Three Histidine Residues in the Active Center of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. 1011: Effects of the Replacement on pH Dependence and Transition-State Stabilization†

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ABSTRACT: Cyclodextrin glucanotransferase (CGTase) catalyzes the formation of cyclodextrins from amylose through an intramolecular transglycosylation reaction. On the basis of the three-dimensional structures of CGTases three histidine residues, which are conserved between CGTases and α-amylases, are located at the active center and are proposed to constitute the substrate binding sites. The three histidine residues (His-140, His-233, and His-327) of CGTase from alkalophilic *Bacillus* sp. 1011 were individually replaced by site-directed mutagenesis to probe their roles in catalysis. Asparagine-replaced CGTases (H140N-, H233N-, and H327N-CGTase) retained cyclization activity but had altered production ratios of α-, β-, and γ-cyclodextrin. Replacement of histidine by asparagine residues strongly affected the k_{cat} for β-cyclodextrin-forming, coupling, and hydrolyzing activities, whereas it barely affected the K_m values. The activation energies for α-cyclodextrin hydrolysis were increased more than 12 kJ/mol by the replacement. Furthermore, the K_i values of acarbose, which is thought to be a transition-state analog of glycosidase catalysis, were 2–3 orders of magnitude larger in asparagine-replaced CGTases than that in wild-type CGTase. Therefore, the three histidine residues participate in the stabilization of the transition state, whereas they participate little in ground-state substrate binding. H327N-CGTase had decreased activity over an alkaline pH range, indicating that His-327 is important for catalysis over an alkaline pH range.

Cyclodextrin glucanotransferase (CGTase,[1] EC 2.4.1.19) converts starch and related α-1,4-glucans to cyclodextrins through an intramolecular transglycosylation reaction. Besides this cyclization reaction, 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 a weak hydrolyzing activity. Since cyclodextrins can form inclusion complexes with many molecules and change the chemical and physical properties of the included molecules, CGTase is an important enzyme in the food and pharmaceutical industries.

CGTases from bacilli contain 680–690 amino acids and have over 60% identity within their amino acid sequences. Although sequence similarities between CGTases and α-amylases are usually below 30%, three highly conserved regions have been identified (Binder et al., 1986; Kimura et al., 1987) and have been designated as the A-, B-, and C-region, respectively (Figure 1). These regions are also found in other amylolytic enzymes such as isoamylase (Amamura et al., 1986), pullulanase (Katsuragi et al., 1987), and α-glucosidases (Hong & Marmur, 1986; James et al., 1989). Three-dimensional structures are available for α-amylases Taka-amylase A (TAA), porcine pancreatic α-amylase (PPA), and acid α-amylase from *Aspergillus niger* (Boel et al., 1990; Buisson et al., 1987; Matsuura et al., 1984). About 400 amino acid residues of the NH_2-terminal regions of α-amylases are folded to give a $(\beta/\alpha)_8$-barrel structure, containing the A-, B-, and C-region, and the conserved regions constitute the active center. Three-dimensional structures of CGTases from *Bacillus circulans* and *Bacillus stearothermophilus* have been elucidated recently (Klein & Schulz, 1991; Kubota et al., 1991). The chain folds of both CGTases are similar. The NH_2-terminal 400 amino acids of CGTases are folded into barrel structures, which closely resemble those of α-amylases and which also contain the conserved regions. The catalytic residues of α-amylases have been proposed from their three-dimensional structures. Although it has been proposed that the catalysts are different pairs among the three α-amylases, they are restricted within the three conserved acidic amino acid residues: aspartate in the B-region; glutamate, about 30 amino acid residues away on the COOH-terminal side of the B-region (designated as the B′-region); and aspartate in the C-region. The one glutamate and the two aspartate residues in the B-, B′-, and C-region are found in all amylolytic enzymes possessing the conserved regions. Site-directed mutagenesis studies have shown that any one of these residues is essential for catalysis in α-amylases (Holm et al., 1990; Nagashima et al., 1992; Takase et al., 1992), neopullulanase (Kuriki et al., 1991), and CGTase (Nakamura et al., 1992). These findings suggest that the catalytic mechanisms among these amylolytic enzymes are similar and that the variations in substrate specificity and products can be ascribed to the relationships between their similar catalytic centers and different subsite structures. It is thought that α-amylase catalyzes the hydrolysis of the α-1,4-glucosidic bond by acid–base catalysis as proposed for lysozyme (Matsuura et al., 1984, 1991); cleavage of the glucosidic bond is catalyzed via an oxocarbonium ion intermediate (Blake et al., 1967).

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[1] Abbreviations: CGTase, cyclodextrin glucanotransferase; kb, kilobase pairs; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PPA, porcine pancreatic α-amylase; SDS, sodium dodecyl sulfate; TAA, Taka-amylase A.

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Enzyme	A-region	B-region	B'-region	C-region	Reference
α-Amylase					
A. oryzae	^{117}DVVAN**H**	^{202}GLRIDTV**K**H	^{230}EVLD	^{292}FVEN**H**D	Toda et al. (1982)
B. subtilis	^{97}DAVIN**H**	^{172}GFRFDAA**K**H	^{208}EILQ	^{264}WVES**H**D	Yamazaki et al. (1983)
B. stearothermophilus	^{101}DVVFD**H**	^{230}GFRLDGL**K**H	^{264}EYWS	^{326}FVDN**H**D	Ihara et al. (1985)
P. stutzeri	^{112}DVVPN**H**	^{189}GFRFDFVRG	^{219}ELWK	^{288}FVDN**H**D	Fujita et al. (1988)
Human	^{99}DAVIN**H**	^{196}GFRIDASK**H**	^{236}EVID	^{298}FVDN**H**D	Nakamura et al. (1984)
Barley	^{101}DIVIN**H**	^{127}DGRLDWGP**H**	^{218}EVWD	^{299}FVDN**H**D	Rogers & Millman (1983)
CGTase					
Bacillus sp. #1011	^{135}DFAPN**H**	^{225}GIRVDAVK**H**	^{257}EWFL	^{323}FIDN**H**D	Kimura et al. (1987)
B. stearothermophilus	^{131}DFAPN**H**	^{221}GIRMDAVK**H**	^{253}EWFL	^{319}FIDN**H**D	Sakai et al. (1987)
B. macerans	^{135}DFAPN**H**	^{225}GIRFDAVK**H**	^{258}EWFL	^{324}FIDN**H**D	Sakai et al. (1987)
B. circulans	^{135}DFAPN**H**	^{225}GIRVDAVK**H**	^{257}EWFL	^{323}FIDN**H**D	Nitschke et al. (1990)
K. pneumoniae	^{130}DYAPN**H**	^{219}AIRIDAI**K**H	^{257}EWFG	^{328}FMDN**H**D	Binder et al. (1986)
Pullulanase					
K. aerogenes	^{600}DVVYN**H**	^{671}GFRFDLMGY	^{704}EGWD	^{827}YVSK**H**D	Katsuragi et al. (1987)
Neopullulanase					
B. stearothermophilus	^{242}DAVFN**H**	^{324}GWRLDVANE	^{357}EIWH	^{419}LLGS**H**D	Kuriki & Imanaka (1989)
α-Amylase-pullulanase					
C. thermohydrosulfuricum	^{488}DGVFN**H**	^{594}GWRLDVANE	^{627}ENWN	^{699}LLGS**H**D	Melasniemi et al. (1990)
Isoamylase					
P. amyloderamosa	^{291}DVVYN**H**	^{370}GFRFDLASV	^{454}EWSV	^{502}FIDV**H**D	Amemura et al. (1988)
α-Glucosidase (Maltase)					
A. aegypti	^{114}DFVPN**H**	^{215}GFRIDAVPY	^{276}EYSK	^{351}VLGN**H**D	James et al. (1989)
S. carlsbergensis	^{106}DLVIN**H**	^{210}GFRIDTAGL	^{276}EVAH	^{344}YIEN**H**D	Hong & Marmur (1986)

FIGURE 1: Comparison of amino acid sequences in the conserved regions (A, B, B′, and C) of various amylolytic enzymes. Amino acids are numbered from the NH_2-terminus of each mature enzyme. The conserved histidine residues are indicated by black highlighting.

The three histidine residues located at the A-, B-, and C-region are found in most α-amylases and in all known CGTases (Figure 1). Moreover, the histidine residues in the A- and C-region are conserved among all amylolytic enzymes possessing the conserved regions. From X-ray crystallographic studies of TAA and *B. stearothermophilus* CGTase, these residues are located near the catalytic triad and are proposed to constitute subsites adjacent to the α-1,4-glucosidic bond that will be split (Kubota et al., 1991; Matsuura et al., 1984). The pH dependence of kinetic parameters in PPA has suggested that one histidine residue of the three plays a role in catalysis (Ishikawa et al., 1990). Chemical modification studies of CGTases from *Klebsiella pneumoniae* and *B. circulans* with diethyl pyrocarbonate have suggested that at least one histidine residue is important for the cyclization reaction (Bender, 1991; Mattson et al., 1992). To elucidate the roles of the three conserved histidine residues in CGTase, the histidine residues located at the A-, B-, and C-region (His-140, His-233, and His-327, respectively) of CGTase from alkalophilic *Bacillus* sp. 1011 were replaced by arginine or asparagine residues, and their effects on the enzymatic properties were analyzed. The activities of the three mutant enzymes in which three histidine residues were individually replaced by arginine residues were too low to analyze the roles of the three histidine residues for the reaction mechanisms. On the other hand, asparagine-replaced CGTases retained sufficiently high activities and were used for more detailed analysis. The kinetic data led us to propose that the three histidine residues participate in the stabilization of the transition state, rather than in substrate binding in the ground state as proposed from the X-ray crystallographic studies. Furthermore, the pH profiles of H327N-CGTase were quite different from those of wild-type CGTase over an alkaline pH range, indicating that His-327 is important for catalysis over an alkaline pH range in addition to transition-state stabilization.

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. Glucoamylase (EC 3.2.1.3) from *Rhizopus niveus* was from Seikagaku Kogyo Co., Ltd. Tetracycline and ampicillin were from Wako Pure Chemical Industry Ltd. Soluble starch was from E. Merck. Amylose (the average degree of polymerization is 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).

Bacterial Strains and Plasmids. Escherichia coli JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac proAB*)/F′:*traD36 proAB lacI*^{q} *Z*ΔM15] was used for recombinant DNA manipulations. *E. coli* BW313 {*HfrKL-16PO*/45[*lysA*(*61*–62)] *dut1 ung1 thi-1 relA1*} was used for site-directed mutagenesis. A protease-deficient mutant, *E. coli* ME8417 [*lon*::Tn*10*(*tet*^{r}) *thr leu lacY*], provided by Dr. H. Takahashi (The University of Tokyo), was used as the host for production of wild-type and mutant CGTases. 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 alkalophilic *Bacillus* sp. 1011, into the *Hin*dIII site of pUC13.

Construction of Mutant CGTase Genes. Synthetic oligonucleotides were prepared on an Applied Biosystems 380A DNA synthesizer and purified on oligonucleotide purification cartridges (Applied Biosystems). 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:

H140N: 5′-CACCGAACAATACATCTC-3′
H140R: 5′-GCACCGAACCGTACATCTCCG-3′
H233N: 5′-GCGGTCAAGAATATGCCA-3′
H233R: 5′-GCGGTCAAGCGTATGCCATTC-3′
H327N: 5′-ATCGACAATAATGACATG-3′
H327R: 5′-ATCGACAATCGTGACATGGAG-3′

Expression and Purification of Wild-Type and Mutant CGTases. The *E. coli* ME8417 strains carrying the constructs 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 of wild-type and mutant CGTases during purification, all buffer and chromatography gels were freshly prepared each time and used only once for all. Protein concentrations were determined by using 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 units/mL) was incubated at 37 °C for appropriate periods with amylose solution (10 g/L) in 10 mM sodium phosphate, pH 6.0, and reactions were terminated by boiling the solutions for 10 min. Each cyclodextrin in the reaction mixtures was identified and quantified by the method of Kobayashi et al. (1984) with modifications. Since cyclodextrins cannot be digested by glucoamylase, reaction mixtures were treated with glucoamylase. The ratios of the α-, β-, and γ-cyclodextrin in reaction mixtures were then analyzed by high-performance liquid chromatography (HPLC). HPLC was performed at 20 °C using a LiChrospher 100 NH_2 column (mean particle size of 5 μm, 4 × 250 mm; Cica-Merck) and a refractive index director. The mobile phase was 65% acetonitrile at a flow rate of 0.8 mL/min. The amounts of cyclodextrins were calculated from the decreased amounts of glucose relative to that at time 0 after the glucoamylase digestion of the reaction mixtures.

Enzyme Assay. All reactions were performed at 37 °C in 10 mM sodium phosphate, pH 6.0. Starch-degrading activity was measured by the blue value method of Fuwa (1954) with slight modifications 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. Determination of β-cyclodextrin-forming activity was based upon the phenolphthalein method of Vikmon (1981). Two milliliters of amylose or soluble starch solution was incubated with the enzymes for appropriate periods. The reaction was stopped by adding 500 μL of 0.2 N hydrochloric acid, after which 1 mL of 500 mM sodium carbonate and 500 μL of 240 μM phenolphthalein were added. The decrease in absorbance at 550 nm, caused by complexing of the dye with the β-cyclodextrin produced, was calibrated with free and β-cyclodextrin-complexed phenolphthalein. The amounts of reducing sugar liberated by the hydrolyzing activity of the enzymes were measured by the methods of Somogyi (1952) and Nelson (1944). The coupling activity assay was based upon the method of Thoma et al. (1965) with the following modifications: (i) instead of α-cyclodextrin, β-cyclodextrin was used as the donor of the coupling reaction; (ii) the reaction was stopped with hydrochloric acid at a final concentration of 0.04 N; and (iii) instead of α-amylase, the reaction mixture was treated with glucoamylase after the coupling reaction was terminated, to digest linear maltooligosaccharides produced by the coupling reaction. The activity was calculated from the consumed amounts of β-cyclodextrin in the reaction mixtures calibrated from the amounts of glucose in glucoamylase-treated reaction mixtures.

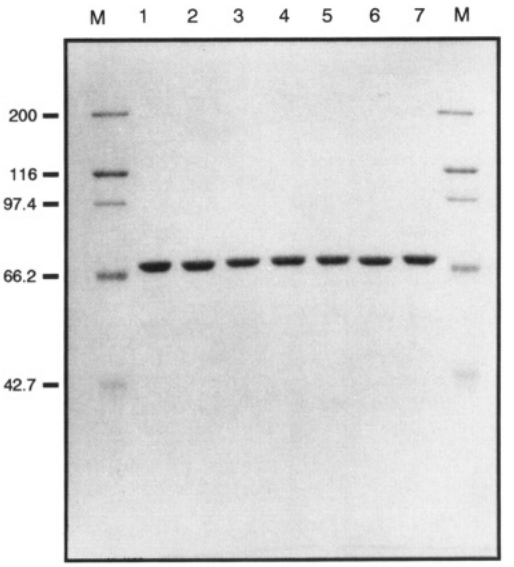

FIGURE 2: SDS–PAGE of the purified wild-type and mutant CGTases. SDS–PAGE was performed using 8.5% gels. Purified proteins (2 μg) were loaded onto an SDS–polyacrylamide gel, electrophoresed, and stained with Coomassie brilliant blue. Lanes M, Bio-Rad high molecular mass protein standards; the sizes of the molecular mass markers are given in kDa. Lane 1, wild-type CGTase; lane 2, H140R-CGTase; lane 3, H140N-CGTase; lane 4, H233R-CGTase; lane 5, H233N-CGTase; lane 6, H327R-CGTase; lane 7, H327N-CGTase.

Kinetic parameters k_{cat} and K_m were determined by the nonlinear least-squares method with the Taylor expansion (Sakoda & Hiromi, 1976). The K_i values of acarbose on β-cyclodextrin-forming activity were determined by Dixon plots (Segel, 1975).

The changes in activation energy [$\Delta(\Delta G)$] for α-cyclodextrin hydrolysis was calculated from the relationship

$$\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}] \quad (1)$$

where $(k_{cat}/K_m)_{mut}$ and $(k_{cat}/K_m)_{wt}$ refer to the catalytic efficiencies (k_{cat}/K_m) of mutant and wild-type enzyme, respectively (Wilkinson et al., 1983).

Other Experimental Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli (1970).

RESULTS

General Characterization of Wild-Type and Mutant CGTases. The genes for wild-type and mutant CGTases were expressed in a protease-deficient mutant, *E. coli* ME8417. All the enzymes produced in the periplasm of the cells were extracted and purified under the same conditions. The purity of the enzymes exceeded 90%, on the basis of SDS–PAGE profiles (Figure 2). The molecular masses of mutant CGTases were estimated to be 70 kDa and were equal to that of the wild-type enzyme. No significant difference was observed in circular dichroism spectra between wild-type and mutant enzymes (data not shown), suggesting that the mutations did not cause a significant conformational change.

Table I: Specific Activity for the Starch-Degrading Activity of Wild-Type and Mutant CGTases[a]

enzyme	starch-degrading activity (units/mg)	enzyme	starch-degrading activity (units/mg)
wild-type	2160	H233N	834
H140R	50.5	H327R	1.0
H140N	546	H327N	889
H233R	11.4		

[a] Starch-degrading activity was measured at 37 °C in 10 mM sodium phosphate, pH 6.0.

The specific activities of the starch-degrading activity of H140R-, H233R-, and H327R-CGTase were reduced to 2.3, 0.5, and 0.05% of that of wild-type CGTase, respectively (Table I). Since these activities were too small to quantify, we could not analyze the role of the histidine residues in enzyme action using the arginine-replaced CGTases. In contrast, three asparagine-replaced CGTases (H140N-, H233N-, and H327N-CGTase) retained 25, 39, and 41%, respectively, of the starch-degrading specific activity found in wild-type CGTase and were therefore used for more detailed analyses.

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 β-cyclodextrin mainly at all incubation periods tested (more than 50% of total cyclodextrins produced). The production of α-cyclodextrin gradually increased in proportion to the reaction time, reaching a level of 35% of total cyclodextrins at 24 h (Figure 3A). Although all asparagine-replaced CGTases retained cyclization activities, the production ratios of α-, β-, and γ-cyclodextrin were changed. The amounts of α-cyclodextrin were quite low in the reaction mixtures of mutant enzymes at all incubation periods tested (up to 5% of total cyclodextrins produced). Especially with H233N-CGTase, no α-cyclodextrin was detected even after longer incubation periods (Figure 3C). Similar results were observed when soluble starch was used as the substrate (data not shown). The formation of reducing sugar from soluble starch was decreased in asparagine-replaced CGTases (data not shown).

Kinetic Parameters of Wild-Type and Mutant CGTases. The major degradation production from amylose by the action of wild-type and mutant CGTases was β-cyclodextrin (Figure 3). We therefore assayed β-cyclodextrin-forming activity to compare the cyclization activity of the wild-type with that of mutant CGTases (Table II). The K_m value in wild-type CGTase was 56.0 μM for amylose. The K_m values were not significantly changed in these mutant CGTases. However, the k_{cat} values for β-cyclodextrin-forming activity in H140N-, H233N-, and H327N-CGTase were reduced to 22, 18, and 63%, respectively, of that in wild-type CGTase. When soluble starch was used as the substrate, the k_{cat} values were also reduced to 9.8, 11, and 21% in H140N-, H233N-, and H327N-CGTases, respectively. The values of k_{cat}, however, were different from those for amylose, suggesting that the efficiency for CGTase cyclization depends on the structures of the substrates.

The k_{cat} value for α-cyclodextrin-hydrolyzing activity of wild-type CGTase was 3.96 s^{-1}. The k_{cat} values in H140N-, H233N-, and H327N-CGTase were reduced to 0.7, 1.5, and 1.8%, respectively, whereas the K_m values for α-cyclodextrin were elevated only 1.6–2.1-fold by the replacement.

Coupling activity between β-cyclodextrin and methyl α-glucoside was assayed to compare the intermolecular transglycosylation activities of wild-type and mutant CGTases. The k_{cat} values were also reduced to 2.8–4.2%, whereas the K_m values for both β-cyclodextrin and methyl α-glucoside were little changed by the replacement.

Effects of a Pseudo-Tetrasaccharide, Acarbose, on Starch-Degrading and β-Cyclodextrin-Forming Activities. The pseudo-tetrasaccharide acarbose (Figure 4) is a competitive inhibitor for small-intestinal sucrase (Hanozet et al., 1981) and mammalian lysosomal acid α-D-glucosidase (Calder & Geddes, 1989). The unsaturated cyclitol ring of acarbose seems to interact with the glucopyranosyl binding site, and its half-chair conformation is thought to act as a transition-state analog of the putative oxocarbonium ion intermediate of glycosidase catalysis (Goldsmith et al., 1987; Schmidt et al., 1977). It has been reported that acarbose inhibits the starch-degrading activity of CGTases (Hidaka et al., 1980; Truscheit et al., 1981). The acarbose concentrations required for 50% inhibition (ID_{50}) of starch-degrading activity in wild-type and mutant CGTases were assayed and are summarized in Table III. The ID_{50} for starch-degrading activity in wild-type CGTase was 0.6 μM and increased 1300-, 210-, and 100-fold in H140N-, H233N-, and H327N-CGTase, respectively.

The effects of acarbose on cyclization activity were also analyzed. Acarbose did not affect the initial production ratio of α-, β-, and γ-cyclodextrin as judged by HPLC (data not shown). However, acarbose inhibited the β-cyclodextrin-forming activity of CGTase in a competitive manner (Figure 5). The K_i values of acarbose, determined by β-cyclodextrin-forming activity, were 0.06 μM in wild-type CGTase. The value of K_i for acarbose in wild-type CGTase was quite low compared with the K_m values for amylose and α- and β-cyclodextrin (Table II). The K_i value of acarbose was increased 1400-, 320-, and 120-fold in H140N-, H233N-, and H327N-CGTase, respectively (Table III). The increases in K_i for the mutant CGTases correlated with those in ID_{50}. These results show that replacement of the conserved histidine residues by asparagine induces a considerable decrease in the affinity of acarbose for CGTase.

Effects of pH on Stability and Activity of Wild-Type and Mutant CGTases. The pH stability of wild-type and mutant CGTases is shown in Figure 6A. 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 were stable over the pH range 6.0–9.0 under the present experimental conditions. When the enzymes were kept at 37 °C for 30 min, all enzymes were stable over the pH range 5.5–12.0 (data not shown).

The pH profiles on starch-degrading activity of wild-type and mutant CGTases are shown in Figure 6B. Wild-type CGTase had more than 80% activity over the pH range 4.5–8.5, the optimum being at pH 5.5. The activity at pH 8.0 was 90% of that at pH 5.5. Similar pH profiles were observed in H140N- and H233N-CGTase. In contrast, the pH dependence of H327N-CGTase was quite difference. H327N-CGTase had decreased activity over an alkaline pH range. Its activity at pH 8.0 was reduced to 30% of that at pH 5.5.

The difference in pH dependence between wild-type CGTase and H327N-CGTase was analyzed in more detail. The pH profiles of k_{cat} for α-cyclodextrin hydrolysis by wild-type CGTase and H327N-CGTases were assayed under conditions of sufficiently high α-cyclodextrin concentrations (70 mM) that k_0 was proportional to k_{cat} (Figure 7). Wild-type CGTase also had more than 80% activity over the pH range 4.3–8.5, the optimum being at pH 6.0 (Figure 7A). H327N-CGTase also had decreased hydrolyzing activity over an alkaline pH range (Figure 7B). These results indicate that replacement

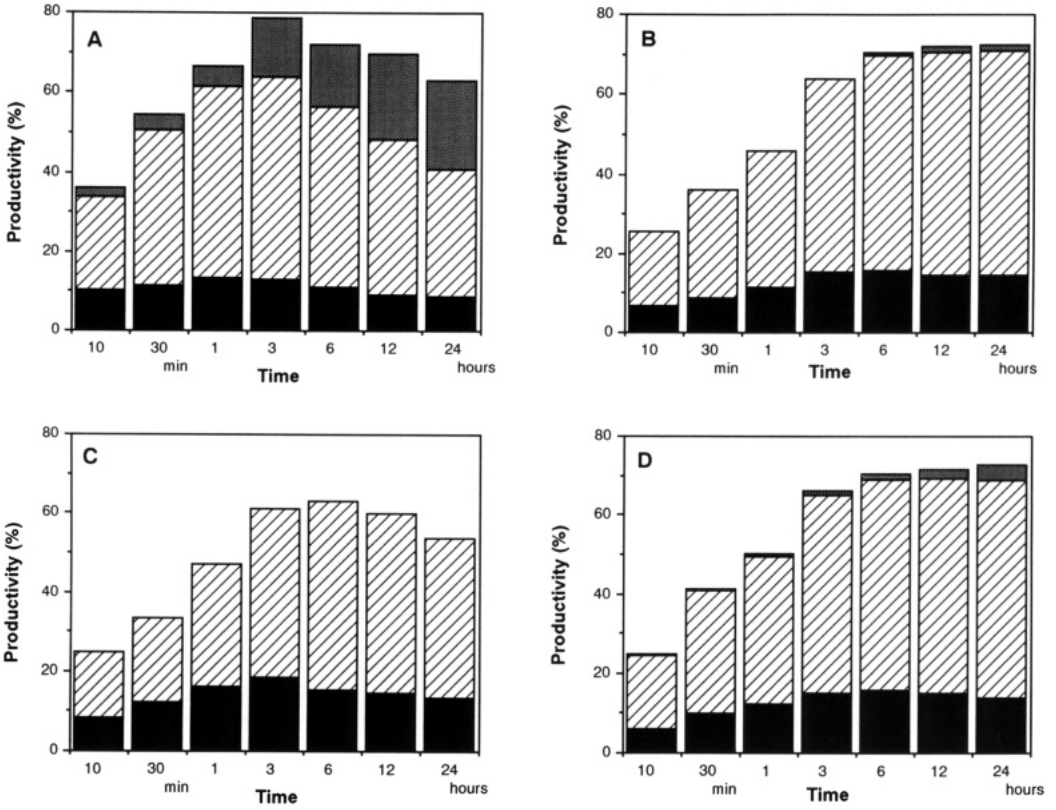

FIGURE 3: Time course of the production of α-, β-, and γ-cyclodextrin from amylose by wild-type CGTase (A), H140N-CGTase (B), H233N-CGTase, (C), and H327N-CGTase (D). The average degree of polymerization of amylose was 17: stippling, α-cyclodextrin; diagonal lines, β-cyclodextrin; solid shading, γ-cyclodextrin.

Table II: Kinetic Parameters of Wild-Type and Mutant CGTases[a]

	β-cyclodextrin-forming activity				hydrolyzing activity of α-cyclodextrin		coupling activity between β-cyclodextrin and methyl α-glucoside		
	amylose[b]		soluble starch						
enzyme	K_m (μM)	k_{cat} (s^{-1})	K_m ($\times 10^{-2}$ g/L)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} ($\times 10^{-3}$ s^{-1})	$K_m^{\beta\text{-CD}}$ (mM)	$K_m^{\text{Methyl-}\alpha\text{-Glc}}$ (mM)	k_{cat} (s^{-1})
wild-type	56.0 ± 1.7	22.2 ± 0.2	4.3 ± 0.5	50.4 ± 1.3	0.61 ± 0.06	3960 ± 121	0.17 ± 0.02	12.0 ± 0.9	68.5 ± 1.5
H140N	56.0 ± 5.5	4.85 ± 0.16	3.2 ± 0.5	4.92 ± 0.14	1.05 ± 0.28	26.0 ± 3.0	0.27 ± 0.09	25.6 ± 1.3	2.11 ± 0.05
H233N	46.1 ± 6.6	4.03 ± 0.16	4.2 ± 0.6	5.48 ± 0.16	0.99 ± 0.09	60.9 ± 2.1	0.22 ± 0.06	9.78 ± 0.71	1.93 ± 0.13
H327N	35.5 ± 1.0	14.0 ± 0.1	4.1 ± 0.9	10.5 ± 0.5	1.28 ± 0.13	71.4 ± 2.7	0.36 ± 0.02	14.7 ± 3.4	2.90 ± 0.27

[a] All assays were performed at 37 °C in 10 mM sodium phosphate, pH 6.0. [b] The average degree of polymerization was 17.

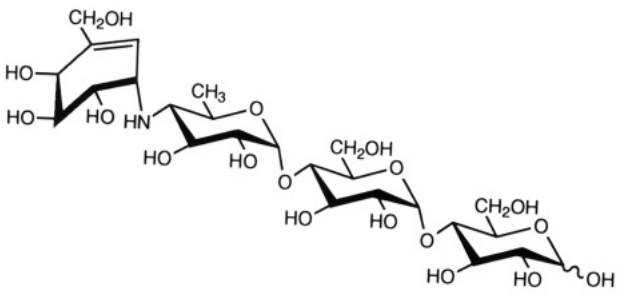

FIGURE 4: Structure of acarbose (Schmidt et al., 1977).

Table III: Acarbose Concentrations Required for 50% Inhibition (ID_{50}) of Starch-Degrading Activity, and K_i Values for Acarbose

enzyme	ID_{50} (μM)	K_i (μM)[a]	enzyme	ID_{50} (μM)	K_i (μM)[a]
wild-type	0.6	0.06	H233N	125	19
H140N	800	85	H327N	62.5	6.9

[a] The K_i of acarbose was determined by β-cyclodextrin-forming activity at 37 °C in 10 mM sodium phosphate, pH 6.0, using a Dixon plot.

of His-327 by an asparagine residue affects the activity over an alkaline pH range.

DISCUSSION

To evaluate the roles of the three histidine residues in the active center of CGTase, we had replaced them with arginine or asparagine residues, and the effects of the replacements were analyzed. Arginine-replaced CGTases had quite low activity. Replacing histidine with arginine seems to retain the positive charge at the replacement position. The pK_a value of the guanidium group of arginine, however, is much higher than that of the imidazole of histidine, and the side chain of arginine is much longer than that of histidine. Therefore, the drastic decreases in the activity of arginine-replaced CGTases could be caused by alterations in the local conformation at the active center. In contrast, asparagine-replaced CGTases retained 25–41% starch-degrading specific activities. Replacement of histidine by asparagine would be sterically conservative, and the hydrogen-bonding capability of the δ-nitrogen of histidine can be mimicked, but that of the ε-nitrogen cannot.

Although the three asparagine-replaced CGTases retained cyclization activity, their β-cyclodextrin-forming activity was reduced 2–10-fold. Chemical modification studies of CGTases

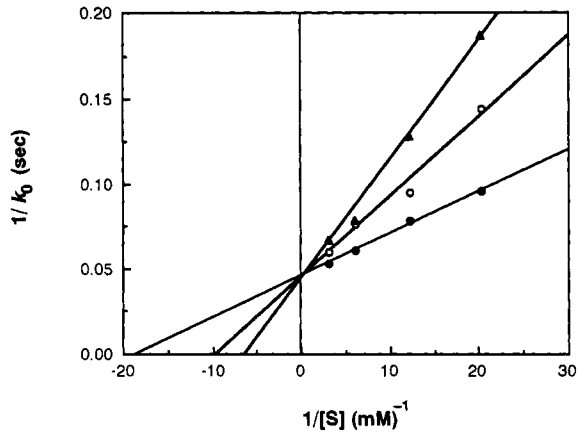

FIGURE 5: Competitive inhibition of CGTase from *Bacillus* sp. 1011 by acarbose. β-Cyclodextrin-forming activity using amylose as the substrate was measured in the presence or absence of acarbose in the reaction mixture. The final concentrations of acarbose were none (●), 0.05 μM (○), and 0.10 μM (▲).

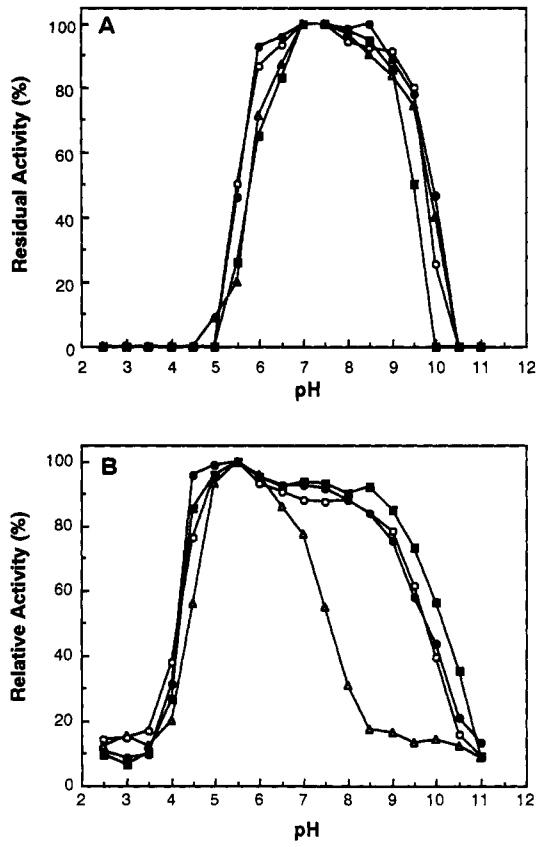

FIGURE 6: Effects of pH on stability (A) and starch-degrading activity (B) of wild-type and mutant CGTases. For measurement of pH stability, each enzyme was incubated at 50 °C for 30 min at the indicated pH and neutralized by 300 mM sodium phosphate, pH 6.0. Residual activity was measured by starch-degrading activity at pH 6.0. In both analyses, McIlvaine buffer (pH 2.5–8.0) and 100 mM H_3BO_3·KCl·Na_2CO_3 buffer (pH 8.0–11.0) were used: wild-type CGTase (●), H140N-CGTase (○), H233N-CGTase (■), and H327N-CGTase (△).

with diethyl pyrocarbonate have also suggested that a histidine residue is important for cyclization (Bender, 1991; Mattson et al., 1992). The histidine residue that is modified is thought to be located near the active site, suggesting that the ethoxyformylated histidine residue was one of the three replaced in this study. However, the following differences in characteristics can be found between ethoxyformylated and asparagine-replaced CGTases: (i) the production ratio of α-,

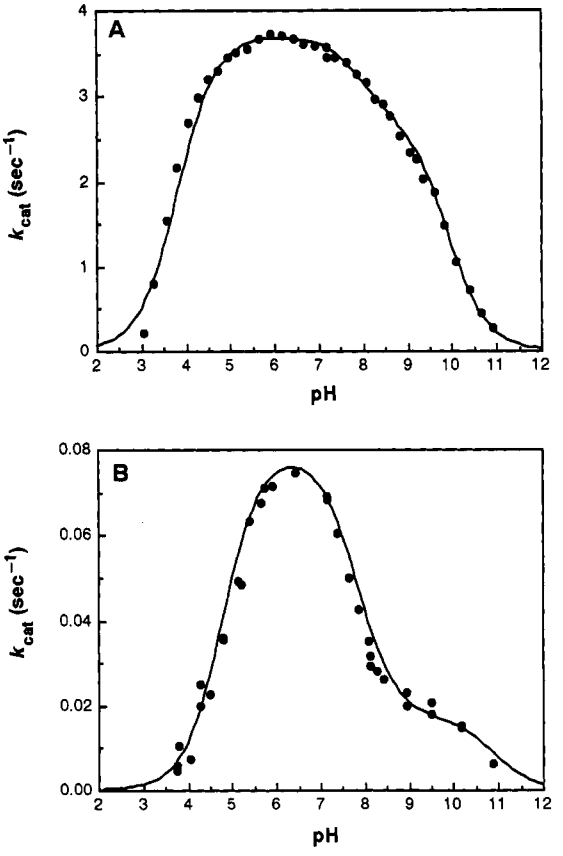

FIGURE 7: pH profiles of k_{cat} for α-cyclodextrin hydrolysis by wild-type CGTase (A) and H327N-CGTase (B). The assays were performed at 37 °C using 10 mM sodium phosphate ranging from pH 3.0 to 11.0. The concentration of α-cyclodextrin was sufficiently high that k_{obs} was proportional to k_{cat}.

β-, and γ-cyclodextrin is affected by replacing histidine with asparagine (Figure 3), whereas it is not affected by ethoxyformylation (Mattson et al., 1992); (ii) the formation of reducing sugar from soluble starch is decreased in asparagine-replaced CGTases (data not shown) but increased by ethoxyformylation of a histidine residue (Bender, 1991; Mattson et al., 1992). Although these conflicts may be caused by the differences in effects between ethoxyformylation and replacement of the histidine on enzymatic properties, we could not clarify whether the ethoxyformylated histidine was one of the three replaced in this study or not.

The values of k_{cat} for β-cyclodextrin-forming activity were different between the two substrates, amylose and soluble starch (Table II). This suggests that the cyclization activity of CGTase depends on the structure of the substrates. We suppose that the k_{cat} value for the cyclization reaction indicates the efficiency of the cyclization reaction between cyclization, disproportionation, coupling, and the hydrolysis of the enzyme rather than the activity of transglycosylation. In addition to this hypothesis, the K_m value for cyclization does not indicate the true affinity for substrate of the enzyme, since cyclization is, in fact, a two-substrate reaction: one substrate is amylose as a donor, and the other is the nonreducing end of the amylose bound to the enzyme as the acceptor. We presume that the roles of each CGTase residue should not be evaluated only from the kinetic perspective when the cyclization activity of the enzyme is studied. Therefore, we assayed the coupling and hydrolyzing activities of wild-type and mutant CGTases, in addition to their β-cyclodextrin-forming activity.

Replacement of histidine by asparagine strongly affected k_{cat} values for β-cyclodextrin-forming (intramolecular trans-

glycosylation), coupling (intermolecular transglycosylation), and hydrolyzing activities, but had little effect on the K_m values for the three activities (Table II). From these observations, participation of the three histidine residues in ground-state substrate binding seems to be very little, although they are proposed to constitute substrate binding sites according to an X-ray crystallographic study (Kubota et al., 1991).

The difference in the activation free energies [$\Delta(\Delta G)$] for α-cyclodextrin hydrolysis between wild-type and mutant CGTases was calculated from the catalytic efficiencies for mutant and wild-type CGTases using eq 1 (Wilkinson et al., 1983). The value of $\Delta(\Delta G)$ reflects the change of the binding strength of the enzyme transition-state complex. The values of $\Delta(\Delta G)$ for α-cyclodextrin hydrolysis were increased 14.4, 12.0, and 12.3 kJ/mol in H140N-, H233N-, and H327N-CGTase, respectively. These values are typical of energy loss resulting from the elimination of a charged group that hydrogen bonds to the substrate (Fersht, 1987). Furthermore, the affinities of acarbose were decreased by 2–3 orders of magnitude by the replacement (Table III). Acarbose is thought to be a transition-state analog in glycosidase catalysis (Goldsmith et al., 1987; Schmidt et al., 1977), and it also seems to act as a transition-state analog toward CGTase from the following observations. First, the structure of the unsaturated cyclitol ring of acarbose (Figure 4) is similar to that of the oxocarbonium ion, a putative intermediate of glycosidase catalysis (Schmidt et al., 1977). Second, the reaction mechanism of CGTase seems to be similar to that of other glycosidases (Nakamura et al., 1992). Third, acarbose inhibits CGTase activity in a competitive manner (Figure 5). Finally, the value of K_i (6×10^{-6} M; Table II) is quite low compared with the K_m values of the substrates (10^{-4}–10^{-2} M; Table II). The elevated activation energies and the relatively low affinities for acarbose in mutant CGTases indicate that all three histidine residues participate in the stabilization of the transition state.

The three-dimensional structure of *B. stearothermophilus* CGTase (Kubota et al., 1991) has suggested that His-140 and His-327 bind the same glucose unit of amylose, whereas His-233 binds a different one. His-327 clearly has another role in catalysis, besides participation in transition-state stabilization, because of the decreased activity at high pH in H327N-CGTase. However, we could not clarify whether His-140 and His-233 have different roles in catalysis. It is noteworthy that the acceleration of starch-degrading activity by maltose was most effective toward H233N-CGTase (data not shown). Maltose acts as an acceptor for intermolecular transglycosylation and hence accelerates the starch degradation of CGTase (Kitahata, 1988). Moreover, despite the small change, only H233N-CGTase had a decreased K_m value for methyl α-glucoside, the acceptor used in the coupling reaction (Table II). We presume that replacement of His-233 by an asparagine residue affected the binding of an acceptor to the enzyme, in addition to affecting transition-state stabilization.

Since *Bacillus* sp. 1011 is an alkalophilic microorganism, wild-type CGTase retained high activity over an alkaline pH range (Figures 6B and 7A). A relatively high activity over an alkaline pH range was also observed in other CGTases from alkalophilic bacteria but not in those from nonalkalophilic bacteria such as *Bacillus macerans* (Kaneko et al., 1989; Kobayashi et al., 1978; Sin et al., 1991). Although it has been proposed that the catalysts in amylolytic enzymes possessing the conserved regions are the three acidic amino acid residues (Asp-229, Glu-257, and Asp-328 in this CGTase), as a result of X-ray crystallography (Boel et al., 1990; Buisson et al., 1987; Matsuura et al., 1984; Klein et al., 1992; Kubota et al., 1991) and site-directed mutagenesis (Holm et al., 1990; Kuriki et al., 1991; Nagashima et al., 1992: Nakamura et al., 1992; Takase et al., 1992), these residues should be deprotonated over an alkaline pH range. Therefore, CGTases from alkalophilic bacteria may use a non-carboxylic acid group as a catalyst under alkaline conditions. H327N-CGTase had clearly decreased activities over an alkaline pH range (Figures 6 and 7B). Since the pH stability over an alkaline pH range was insignificantly changed by replacing His-327 with asparagine (Figure 6), the reduced activity of H327N-CGTase at high pH values indicates that His-327 contributes, directly or indirectly, to the catalysis over an alkaline pH range.

In *B. circulans* CGTase, the ϵ-nitrogen of His-327 hydrogen bonds to the carboxylate of Glu-257, a putative proton donor of this CGTase (Klein et al., 1992). This hydrogen bond would also exist in *Bacillus* sp. 1011 CGTase, due to the high homology in the primary structures between the two CGTases (72.2% amino acid identity). As mentioned above, an asparagine residue cannot mimic the hydrogen-bonding capability of the ϵ-nitrogen of histidine, indicating that the hydrogen bond between His-327 and Glu-257 was eliminated by replacing His-327 with asparagine. The hydrogen bond between His-327 and Glu-257 may be important for catalysis over an alkaline pH range.

Participation of histidine in catalysis was also observed in other amylolytic enzymes. In PPA, one histidine residue is proposed to act as a proton donor (Ishikawa et al., 1990). Site-directed mutagenesis experiments on the conserved histidine residues in human pancreatic α-amylase have suggested that the conserved histidine residues participate, directly or indirectly, in catalysis (Ishikawa et al., 1992). The substrate-dependent shift of optimum pH, which is observed in wild-type human pancreatic α-amylases, was eliminated in histidine-replaced mutants. Replacement of the conserved histidine residues in neopullulanase from *B. stearothermophilus* induced both a decrease in its hydrolyzing activity and a change in the ratio of products (Kuriki et al., 1991). These observations are quite similar to the case of this CGTase and suggest that the roles of the histidine residues in catalysis are conserved among amylolytic enzymes.

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