Identification of Catalytic Amino Acids of Cyclodextran Glucanotransferase from *Bacillus circulans* T-3040

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In glycoside hydrolase family 66 (see http://afmb.cnrs-mrs.fr/CAZY/), cyclodextran glucanotransferase (CITase) is the only transglycosylation enzyme, all the other family 66 enzymes being dextranases. To analyze the catalytic amino acids of CITase, we modified CITase chemically from the T-3040 strain of *Bacillus circulans* with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). EDC inactivated the enzyme by following pseudo-first order kinetics. In addition, the substrates of an isomaltooligosaccharide and a cyclodextran inhibited EDC-induced enzyme inactivation, implicating the carboxyl groups of CITase as the catalytic amino acids of the enzyme. When two conserved aspartic acid residues, Asp145 and Asp270, were replaced with Asn in T-3040 mature CITase, CIT-D270N had reduced activity. The $V_{\text{max}}$ of CIT-D145N was 1% of that of wild-type CITase, whereas the $K_m$ of CIT-D145N was about the same as that of the wild-type enzyme. These findings indicate that Asp145 and Asp270 play an important role in the enzymatic reaction of T-3040 CITase.

**Key words:** cyclodextran; cyclodextran glucanotransferase; catalytic amino acid; aspartate residue

Cyclodextran glucanotransferase (CITase) is an enzyme that catalyzes the intramolecular transglycosylation of dextran to produce cyclic oligosaccharides of α-1,6 linked glucose residues, known as cycloisomaltooligosaccharides or cyclodextrins (CIs). Our group has reported three types of CIs, CI-7, -8, and -9, named according to the numbers of glucose units.13 Only two enzymes have ever been found to produce cyclic α-glucooligosaccharides as the major product by a single enzyme molecule. One is cyclodextrin glucanotransferase (CGTase to produce cyclodextrin: CD)2 and the other is CITase.15 CITase acts on α-1,6 glucan (dextran), but not on α-1,4 glucan (amylose) or α-1,4/α-1,6 glucans (amylopectin and pullulan).

CIs are extremely water-soluble cyclooligosaccharides, more soluble than α-, β-, or γ-CD. They have been reported strongly to decrease glucansucrase activity, a dental-caries formation element, by competitive inhibition, with $K_i$ values of 0.2 to 0.6 mM, whereas the $K_m$ value of glucansucrase for a substrate (sucrose) was 24 mM, suggesting that CIs might prevent dental caries when used as cariostatic food additives.3 In addition, hydrophobic substance solubilized action, anti-Human Immunodeficiency Virus action, and anti-ulcer action have also been observed in a CI and its derivatives.5 CIs are likely to be multifunctional oligosaccharides. Apart from CITase, however, no other enzymes have been reported to produce CIs, and little is known about the reaction mechanism of this unique enzyme.

Cultivation of *Bacillus circulans* strain T-3040 together with dextran has yielded CITase and its product CIs in the culture supernatant.10 Subsequently, the CITase gene (*cit*) was cloned and its nucleotide sequence was determined.5 CITase belongs to glycoside hydrolase family 66.6 The other enzymes in this family are dextranases, which hydrolyze dextran to produce isomaltooligosaccharides (IGs) but no CIs. The amino acid sequence of CITase was found to be only about 20 to 30% identical to those of other family 66 enzymes, even in the N-terminal conserved region. Although Asp385 in *Streptococcus mutans* dextranase has been reported to exist in the catalytic site of the enzyme,7 there has been no further information about the catalytic center or the structure of other family 66 enzymes.

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**Abbreviations:** CGTase, cyclodextrin glucanotransferase; CI, cyclodextran (or cycloisomaltooligosaccharide); CITase, cyclodextran glucanotransferase; CIT-D145N, recombinant T-3040 CITase mutant whose Asp145 has been replaced with Asn; CIT-D270N, recombinant T-3040 CITase mutant whose Asp270 has been replaced with Asn; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GEE, glycine ethyl ester; HBDase, highly branched dextran hydrolase; IG, isomaltooligosaccharide; PCR, polymerase chain reaction

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The major products of CITase are CIs, such as CI-7, CI-8, and CI-9. CITase has been shown to produce linear isomaltonozae from CI-8 and glucose by an intermolecular transglucosylation reaction (coupling), as well as to produce several linear isomaltooligosaccharides from isomaltoptenate by the same reaction (disproportionation). These features resemble those of cyclodextrin glucanotransferase (CGTase), but there are no amino acid sequence similarities between CITase and CGTase. In contrast, the amino acid sequence of T-3040 CITase is similar to those of dextranases from Streptococcus downei, S. mutans, and S. salivarius, all of which belong to family 66. In CITase, but not in dextranases, there is an insertion of 89 amino acids, but the function of this insertion is not clear. Although site-directed mutagenesis has proven to yield mutant CITases with increased reaction velocity, there is little information about the catalytic site of CITase. Hence we have sought to determine the catalytic site of T-3040 CITase. We identified the two aspartic acid residues, which play an important role in the enzyme reaction, by chemical modification and by site-directed mutagenesis toward the acidic residues conserved in family 66 enzymes.

Materials and Methods

Construction of CITase expression plasmid. cit was amplified using primers T-3040-5 (5′-CTC ATG AGC GGC TCT GGC GGC ATC GAG CG-3′, in which the inserted BspHI site is indicated by underlining) and T-3040-6 (5′-C TTC GAG CTA GCTCAC ATT GAT CCC-3′, in which the inserted XhoI site is indicated by underlining). The N-terminal amino acid of the mature T-3040 CITase has been reported to be serine, encoded by the codon TCA. To express T-3040 CITase better, this TCA was replaced by AGC using primer T-3040-5. Polymerase chain reaction (PCR) was performed using KOD plus polymerase (Toyobo, Tokyo) and, as a template, pCI429, which contained the cit encoding CITase from B. circulans T-3040 cloned into the plasmid vector pUC118. The PCR product was digested with BspHI and XhoI and the resulting fragment of 2,814 bp was ligated into the NcoI and XhoI sites of pET-23d. The 1,976 bp EcoRI–SmaI fragment of cit DNA obtained from pCI429 was substituted into the corresponding position of this plasmid. To confirm that no mutation had been introduced into PCR-amplified regions (BspHI–EcoRI and SmaI–XhoI), the nucleotide sequences of both regions were determined with a model 310 capillary DNA sequencer (Applied Biosystems, Foster City, CA) using a dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Expression and purification of recombinant CITase. E. coli BL21 (DE3) was transformed with pCIT, and the transformed cells were grown in Luria-Bertani medium (Becton, Dickinson and Company, Sparks, MD) containing ampicillin (50 μg/ml) at 37°C. When the optical density at 600 nm reached 0.9, 0.1 mM isopropyl β-D-thiogalactopyranoside was added as an inducer and the bacteria were cultivated for an additional 21 h at 18°C. The cells were harvested by centrifugation at 6,000 × g for 10 min, washed once with buffer A (50 mM Na-phosphate buffer, pH 7.0), resuspended in buffer A, and disrupted by sonication. After centrifugation (5,000 × g for 10 min), the supernatant was brought to 80% saturation with ammonium sulfate, allowed to stand overnight at 4°C, and centrifuged at 6,000 × g for 20 min. The resulting pellet was suspended in an appropriate volume of buffer A and dialyzed against the same buffer. The supernatants were collected by centrifugation (6,000 × g for 20 min) and loaded onto a Resource Q column (1 ml, Amersham Biosciences, Uppsala, Sweden) equilibrated with buffer B (1.5 mM ammonium sulfate, 50 mM Na–phosphate buffer, pH 7.0). The column was washed with buffer B, and the enzyme was eluted with a 1.5–0 M linear ammonium sulfate gradient. The CITase-containing fractions were dialyzed against buffer C (50 mM Na–phosphate buffer, pH 8.0) and loaded onto a Resource Q column equilibrated with buffer C. The column was washed with buffer C and the enzyme was eluted with a 0–1.0 M linear NaCl gradient. The eluted fraction was loaded onto a gel filtration column (Superose 12, 1.0 × 30 cm, Amersham Biosciences) equilibrated with buffer A containing 0.15 M NaCl. The purifying enzyme was defined as wild-type T-3040 recombinant CITase.

Enzyme assay. Enzyme solution (50 μl) was added to an equal volume of substrate solution containing 2% (W/V) Dextran T-40 (Amersham Biosciences) in 80 mM Na–acetate buffer (pH 5.5). The reaction mixtures were incubated at 40°C for 2 h, and the reactions were stopped by boiling for 10 min. To each mixture, was added 50 μl of Rhizopus sp. glucoamylase (Wako Pure Chemical Industries, Osaka, Japan) and 25 μl of highly branched dextran hydrolase (HBDase) (the kind gift of Dr. Oguma and Mr. Kawamoto of the Noda Institute for Science Research, Chiba, Japan) in 40 mM Na–acetate buffer (pH 5.5) to digest linear oligosaccharides to glucose at 40°C for 2 h. The reactions were stopped by boiling for 10 min, and each sample was mixed with an equal volume of 100% acetonitrile. CI synthesis activity was determined by measuring liberated CIs (sum of the amounts of CI-7, CI-8, and CI-9) by HPLC (Intelligent HPLC System, Jasco International, Tokyo) with a TSKgel Amide-80 column (Tohos, Tokyo). The mobile phase was acetonitrile–water (55:45, vol/vol), and the flow rate was 1 ml/min. The products were detected with a refractive index detector (RI-2031 Plus detector, Jasco International, Tokyo). To determine the kinetic parameters of CITase and the inhibition constant for 1-ethyl-3-(3-dimethylaminopropyl)-carbosemiude (EDC), purified enzyme (1.32 μl, 1.41 μg) was incubated with 2.8 × 10⁻⁶–7.1 × 10⁻⁴ M substrate dextran T-40, in the presence or absence of 5 mM EDC, in 30 mM Na–
phosphate buffer (pH 5.8) in a 50 µl reaction mixture. CI synthesis activity was assayed as described above.

One unit of CI synthesis activity was defined as the amount of enzyme that produced 1 µmol of cyclodextrins (sum of CI-7, -8, and -9) from 0.5 mM dextran T-40 at 40 °C in 40 mM Na–acetate buffer (pH 5.5). One unit of glucoamylase activity was defined as the amount of enzyme that produced 1 µmol of glucose per min from 0.25 mM dextran T-40 at 40 °C in 40 mM Na–acetate buffer (pH 5.5). One unit of HBDase activity was defined as the amount of enzyme that produced 1 µmol of glucose from 100 mM maltose at 40 °C in 40 mM Na–acetate buffer (pH 5.5).

**Chemical modification of CITase with EDC.** The purified enzyme (1.43 mM, 1.53 µg protein) was incubated at 30 °C with various concentrations of EDC containing 80 mM glycine ethyl ester (GEE) in 40 mM Na–phosphate buffer (pH 5.8) in a total volume of 25 µl. To each was added 20 µl of 2 M Na–acetate buffer (pH 5.5) to stop the reaction. The enzyme was isolated by gel filtration using 0.3 ml of Bio-Gel P6 packed in a 1 ml syringe column.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed using a Mutan-Super Express Km Kit (Takara, Kyoto, Japan) and primers T-3040-39 (5’-CGC CTG GCA GTT CTA CAA CTG GAT G TG GCG-’3’) to replace Asp145 with Asn and T-3040-40 (5’-GAC GGC ATC CAT CAC GTG AAC CAG ATG GGC CAG-’3’) for replace Asp270 with Asn. The EcoRI-KpnI (1.1 kbp) fragments containing the D145N and D270N mutations were introduced into the corresponding sites of pCIT, and the resulting plasmids, designated pCIT-D145N and pCIT-D270N respectively, were used for expression.

**Protein analysis.** Protein concentration was determined by Coomassie protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard.

**Results and Discussion**

**Chemical modification of CITase by EDC.**

From the nucleotide sequence of the CITase gene,5) we estimated the molecular mass of the deduced mature CITase, without the N-terminal 38 amino acids constituting the secretion signal, to be 103,103 Da. Following construction of pCIT and its expression in E. coli, we purified the recombinant mature CITase, as described in “Materials and Methods.” Most of the CITase protein was present in the soluble fractions. The purified recombinant CITase was homogeneous, as determined from SDS–PAGE, and its molecular mass was about 100 kDa, similar to that of the native form and to its deduced molecular mass.

We modified the carboxyl groups of purified recombinant CITase using EDC. First, an EDC activates the carboxyl group to form an ester bond, to which glycine ethyl ester (GEE) further reacts to form a stable amide bond. Although EDC modification is usually performed in TEMED–HCl (pH 4.75) buffer,12) this buffer was observed to inactivate about 50% of the enzyme within 30 min in the absence of EDC, even at pH 5.5, suggesting inactivation of CITase by TEMED. To avoid a reaction between EDC and the carboxylic residue of the buffer component, we used an Na–phosphate buffer (pH 5.8), which did not inactivate CITase at 30 °C over long incubation. When we assayed the time course of CITase inactivation with 20 mM EDC, 40% of the enzymatic activity remained at 15 min, and finally CITase was completely inactivated after 60 min. A semilogarithmic plot of remaining activity versus incubation time was essentially linear, indicating that inactivation followed pseudo-first-order kinetics (Fig. 1A). The relationship between log [EDC (mM)] and log [k (min⁻¹)] gave a slope with n = 0.45 (Fig. 1A, insert) which apparently indicates that inactivation of CITase did not occur at a 1:1 mol/mol ratio of enzyme and EDC.

CITase produces CIs from dextran, and small amounts of isomaltooligosaccharides are also produced, at a ratio of 95:1. The CIs produced in this reaction are slowly hydrolyzed to smaller isomaltooligosaccharides over time. In addition, CI and isomaltooligosaccharides are substrates for coupling and disproportionation reactions catalyzed by CITase. We found that CI-8 and isomaltoolotetroase (IG4) inhibited the EDC-induced decrease in CI synthesis activity (Fig. 1B). For example, when 7.7 mM CI-8 or 15.0 mM IG4 was incubated with CITase and 20 mM EDC containing 80 mM GEE, the remaining enzymatic activity after 15 min was 70% of the original activity, whereas the activity was only 40% in the absence of CI-8 or IG4. The ability of substrates to protect against inactivation by EDC suggests that the catalytic amino acids of the enzyme were modified by EDC.

When we incubated CITase with 2.8 × 10⁻⁶–7.1 × 10⁻⁴ M substrates in the presence or absence of 5 mM EDC, as described in “Materials and Methods,” GEE was not added to the reaction mixture to avoid masking carboxylic acid irreversibly. The resulting Lineweaver–Burk plots (standard error of each plot, ±5%), shown in Fig. 1C indicated that EDC functioned as a competitive inhibitor of T-3040 CITase with a Kᵢ value of 5.61 mM (standard deviation = ±5%). EDC is a “bi-functional” chemical acting as a chemical modification reagent as well as a competitive inhibitor. Generally, the competitive inhibitor prevents the chemical modification reagent from binding to a catalytic amino acid. It is thought that the EDC-mediated inactivation mechanism is a combination of the reactions of modification and protection that occur simultaneously. When one molecule of EDC covers an active site (as a simple competitive inhibitor), it protects another EDC molecule (acting as a modification reagent) from attacking the
amino acid in the active site, resulting in a reduction of inactivation. The typical stoichiometrical inactivation
of “1:0.45 mol/mol ratio of enzyme and EDC” was estimated from the slope (n = 0.45) in the inlet panel of
Fig. 1A. This gradual slope, less than 1.0, is caused by a decrease in the inactivation velocity due to the self-
protecting function of EDC.

The findings above-mentioned indicate that EDC modified the carboxylic residues of aspartate and/or glutamate, which might play an important role in the CITase-catalyzing formation of cyclodextran.

Selection of the positions for mutagenesis and mutant constructions

When a fluorescent reagent, N-(1-naphthyl)-ethylenediamine, was used as a nucleophile instead of GEE, incorporation of fluorescence into the protein was observed. To determine the location of the carboxylic amino acids modified, the labeled CITase was digested with lysyl endopeptidase or V8 protease and the peptides were separated by HPLC, but no peptide peak with high fluorescent intensity was obtained. Site-directed mutagenesis was then used to investigate the essential fluorescent intensity was obtained. Site-directed muta-
tions were separated by HPLC, but no peptide peak with high fluorescent intensity was observed. To determine the location of the carboxylic amino acids (Fig. 2), Asp265 and Asp270 showed that three aspartic acids (Asp145, Asp265, and Asp270) in T-3040 mature CITase were conserved
in all family 66 enzymes. To determine the catalytic amino acids, we replaced each of these Asp residues with lysyl endopeptidase or V8 protease and the peptides were separated by HPLC, but no peptide peak with high fluorescent intensity was obtained. Site-directed muta-
genesis was then used to investigate the essential carboxylic amino acid residues for CITase activity.

CITase belongs to glycoside hydrolase family 66.6) The deduced molecular size of mature T-3040 CITase was 934 amino acids, whereas those of other enzymes vary from 590 to 1,400 amino acids. When we compared the amino acid sequence of T-3040 CITase with those of seven family 66 dextranases13–19) (Fig. 2), we observed the sequence FDGIIHVQNGQR at the catalytic site of T-3040 CITase, which was 40 to 75% identical to the conserved sequences of other family 66 enzymes (see the single-asterisk regions in Fig. 2).7,17) The dextran-
binding domain7) deduced in other family 66 dextran-
ases was also present in T-3040 CITase (see the double-asterisk regions in Fig. 2). Of the family 66 enzymes, however, only Bacillus circulans U-155 CITase (accession no. D88360, unpublished data) was highly similar to T-3040 CITase, with a 66% overall identity in amino acid sequence. In contrast, T-3040 CITase had only a 20 to 30% sequence identity to other family 66 enzymes (Fig. 2), even at their catalytic sites containing N-terminal conserved regions. In addition, CITase has a long insertion between Asn380 and Thr506, which is not present in any known family 66 dextranases.9) Alignment of amino acid sequences showed that three aspartic acids (Asp145, Asp265, and Asp270) in T-3040 mature CITase were conserved carboxylic amino acids (Fig. 2), Asp265 and Asp270 being in the conserved catalytic site of family 66 dextranases. Of the three Asp residues, at positions 380, 385, and 389, in the catalytic site of Streptococcus mutans dextranase, only Asp385 was essential, whereas the others did not affect any enzyme activity.7) Thus Asp270 corresponded to S. mutans Asp385 and Asp145 was conserved in all family 66 enzymes. To determine the catalytic amino acids, we replaced each of these Asp residues with Asn and constructed mutant enzyme plasmids pCIT-D145N and pCIT-D270N.

Both mutant plasmids expressed soluble enzyme frsnes. Although CIT-D145N was successfully puri-

Fig. 1. Inactivation of CITase by EDC.

A, Semilogarithmic plots of remaining CITase activity versus incubation time with various EDC concentrations. The purified CITase (1.43 mU, 1.53 μg protein) was inactivated with the indicated concentrations of EDC (0–20 mM) and 80 mM GEE in 40 mM Na-phosphate buffer (pH 5.8) in 25 μl reaction mixture at 30 °C for 0–15 min. After termination of the reaction, the enzyme was purified, and then the remaining CI synthesis activity was measured, as described in “Materials and Methods.” . EDC 0 mM; ○, 2 mM; ■, 5 mM; □, 10 mM; △, 20 mM. The insert shows the relationship between log [EDC (mM)] and log k, where k is a pseudo-first-order rate constant obtained from the slopes of the plots in panel A. B, Substrate inhibition of EDC-inactivation of CITase. Purified CITase (1.43 mU, 1.53 μg protein) was inactivated with 20 mM EDC and 80 mM GEE in 40 mM Na-phosphate buffer (pH 5.8), in the presence or absence of 7.7 mM CI-8 or 15.0 mM IG4, in 50 μl reaction mixture at 30 °C for 0–16 min. The remaining CI synthesis activity of the modified enzyme was measured as described above. . 0 mM EDC, 15.0 mM IG4; ○, 0 mM EDC, 7.7 mM CI-8; △, 20 mM EDC, 15.0 mM IG4; △, 20 mM EDC, 7.7 mM CI-8; □, EDC 20 mM. C, EDC as competitive inhibitor of the CITase reaction. Purified CITase (1.32 mU, 1.41 μg protein) was incubated at 40 °C in the presence or absence of EDC (final 5 mM), with 2.8 × 10−5–7.1 × 10−4 M dextran T-40 in 50 μl reaction mixture. After the reaction was stopped by boiling, the CI synthesis activity was measured, as described in “Materials and Methods.” ○, 0 mM EDC; ●, 5 mM EDC.
fied by the method used for wild-type CITase, the rapid proteolytic digestion of CIT-D270N after a Resource Q fractionation step precluded its purification by this method. Since the stability of T-3040 CITase has been reported to increase in the presence of Ca\(^{2+}\),\(^{10,20}\) we added 1 mM CaCl\(_2\) to all the purification steps to prevent CITase proteolysis. Both CIT-D145N and CIT-D270N mutant enzymes could be purified to homogeneity, as assessed by SDS–PAGE, and their molecular sizes were the same as that of wild-type recombinant CITase.

Characterization of the mutant enzyme

CIT-D270N was completely inactive and did not produce CIs or other oligosaccharides from dextran. These findings indicate that Asp270 is involved in the catalytic activity of CITase, similarly to that of Streptococcus mutans dextranase. In contrast, the activity of CIT-D145N decreased drastically, but it produced a trace amount of CI. When we compared the kinetic characteristics of wild-type CITase and CIT-D145N, we found that the \(V_{\text{max}}\) value of CIT-D145N (0.941 pmol·mg\(^{-1}\)·min\(^{-1}\)) was about 1% of that of the wild-type enzyme (0.872 pmol·mg\(^{-1}\)·min\(^{-1}\)), whereas the \(K_m\) value of CIT-D145N (6.02 mM) was almost identical to that of the wild-type enzyme (6.30 mM) (Table 1). These results suggest that the aspartic acid residue at position 145 is critical for CI synthesis reactions but does not affect substrate binding or stabilization of the intermediate.

<table>
<thead>
<tr>
<th>CITase</th>
<th>(K_m) (mM)</th>
<th>(V_{\text{max}}) (pmol·mg(^{-1})·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.30 \times 10^{-2}</td>
<td>0.872</td>
</tr>
<tr>
<td>CIT-D145N</td>
<td>6.02 \times 10^{-2}</td>
<td>0.941 \times 10^{-2}</td>
</tr>
<tr>
<td>CIT-D270N</td>
<td>N.D.*</td>
<td>N.D.*</td>
</tr>
</tbody>
</table>

*Not determined. CIT-D270N did not exhibit any activity producing CIs or isomaltooligosaccharides.
The enzymatic activities of CITase are similar to those of CGTase in that, both enzymes catalyze intramolecular transglycosylation as well as three other reactions, disproportionation, coupling, and hydrolysis. Despite their similar activity, however, these enzymes are not similar in amino acid sequence; that is, CGTase belongs to family 13, whereas CITase belongs to family 66. When we compared the amino acid sequences at the catalytic site of family 66 enzymes, we found that those from streptococcal dextranases were highly conserved, whereas dextranases from Bacterioides thetaiotaomicron and Paenibacillus sp. and CITase from Bacillus circulans T-3040 showed less similarity. The amino acid sequence of T-3040 CITase is about 20% identical to those of the streptococcal dextranases and about 30% identical to those of Bacterioides and Paenibacillus dextranases. In addition, Bacterioides and Paenibacillus dextranases are only about 20% identical to those of streptococcal dextranases. However, the enzyme reaction catalyzed by Bacterioides and Penicillium dextranases is the hydrolysis of dextran to produce isomaltooligosaccharides, an activity identical to that of the streptococcal dextranases rather than T-3040 CITase. These results indicate that, for family 66 enzymes, the enzymatic reaction patterns are difficult to predict simply by amino acid sequences.

Modification of CITase with EDC indicated the importance of carboxyl amino acids in ClI synthesis reactions, and site-directed mutagenesis showed that the Asp145 and Asp270 residues of T-3040 CITase were essential for these reactions. Our finding, that the D270N mutant enzyme was completely inactive, suggests that the catalytic site of CITase is most probably the same as that of streptococcal dextranases and that Asp270 plays a role similar to that of the Asp385 of S. mutans dextranase. We also found that Asp145 in T-3040 CITase was important for enzyme activity. Both Asp145 and Asp270 play roles in the catalytic reactions of this enzyme, including hydrolysis, intramolecular transglycosylation, disproportionation, and coupling. These Asp residues are also conserved in all other family 66 enzymes, suggesting their importance in catalytic activity throughout this family of enzymes. The ability of T-3040 CITase to catalyze intramolecular transglycosylation, while other family 66 enzymes catalyze only hydrolysis reactions, requires further investigation of the structure and functions of the regions around the two catalytic aspartic acids and the role of unique long insertion in CITase. Another intramolecular transglycosylation catalyzing enzyme, CTTase, contains two Asp residues and a Glu residue highly conserved in family 13 enzyme (Asp229, Glu257, and Asp328 in Bacillus sp. #1011). In the proposed catalytic mechanism of CGTase, Glu257 is the acid/base catalyst and Asp229 the catalytic nucleophile, and Asp328 stabilizes substrate binding and elevates the pKa of Glu257. It is unclear whether there is a similar mechanism in CITase. Currently we are analyzing the function of Asp265 and Glu342, both of which are conserved in this family of enzymes.

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