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Calcium Ion Contribution to Thermostability of Cyclodextrin Glycosyltransferase Is Closely Related to Calcium-Binding Site CaIII

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ABSTRACT: In the study, we investigated the contribution of Ca^{2+} to the thermostability of α-cyclodextrin glycosyltransferase (α-CGTase) from *Paenibacillus macerans*, which has two calcium-binding sites (CaI and CaII), and β-CGTase from *Bacillus circulans*, which contains an additional calcium-binding site (CaIII), consisting of Ala315 and Asp577. It was found that the contribution of Ca^{2+} to the thermostability of two CGTases displayed a marked difference. Ca^{2+} affected β-CGTase thermostability significantly. After Ca^{2+} was added to β-CGTase solution to a final concentration of 5 mM followed by incubation for 120 min at 60 °C, residual activity of β-CGTase was 88.3%, which was much higher than that without Ca^{2+}. However, Ca^{2+} had a small contribution to α-CGTase thermostability. Furthermore, A315D and D577K mutations at CaIII could significantly change the contribution of Ca^{2+} to β-CGTase thermostability. These results suggested that the contribution of Ca^{2+} to CGTase thermostability was closely related to CaIII.

KEYWORDS: *CGTase, thermostability, site-directed mutagenesis, calcium-binding site, calcium ion*

INTRODUCTION

Thermostability of an enzyme is one of the most important parameters that determines its utility and commercial applicability.[1−3] Thermostable enzymes allow for higher operating temperatures, which can have positive effects on reducing the risk of microbial contamination and influencing the solubility and bioavailability of organic compounds.[4] Other advantages of high process temperatures include greater reactivity, owing to an increase in the diffusion coefficient and a decrease in the viscosity of substrates, greater stability, and increased process yield.[5] Thus, the thermostability of an enzyme is very important for its potential use in biotechnological processes.

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is an important industrial enzyme used to convert starch or starch derivatives into cyclodextrins by intramolecular transglycosylation. CGTase catalyzes three other reactions simultaneously: hydrolysis, disproportionation, and coupling reactions, and the cyclization reaction is the major activity of CGTase.[6] On the basis of the main cyclodextrin product during the preliminary stage of substrate conversion, CGTases have been classified into three main groups: α-, β-, and γ-CGTases.[7]

Cyclodextrins are cyclic oligomers of glucose linked via α(1,4)-glycosidic bonds. The most common types are α-, β-, and γ-cyclodextrin, which consist of six, seven, and eight glucose residues, respectively.[8] These molecules can form inclusion complexes with a variety of small hydrophobic guest molecules, altering their physical and chemical properties. Because of these features, cyclodextrins have numerous potential applications in the food, agriculture, cosmetic, pharmaceutical, and chemical industries.[9−11] During the industrial production of cyclodextrins, the operating temperature of the enzymatic conversion is normally 60−65 °C, which requires the CGTases to have excellent stability at these temperatures. Therefore, it is important to establish the thermostability of CGTases with potential industrial roles.

The thermostability of an enzyme depends upon various intrinsic (e.g., amino acid composition) and extrinsic (e.g., metal ions, presence of cofactors, pH, etc.) factors.[12,13] All known calcium ions have a key role in promoting enzyme activity and stability, especially for α-amylase,[1] which might relate to the presence of calcium-binding sites in this group of enzymes.[14] CGTase is a member of α-amylase family 13; these enzymes use an α-retaining mechanism to catalyze a variety of reactions.[15] CGTases and α-amylases have one or more calcium-binding sites composed of different amino acid residues.[14,16] Many site-directed mutations at calcium-binding sites affecting α-amylase thermostability and catalytic activity have been made.[17,18] Recently, the thermostability of *Bacillus* sp. G1 CGTase was enhanced by rational mutagenesis at the calcium-binding sites.[16] To the best of our knowledge, this is the only published work on mutating CGTase at calcium-binding sites to improve thermostability. Comparative studies related to calcium ions and the contribution of calcium ions to the thermostability of (mutant) CGTases from different strains are scarce.

Our earlier work found that CGTases from *Paenibacillus macerans* JFB05-01 and *Bacillus circulans* STB01 had relatively low thermostability (half-lives of about 10 min at 60 °C),[19] which severely limit their industrial application. In this study, these two enzymes were used for comparative studies of thermostability by adding different concentrations of calcium ions and the relative mechanisms were analyzed. Because the structural basis for thermostability of CGTases is not well-

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understood, the data presented here provide important information for further studies related to the thermal denaturation mechanism of CGTases and provide an effective approach for improving the thermostability of CGTases or other related enzymes.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* JM109 was used for recombinant DNA manipulations. The *cgt* genes encoding wild-type α-CGTase and β-CGTase were from *P. macerans* JFB05-01 and *B. circulans* STB01, respectively. The α-CGTase was produced with *E. coli* BL21(DE3) harboring plasmid *cgt*/pET-20b(+), while the (mutant) β-CGTases were produced with *Bacillus subtilis* WB600 harboring (mutant) plasmid *cgt*/pST. Plasmid *cgt*/pST was used for site-directed mutagenesis, sequencing, and expression of the (mutant) β-CGTase proteins.

DNA Manipulations and Sequencing. Prime STAR HS DNA polymerase, restriction endonucleases, and polymerase chain reaction (PCR) reagents were purchased from TaKaRa Shuzo (Otsu, Japan) and used following the instructions of the manufacturer. DNA manipulations and calcium chloride transformation of *E. coli* were performed as described.[20] Transformation of *B. subtilis* was performed according to the method by Spizizen.[21] DNA sequences were determined by cycle sequencing with an ABI PRISM BigDye primer cycle sequencing kit with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). Multiple sequence alignment was carried out with the CLUSTAL W program.

Site-Directed Mutagenesis. The CGTase mutants were generated by a one-step PCR method using plasmid *cgt*/pST as the template and a pair of complementary primers (Table 1). After amplification, the PCR products were digested with *Dpn*I and transformed into *E. coli* JM109 cells. The resulting (mutant) plasmids were transformed into *B. subtilis* WB600 competent cells. All of the intended mutations were confirmed by DNA sequencing.

Table 1. Primers Used for Site-Directed Mutagenesis

desired mutation	primer sequence (5′ → 3′)[a]	DNA template
A315D	CAGCCGATTACGACCAGGTGGA	*cgt*/pST
	TCCACCTGGTCGTAATCGGCTG	
D577K	GCAATGTGTATAAGAACTTCGAG	*cgt*/pST
	CTCGAAGTTCTTATACACATTGC	

[a]Nucleotide sequences corresponding to the mutated amino acids are underlined.

Production and Purification of CGTase Proteins. In the case of α-CGTase production, a single colony of *E. coli* BL21(DE3) carrying (mutant) plasmid from *cgt*/pET-20b(+) was inoculated into 50 mL of Luria−Bertani (LB) medium supplemented with 100 μg/mL ampicillin and grown at 37 °C overnight. The overnight culture (2 mL) was then diluted into 50 mL of terrific broth with 100 μg/mL ampicillin, incubated on a rotary shaker (200 rpm) at 25 °C for 36 h, and then continued at 30 °C for another 36 h.

In the case of (mutant) β-CGTase production, a single colony of *B. subtilis* WB600 carrying (mutant) plasmid from *cgt*/pST was inoculated into 50 mL of LB medium containing kanamycin and gibberellin both in 5 μg/mL and grown at 37 °C overnight. The overnight culture (2 mL) was then diluted into 50 mL of terrific broth with 5 μg/mL kanamycin and 10 μg/mL gibberellin and incubated on a rotary shaker (200 rpm) at 37 °C for 48 h.

The extracellular crude enzymes were harvested by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant was collected and loaded onto a Q-Sepharose anion-exchange column (Amersham Biosciences, Piscataway, NJ) using a 0−400 mM KCl gradient in buffer A (20 mM Tris/HCl at pH 7.5). The active fractions were combined and applied to the next step of the purification onto a phenyl-Superose column (HR 10/10) (Amersham Biosciences, Piscataway, NJ), pre-equilibrated with 20% $(NH_4)_2SO_4$ in buffer A. The column was washed with a reverse gradient from 20 to 0% $(NH_4)_2SO_4$ in buffer A at a flow rate of 1 mL/min.[19] The active fractions were pooled and concentrated by ultrafiltration (Amicon, Millipore, Billerica, MA) and then dialyzed against 10 mM phosphate buffer (pH 6.5) at 4 °C for 48 h. During these experiments, an AKTA purifier 10 system (Amersham Biosciences, Uppsala, Sweden) was used with both of the columns. The purity of enzymes was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 10% gel). The purified enzymes were aliquoted and stored at −80 °C.

Protein Concentration Determination. The protein concentration was quantified by the Bradford method,[22] using the Bio-Rad protein assay reagent kit with bovine serum albumin as the standard.

Enzyme Assays. All enzyme assays were performed by incubating 0.1 mL of appropriately diluted purified enzyme with 0.9 mL of 1% (w/v) maltodextrin (DE = 5, Roquette Freres) in 10 mM phosphate buffer (pH 6.5) at 40 and 50 °C for 10 min for α- and β-CGTases, respectively.

The α- and β-cyclization activities were determined by the methyl orange[23,24] and phenolphthalein methods,[25] respectively. One unit of each activity was defined as the amount of enzyme that produced 1 μmol of the corresponding cyclodextrin per minute under the assay conditions.

Thermostability Determination. The thermostability of the α- and (mutant) β-CGTases were determined by incubation of the purified enzymes (~3.2×10^{-8} M) in 10 mM phosphate buffer (pH 6.5) at 60 °C. Samples were taken at several time intervals, and the residual cyclization activity was determined.

Structure Modeling of the CGTases. The three-dimensional (3D) homology model of the CGTases was generated using SWISS-MODEL protein-modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html).[26] The proposed complex structures of the wild-type CGTases were performed using the crystal structure of *B. circulans* strain 251 (PDB accession code 1EO5). The PyMol molecular Graphics System (http://www.pymol.org) was used to visualize and analyze the generated model structure, and the UCSF Chimera 1.6.1 was used to construct graphical presentations and illustrative figures.

RESULTS AND DISCUSSION

Activation of the Cyclization Activity of α- and β-CGTases by Calcium Ions. The presence of calcium ions had a marked effect on the cyclization activity of both CGTases (Figure 1). Wild-type α- and β-CGTases were obtained in completely Ca^{2+}-free forms, and no difference in cyclization activity or thermostability was observed in the presence or absence of ethylenediaminetetraacetic acid (EDTA) after treatment with Chelex 100 (data not shown). α-CGTase cyclization activity was increased gradually with an increased concentration of Ca^{2+}, reaching a maximum of 37.3 units/mL in the presence of 5 mM Ca^{2+} (increase of nearly 15% compared to the absence of Ca^{2+}). Further increases in the concentration of Ca^{2+} to 10 mM did not enhance the α-CGTase cyclization activity, which remained at the maximum. Similar results were found for β-CGTase; the maximum increase of cyclization activity was achieved at 1 mM Ca^{2+} (increase of ~10% compared to the absence of Ca^{2+}).

Calcium ions activate CGTases from different strains, which might be mainly due to an increased affinity of the enzyme toward the substrate for the positioning of Ca^{2+} relative to the binding sites for stabilizing the substrate-binding groove.[27] In addition, binding Ca^{2+} could increase the electrostatic potential of the protein surface for substrate binding at the active site.[14]

Contribution to the Thermostability of Wild-Type α- and β-CGTases by Calcium Ions. We asked whether calcium ions could not only activate CGTases but also enhance their thermostability. Calcium was added to the α- and β-CGTase

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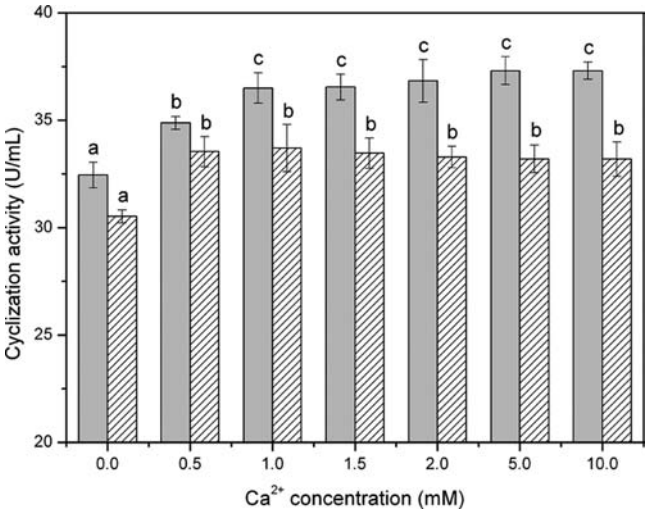

Figure 1. Effects of different concentrations of Ca^{2+} on the cyclization activity of wild-type α- and β-CGTases. The α- and β-cyclization activities of α-CGTase (gray bars) and β-CGTase (hatched bars) were determined after incubation at 4 °C for 1 h with different concentrations of Ca^{2+}. Each value represents the mean of three independent measurements, and bars with different letters on the top are significantly different ($p < 0.05$).

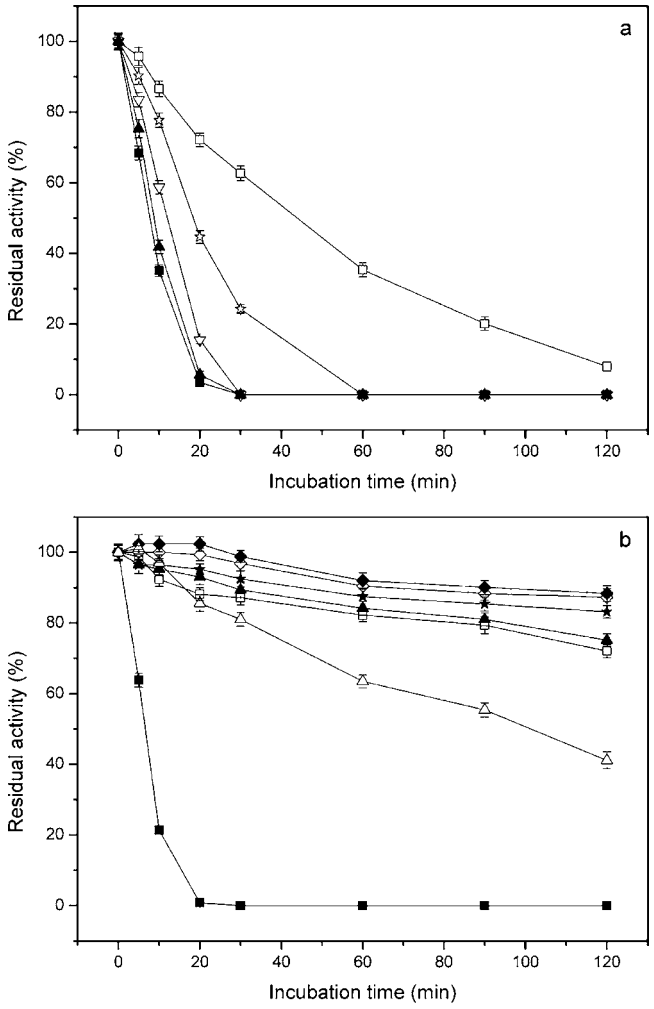

Figure 2. Effects of different concentrations of Ca^{2+} on the thermostability of wild-type (a) α-CGTase and (b) β-CGTase. The native α-CGTases (■) were incubated at 60 °C for up to 120 min with concentrations of Ca^{2+} of (▽) 0.1 mM, (☆) 0.2 mM, (□) 0.5 mM, or (▲) 1 mM. The native β-CGTases (■) were incubated at 60 °C for up to 120 min with concentrations of Ca^{2+} of (□) 0.5 mM, (▲) 1 mM, (★) 1.5 mM, (◇) 2 mM, (◆) 5 mM, or (△) 10 mM. The level of thermostability before incubation was set as 100%. Each value represents the mean of three independent measurements, and the deviation from the mean is <5%.

solutions to different final concentrations followed by incubation at 60 °C for 120 min (Figure 2). The thermostability of wild-type α- and β-CGTases increased in the presence of Ca^{2+}. It was noticeable, however, that the increase was much lower for the α-CGTase compared to the β-CGTase. The α-CGTase (Figure 2a) showed slightly increased thermostability in the presence of 0.1−0.2 mM Ca^{2+} and a maximum increase at 0.5 mM Ca^{2+}, with the half-life prolonged 5.5-fold at 60 °C. A further increase of the concentration of Ca^{2+} to 1 mM decreased the thermostability to the control level. The thermostability of the β-CGTase (Figure 2b) increased as the concentration of Ca^{2+} increased from 0.5 to 5 mM, and the β-cyclodextrin-forming activity after incubation at 60 °C for 120 min was 72.0−88.3% of the untreated enzyme remaining, which is much higher than that in the absence of Ca^{2+}. Any further increase in the concentration of Ca^{2+} resulted in decreased thermostability.

The thermal denaturation of CGTases is accompanied by alteration of the conformation of proteins, which has significant physicochemical consequences.[1] Calcium is essential for maintenance of the structural integrity of α-amylase,[14] and it could be assumed that the increase of thermostability at low concentrations of Ca^{2+} is related to the tight binding of Ca^{2+} at the calcium-binding sites, which might ligate relevant residues and bridge the different parts of the protein, stabilizing the fold. Indeed, the increase of thermostability with increasing Ca^{2+} suggested that the binding was still occurring. When the concentration of Ca^{2+} is high, the ionic strength of the solution might reduce thermostability, owing to extensive non-specific aggregation.[28] Moreover, extensive interaction of Ca^{2+} might screen the negative charge density in the catalytic active sites and result in a slight structural change rendering the enzyme less active.[1] Shielding electrostatic interactions at high concentrations of salt might decrease calcium affinity severalfold.[29]

Structure Comparison of α- and β-CGTases. To determine whether any structural differences might cause the different contributions on thermostability with Ca^{2+}, the structures of α- and β-CGTases were compared. The α-CGTase shared 68% identity with the β-CGTase, and the two CGTases had almost identical active sites, with only one residue different in the 15 active sites. Many α-amylases, including CGTases, have one or more calcium-binding sites.[16] Two calcium-binding sites (CaI and CaII; Figure 3a) have been identified in the *P. macerans* CGTase (Swiss-Prot P04830), and a third calcium-binding site (CaIII; Figure 3b) has been identified in the *B. circulans* CGTase (Swiss-Prot P43379). CaI is located in domain A and contains calcium-interacting residues Asp27, Asn29, Asn32, Asn33, Gly51, and Asp53 (Figure 3c; numbering is based on the parent β-CGTase). CaII is located at the A/B domain interface, containing calcium-interacting residues Asn139, Ile190, Asp199, and His233 (Figure 3d). CaIII, which is uncommon in CGTases, is located at the A/D domain interface, containing calcium-interacting residues Ala315 and Asp577 (Figure 3e). However, their

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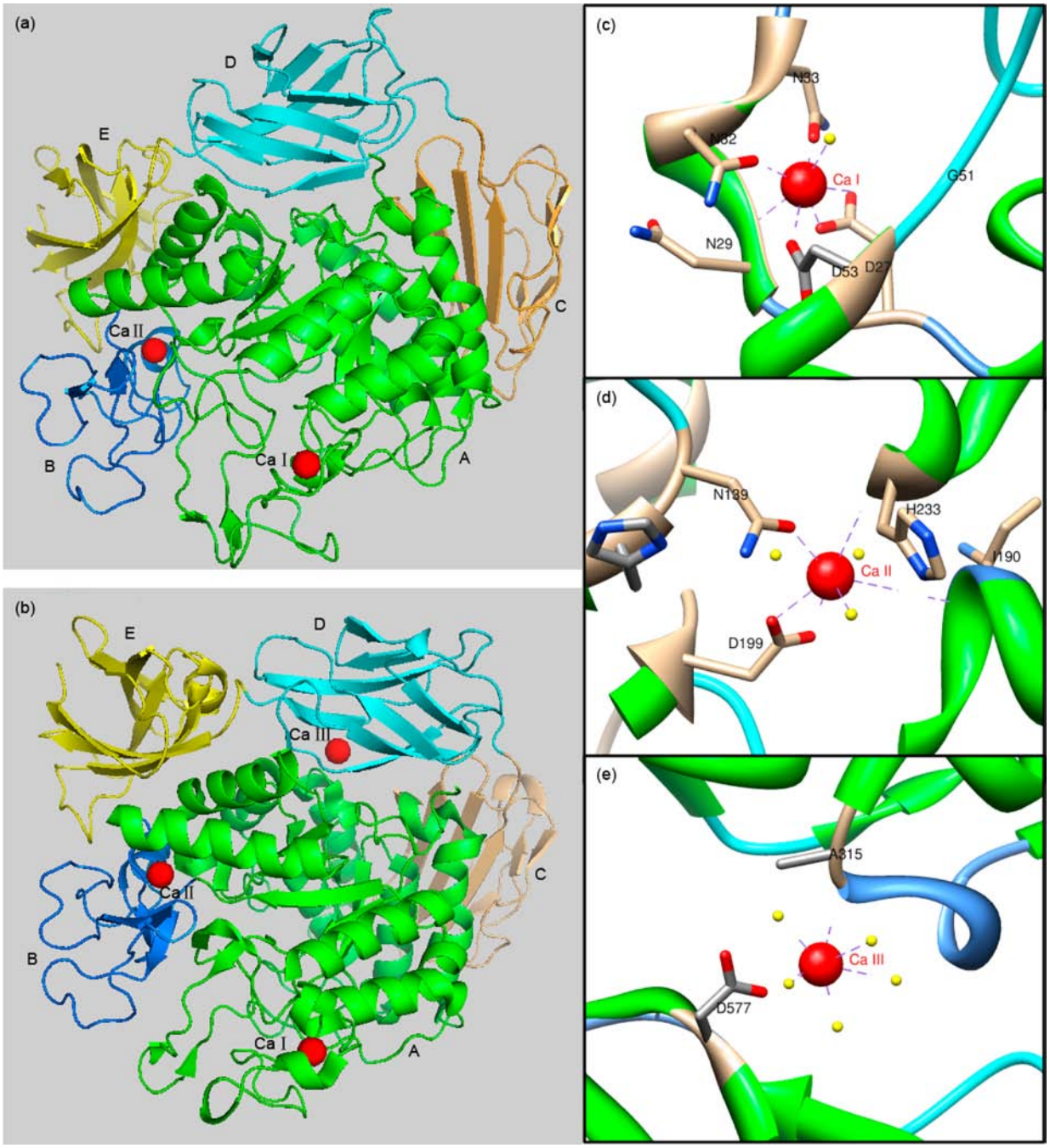

Figure 3. Crystal structure modeling of α- and β-CGTases. A–E, the different CGTase domains. Red ball, Ca^{2+}; yellow ball, water molecule. (a) α-CGTase, (b) β-CGTase, (c) CaI, (d) CaII, and (e) CaIII.

importance for either enzymatic activity or thermostability is not known. Earlier studies showed that domain A possesses a catalytic $(\beta/\alpha)_8$ barrel,[30] and domain D is configured for correct positioning of domain E,[31] which is important for the adsorption of starch granules.[32] Thus, CaIII bridging the two domains is likely to be very important for the correct structural conformation. Sequence alignment with CaIII of β-CGTase reveals that the corresponding residues in α-CGTase are Asp and Lys, respectively. Thus, Ala315 and Asp577 of β-CGTase were replaced by Asp and Lys, respectively, to construct mutants A315D and D577K for further investigating the contribution of CaIII to thermostability of the enzymes with or without the participation of Ca^{2+}.

Thermostability Analysis of Mutants at CaIII of β-CGTase with or without Calcium Ions. Mutants A315D and D577K of β-CGTase were constructed by site-directed mutagenesis via one-step PCR with *cgt*/pST as the DNA template (verified by DNA sequencing). The wild-type and mutant β-CGTases were expressed in *B. subtilis* WB600 and then purified successively.

The effects of different concentrations of Ca^{2+} on thermostability of the mutants A315D and D577K during incubation at 60 °C for 120 min are shown in Figure 4. The thermostability of mutants A315D and D577K was improved significantly compared to the wild type, resulting in 80 and 130% increases in the half-life at 60 °C, respectively. These

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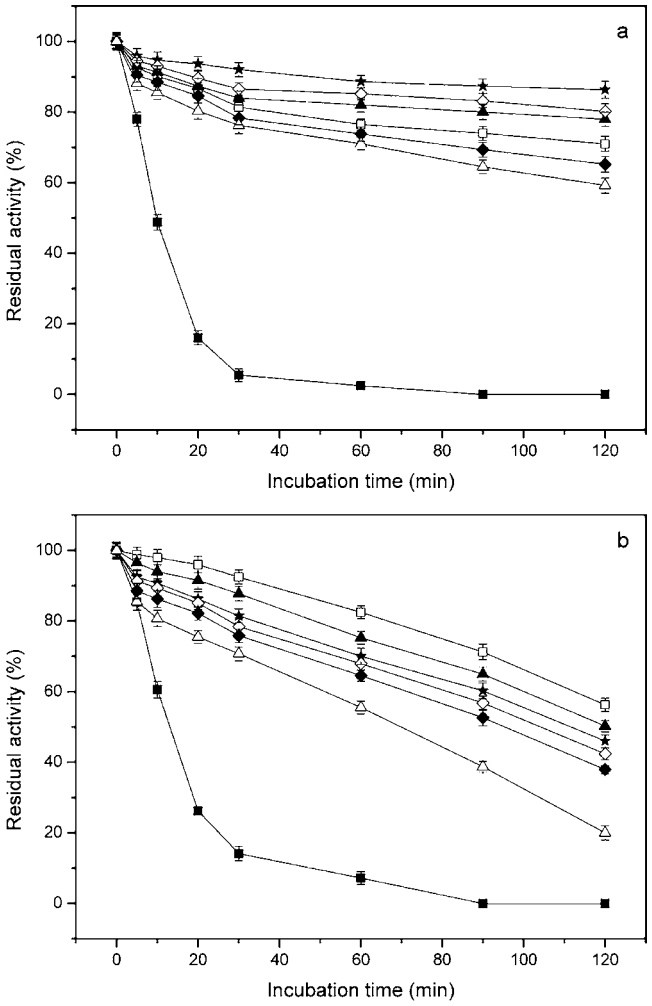

Figure 4. Effect of different concentrations of Ca^{2+} on the thermostability of β-CGTase mutants (a) A315D and (b) D577K. The mutants (■) were incubated at 60 °C up to 120 min with added concentrations of Ca^{2+} of (□) 0.5 mM, (▲) 1 mM, (★) 1.5 mM, (◇) 2 mM, (◆) 5 mM, or (△) 10 mM. The thermostability before incubation was set as 100%. Each value represents the mean of three independent measurements, and the deviation from the mean is <5%.

results indicate that mutants A315D and D577K at the CaIII binding site could enhance the conformational stability of the enzyme, owing mainly to the formation of more hydrogen bonds and increasing electrostatic interactions.

The contribution of Ca^{2+} to the thermostability of mutations A315D and D577K was varied. All concentrations of Ca^{2+} tested improved the thermostability of mutant A315D at 60 °C (Figure 4a). In comparison to the wild-type enzyme, the thermostable effect of Ca^{2+} decreased from a maximum at 5 to 1.5 mM; 86.3% of the β-cyclodextrin-forming activity remained after 120 min of incubation at the same temperature, suggesting that A315D bound to Ca^{2+} more tightly as the microenvironment changed. It appears that subtle steric differences rather than any gross change in structure modulate these differences in calcium affinity and stability. From a structural point of view, Asp appeared to be more important for binding Ca^{2+} than Ala because of the negative charge group (COO^-) on the side chain. Although higher concentrations of Ca^{2+} led to a substantial decrease in thermostability at 60 °C, mutant A315D appeared to be more stable than the wild-type β-CGTase at 10 mM Ca^{2+}, possibly because of the negative charge induced in the mutant and subsequent structural rearrangement.

The overall effects of different concentrations of Ca^{2+} on the thermostability of the D577K mutant were decreased in comparison to the wild-type enzyme and mutant A315D (Figure 4b), and the shift in the curve reflects a change in Ca^{2+} affinity. The optimum concentration of Ca^{2+} for thermostability of the D577K mutant was 0.5 mM, which retained 56.3% of the original activity after incubation at 60 °C for 120 min.

Changes in the structural characteristics of the enzyme in response to Ca^{2+} suggest involvement of intramolecular interactions in the protein structure conferring a more stable conformation. It is known that the presence of metal ions alters the electrostatics of proteins in several ways. There is evidence suggesting that, at higher concentrations, calcium ions bind extensively to the enzyme and occupy sites other than the known calcium-binding sites.[33] Calcium binding is affected by electrostatic forces; in the case of the A315D mutant, introduction of an amino acid with a negative charge group appeared to increase the number of electrostatic interactions and the Ca^{2+} dependence and thermostability of the mutant increased. In the case of the D577K mutant, the Lys side chain contains three more aliphatic carbon atoms than Asp, which might cause steric clashing between the β carbon and the peptide backbone, hindering the combination with Ca^{2+}.[34] In addition, Lys involves a positively charged group in the side chain, which might further restrict the binding of Ca^{2+} with positive charges. In conclusion, calcium-binding site CaIII and the contributions of Ca^{2+} are very important for the thermostability of CGTases.

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Notes

The authors declare no competing financial interest.

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