeeded in crystallizing *Bacillus* sp. 1011 CGTase (Haga et al., 1994), and its X-ray crystallographic study is now in progress. Roles of these aromatic residues in CGTase catalysis will be clarified more precisely from structural analysis of the mutant enzymes complexed with inhibitors or substrate analogs.

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