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Mutations enhance β-cyclodextrin specificity of cyclodextrin glycosyltransferase from *Bacillus circulans*

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ABSTRACT

The effects of amino acid residue at position 31 in the neighborhood of calcium binding site I (CaI) on product specificity of cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) were investigated by replacing Ala31 in the CGTase from *Bacillus circulans* STB01 with arginine, proline, threonine, serine and glycine. The results showed that the mutations A31R, A31P, and A31T resulted in the increases in β-cyclodextrin-forming activity and β-cyclodextrin production, indicating that these mutations enhanced β-cyclodextrin specificity of the CGTase. Especially the mutant A31R displayed approximately 26% increase in β-cyclodextrin production with a concomitant 41% decrease in α-cyclodextrin production when compared to the wild-type CGTase. Thus, it was much more suitable for the industrial production of β-cyclodextrin than the wild-type enzyme. The enhanced β-cyclodextrin specificity of the mutants might be a result of stabilizing CaI, which also suggested that CaI might play an important role in cyclodextrin product specificity of CGTase.

1. Introduction

The cyclodextrins are a family of cyclic oligosaccharides that typically contain 6 (α-cyclodextrin), 7 (β-cyclodextrin), or 8 (γ-cyclodextrin) α-1,4-linked D-glucose molecules (van der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000). They adopt the shape of a hollow truncated cone that has a hydrophilic outside surface and a hydrophobic internal cavity. The hydrophilic outer surface allows the cyclodextrins to dissolve readily in water, while the hydrophobic internal cavity enables them to form inclusion complexes with a variety of hydrophobic guest molecules (Li et al., 2007). Since complex formation can improve the properties of the guest molecule, cyclodextrins have proven useful in a variety of industries, including those related to agriculture and food (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero & Simal-Gandara, 2009; Del Valle, 2004; Ding, Wu, Guo, Yang & Zhang, 2013; Szente & Szejtli, 2004; van der Veen et al., 2000). They are highly recommended as additives to protect lipophilic food components, stabilize flavors or other sensitive ingredients, suppress unpleasant odors or tastes, and achieve controlled release of certain food constituents (Astray et al., 2009; Howard, Brownmiller, Prior & Mauromoustakos, 2013; Leemhuis, Dijkstra & Dijkhuizen, 2002; Marcolino, Zanin, Durrant, Benassi & Matioli, 2011).

Cyclodextrins are commonly produced from starch through an enzymatic process that includes a cyclization reaction catalyzed by cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) (Schoffer Jda, Klein, Rodrigues, & Hertz, 2013; Wang, Wu, Chen & Wu, 2013). A major issue during cyclodextrin production is that all known wild-type CGTases produce a mixture of α-, β-, and γ-cyclodextrin (Kelly, Dijkhuizen & Leemhuis, 2009; Li et al., 2007). These cyclodextrin mixtures can be separated by selective crystallization of β-cyclodextrin or by selective complexation with organic solvents. When performed in concert with cyclodextrin production, these additional processes increase the cost of cyclodextrin production, although resulting in decreased product inhibition and enhanced conversion of starch to cyclodextrin (Li et al., 2007; van der Veen et al., 2000; Wind, Uitdehaag, Buitelaar, Dijkstra & Dijkhuizen, 1998). Moreover, the use of organic solvents also limits the application of cyclodextrins in some fields, such as food and pharmaceuticals (Li, Zhang, Sun, et al., 2009; van der Veen et al., 2000). CGTase mutants that produce a preponderance of one particular type of cyclodextrin would simplify the separation procedure,

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thereby lowering the production cost of cyclodextrin and expanding their application.

The enzyme used in our studies, CGTase from *Bacillus circulans*, produces mainly β-cyclodextrin during the initial stage of starch conversion and thus is identified as a β-CGTase. Although this CGTase can be used in the industrial production of β-cyclodextrin, considerable amounts of α- and γ-cyclodextrin are also produced during the prolonged incubation with starch under conditions resembling industrial production non-solvent process. Thus, mutants of this CGTase capable of producing an increased ratio of β-cyclodextrin are of high industrial interest.

Many efforts have been made to improve CGTase product specificity through site-directed mutagenesis (Costa et al., 2012; Kelly et al., 2009; Leemhuis, Kelly & Dijkhuizen, 2010; Li, Zhang, Sun, et al., 2009; Li, Zhang, Wang, et al., 2009; Nakagawa, Takada, Ogawa, Hatada & Horikoshi, 2006; Uitdehaag, van der Veen, Dijkhuizen & Dijkstra, 2002; van der Veen et al., 2000). Most of these mutations have targeted amino acid residues located in the active site cleft, which contains at least nine sugar-binding subsites numbered −1 to −7 (donor subsites) and +1 and +2 (acceptor subsites) (Costa et al., 2012; Qi & Zimmermann, 2005; Uitdehaag, Kalk, van der Veen, Dijkhuizen & Dijkstra, 1999). However, since CGTases belong to the α-amylase family of glycosyl hydrolases (family 13) (Kumar, 2011; van der Veen et al., 2000), they have two or more calcium binding sites similar to α-amylases (Li, Ban, Gu & Li, 2013; Uitdehaag, Mosi, et al., 1999). Mutation of His233, which is located at a calcium binding site of the CGTase from *Bacillus sp.* 1011, to Asn changed the nature of this CGTase such that it no longer produced α-cyclodextrin (Nakamura, Haga & Yamane, 1993). This suggests that the calcium binding sites may have an important role in determining the product specificity of CGTase, and that the calcium binding sites of the CGTase from *B. circulans* are reasonable targets for our mutagenesis efforts.

The CGTase from *B. circulans* STB01 has three calcium binding sites, referred to as CaI, CaII and CaII (Li et al., 2013; Uitdehaag, van Alebeek, van Der Veen, Dijkhuizen & Dijkstra, 2000). CaI, which is not in the active site cleft, is composed of six amino acid residues: Asp 27, Asn 29, Asn 32, Asn 33, Gly 51 and Asp 53. These residues are partially conserved in the CGTases from different sources (Fig. 1). Interestingly, the identity of the amino acid at position 31 (*B. circulans* CGTase numbering), which is located in the neighborhood of CaI, shows a clear discrimination between CGTases from different sources (Fig. 1).

Structural analysis of the CGTase from *B. circulans*, which has an Ala at position 31, showed a hydrogen-bonding interaction between Asn 29 and Ala31 (Fig. 2), suggesting that Ala31 might help stabilize the CaI. In light of these observations, we formed the hypothesis that the identity of amino acid residue at position 31 might affect the cyclodextrin product specificity of the CGTase. Therefore, we performed site-directed mutagenesis on Ala31 of the CGTase from *B. circulans* STB01 and investigated the effects of amino acid side chains at position 31 on its cyclodextrin product specificity. The relative mechanisms were also analyzed.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli JM109 [F′ (traD36, *pro*AB+ *lac*I^q, Δ(*lac*Z)M15) *end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *mcr*A *sup*E44 λ$^-$ *gyr*A96 *rel*A1 Δ(*lac-pro*AB) *thi*-1] (Yanisch-Perron, Vieira & Messing, 1985) was used for recombinant DNA manipulations. The *cgt* gene encoding wild-type CGTase was from *B. circulans* STB01. Plasmid *cgt*/pST was constructed for site-directed mutagenesis, sequencing, and expression

	Bacterial source	Partial sequence 27-34	Partial sequence 51-55	Accession No.
α-CGTase	*P. macerans*	DGDR7**N**NP	GGDWQ	P04830
	Bacillus macerans	DGN*S***A**NNP	GGDWQ	P31835
	Klebsiella pneumoniae M5a1	DGDP*S***N**NA	GGDLR	P08704
	Thermococcus sp. B1001	DGNT*S***N**NK	GGDLE	BAB18101
α/β-CGTase	*T. thermosulfurigenes* EM1	DGNT*S***N**NP	GGDWQ	AAB00845
	B. licheniformis	DGNP*S***N**NP	GGDWQ	P14014
	B. circulans 8	DGNP*S***N**NP	GGDWQ	P30920
	Bacillus sp. 6.6.3	DGNP*S***N**NP	GGDWQ	CAA46901
β-CGTase	Alkalophilic *B. sp.* 38-2	DGNP*A***N**NP	GGDWQ	P09121
	Bacillus sp. B1018	DGNP*A***N**NP	GGDWQ	P17692
	Alkalophilic *B. sp.* 1011	DGNP*A***N**NP	GGDWQ	P05618
	B. circulans 251	DGNP*A***N**NP	GGDWQ	P43379
β/γ-CGTase	*Bacillus sp.* KC201	DGNP*G***N**NP	GGDWQ	BAA02380
	Alkalophilic *B. sp.* N 1.1	DGNP*G***N**NP	GGDWQ	P31746
	Brevibacillus brevis CD162	DGNP*A***N**NP	GGDWQ	AAB65420
γ-CGTase	*B. clarkii* 7364	DGDP*D***N**NP	GGDWQ	BAB91217
	Bacillus sp. G-825-6	DGDP7**N**NP	GGDWQ	BAE87038

Fig. 1. Multiple sequence alignment of the region around calcium binding site I in the CGTases from different sources. Amino acid residues at calcium binding site I are shown in bold. The position of residue 31 is shown in italics. Two cyclodextrins are indicated in those cases where both cyclodextrins were formed in comparable amounts, but with (slight) preference for the first one mentioned.

of the (mutant) CGTase proteins (Li et al., 2013). The mutant β-CGTases were produced in *B. subtilis* WB600 [*trp*C2 *npr*E *apr*E *epr bpr mpr npr*B; Em^r] (Wu, Lee, Tran & Wong, 1991) harboring mutant plasmid *cgt*/pST.

2.2. DNA manipulation and sequencing

Prime STAR HS DNA polymerase, restriction endonucleases and PCR reagents were purchased from TaKaRa Shuzo (Otsu, Japan) and used according to the manufacturer's instructions. DNA manipulations and calcium chloride transformation of *E. coli* were performed as previously described (Sambrook, Russell & Irwin, 2001). Transformation of *B. subtilis* was performed according to the method of Spizizen (Spizizen, 1958). DNA sequences were determined by cycle sequencing with an ABI PRISM BigDye primer cycle sequencing kit and AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA, USA).

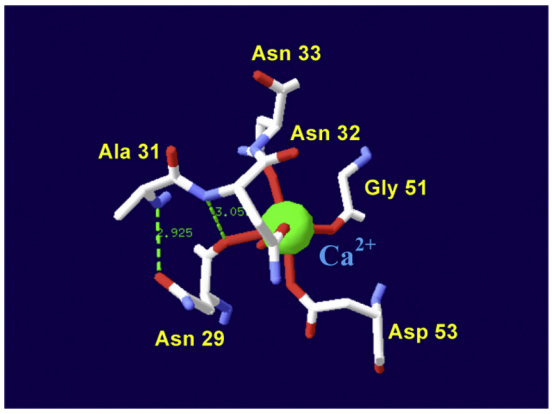

Fig. 2. The conformations of amino acid side chains at calcium binding site CaI in the X-ray crystal structure of wild-type CGTase from *B. circulans*. The structure was obtained from the RCSB Protein Data Bank (PDB accession code 1CXI). Ala31 is located in the neighborhood of CaI.

Table 1
Primers used for site-directed mutagenesis.

Desired mutation	Primer sequence (5′–3′)[a]
A31R	GACGGCAATCCCCGCAACAATCC
	GGATTGTTGCGGGGATTGCCGTC
A31P	GACGGCAATCCCCCCAACAATCC
	GGATTGTTGGGGGGATTGCCGTC
A31T	GACGGCAATCCCACCAACAATCC
	GGATTGTTGGTGGGATTGCCGTC
A31S	GACGGCAATCCCAGCAACAATCC
	GGATTGTTGCTGGGATTGCCGTC
A31G	GACGGCAATCCCGGCAACAATCC
	GGATTGTTGCCGGGATTGCCGTC

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

2.3. Site-directed mutagenesis

The CGTase mutants were generated using a one-step PCR method with plasmid *cgt*/pST as the template and a pair of complementary primers (Table 1). After amplification, the PCR reaction mixtures were treated with *Dpn* I to completely digest the template and then transformed into *E. coli* JM109 cells. All the intended mutations were confirmed by DNA sequencing. The plasmids were transformed into *B. subtilis* WB600 competent cells for expression studies.

2.4. CGTase production and purification

A single colony of *B. subtilis* WB600 harboring *cgt*/pST (wild-type or mutant) was inoculated into 50 mL of LB medium containing 5 μg/mL kanamycin and grown at 37 °C overnight. A 2 mL portion of this overnight culture was diluted into 50 mL of terrific broth containing 5 μg/mL kanamycin and incubated on a rotary shaker (200 rpm) at 37 °C for 48 h. The CGTase present in the supernatant was purified to homogeneity, as previously described (Li et al., 2013). The purified enzymes were aliquoted and stored at −80 °C.

2.5. Protein concentration determination

Protein concentration was determined by the Bradford method (Bradford, 1976), using the Bio-Rad protein assay reagent kit and bovine serum albumin as the standard.

2.6. CGTase assays

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 frères, Lestrem, FR) in 10 mM phosphate buffer (pH 6.5) at 50 °C for 10 min. α-, β-, and γ-Cyclodextrin-forming activities were determined by the methyl orange (Lejeune, Sakaguchi & Imanaka, 1989), phenolphthalein (Makela, Korpela & Laakso, 1987), and bromocresol green (Kato & Horikoshi, 1984) methods, respectively. One unit of each activity was defined as the amount of enzyme needed to produce 1 μmol of the corresponding cyclodextrin per min.

2.7. HPLC product analysis

The formation of cyclodextrins under conditions resembling an industrial production process was measured by incubating 1 unit/mL CGTase (total cyclization activity) with 5% (wet basis, w/v) maltodextrin solution in 10 mM phosphate buffer (pH 6.5) at 50 °C for 10 min. At regular time intervals, samples were taken and boiled for 10 min to terminate the reaction. To eliminate contaminating oligosaccharides, glucoamylase was added to the boiled sample at a final concentration of 1 unit/mL, and then the mixture was incubated at 30 °C for 2 h, followed by boiling for 10 min. The concentrations of α-, β-, and γ-cyclodextrin in the final sample were determined by HPLC on a Lichrosorb NH_2 column (Merck, Darmstadt, FRG) eluted with acetonitrile/water (65:45) at a flow rate of 1 mL/min. Products were detected using a Waters 410 refractive index detector (Waters Corp., Milford, MA).

3. Results

3.1. Preparation of site-directed mutants at Ala31

To test our hypothesis that mutations at position 31 of the CGTase from *B. circulans* STB01 would affect the enzyme's cyclodextrin product specificity, we prepared the wild-type CGTase and five mutants: A31G, A31S, A31T, A31P and A31R. The mutants were constructed by PCR-mediated site-directed mutagenesis and verified by DNA sequencing. The wild-type and mutant enzymes were expressed in *B. subtilis* WB600 as excreted proteins. There was no significant difference in the expression levels between the recombinant wild-type and the mutant CGTases, as 20–25 mg of each of the CGTase proteins were produced per liter in shake flask cultures. The CGTase variants were purified to apparent homogeneity using a combination of anion exchange and hydrophobic chromatographies. The purity and molecular weight of the wild-type and mutant CGTase proteins were verified using SDS-polyacrylamide gel electrophoresis (data not shown).

3.2. Cyclization activities of wild-type and mutant CGTases

The cyclodextrin-forming activities of the wild-type and mutant CGTases, measured during the initial stages of cyclodextrin production (10 min at 50 °C), are shown in Table 2. The A31R, A31P, A31T and A31G mutants showed overall activity nearly identical to that of wild type (260–270 U/mg), while the activity of the A31S mutant was substantially decreased. The relative proportions of α-, β-, and γ-cyclodextrins produced in the initial stages were also affected by these mutations. The A31R and A31P mutants produced a meaningfully higher proportion of β-cyclodextrin and a slight increase in γ-cyclodextrin at the expense of α-cyclodextrin production. Compared to the wild-type enzyme, the mutations A31R and A31P resulted in approximately 25% and 12% increases in β-cyclodextrin-forming activity, respectively. In contrast, the mutant A31T showed a modest decrease in β-cyclodextrin-forming activity, while the mutant A31G had only slight effects on the cyclodextrin-forming activities. Finally, the less active mutant A31S increased the proportion of α-cyclodextrin (by approximately 60%) at the expense of both β- and γ-cyclodextrin production.

3.3. Cyclodextrin product ratios of the wild-type and mutant CGTases under conditions resembling industrial production processes

The production of α-, β- and γ-cyclodextrins was analyzed at regular intervals under conditions that resemble an industrial process—5% (w/v, wet basis) maltodextrin at 50 °C. The results are showed graphically in Fig. 3, and the data at the final time point (9 h) are presented in Table 3. Compared with the wild-type enzyme (Fig. 3A), the mutant A31S (Fig. 3E) converted substantially less of the substrate maltodextrin to cyclodextrin products, and produced a more balanced product profile. The A31G mutant (Fig. 3F) was very similar to the wild-type enzyme in product profile, although it converted somewhat more of the substrate to cyclodextrins (Table 3). The A31R, A31P and A31T mutants (Fig. 3B–D), however, not only converted somewhat more of the substrate to cyclodextrins, they produced higher β:α and β:γ ratios than the wild-type enzyme (Table 3). Especially, the β:α ratios were

Table 2
Cyclization activities of the wild-type and mutant CGTases from *B. circulans* STB01.[a]

CGTase	α-Cyclodextrin-forming activity (U/mg)	β-Cyclodextrin-forming activity (U/mg)	γ-Cyclodextrin-forming activity (U/mg)	Total activity (U/mg)
Wild-type	69.4 ± 1.1 (26.0)	159.9 ± 2.5 (59.8)	38.1 ± 1.0 (14.2)	267.4
A31R	20.5 ± 0.4 (7.7)	199.6 ± 2.8 (74.9)	46.4 ± 0.9 (17.4)	266.5
A31P	40.6 ± 0.5 (15.4)	179.7 ± 2.2 (68.4)	42.6 ± 0.8 (16.2)	262.9
A31T	55.4 ± 0.5 (20.8)	169.8 ± 2.4 (63.8)	40.9 ± 0.7 (15.4)	266.1
A31S	91.2 ± 0.8 (41.8)	102.7 ± 1.9 (47.0)	24.5 ± 0.3 (11.2)	218.6
A31G	63.5 ± 0.7 (24.4)	161.1 ± 2.0 (61.9)	35.7 ± 0.4 (13.7)	260.3

[a] Each value represents the mean of three independent measurements. Numbers between brackets indicate the ratio in specific activities for the formation of the different cyclodextrins.

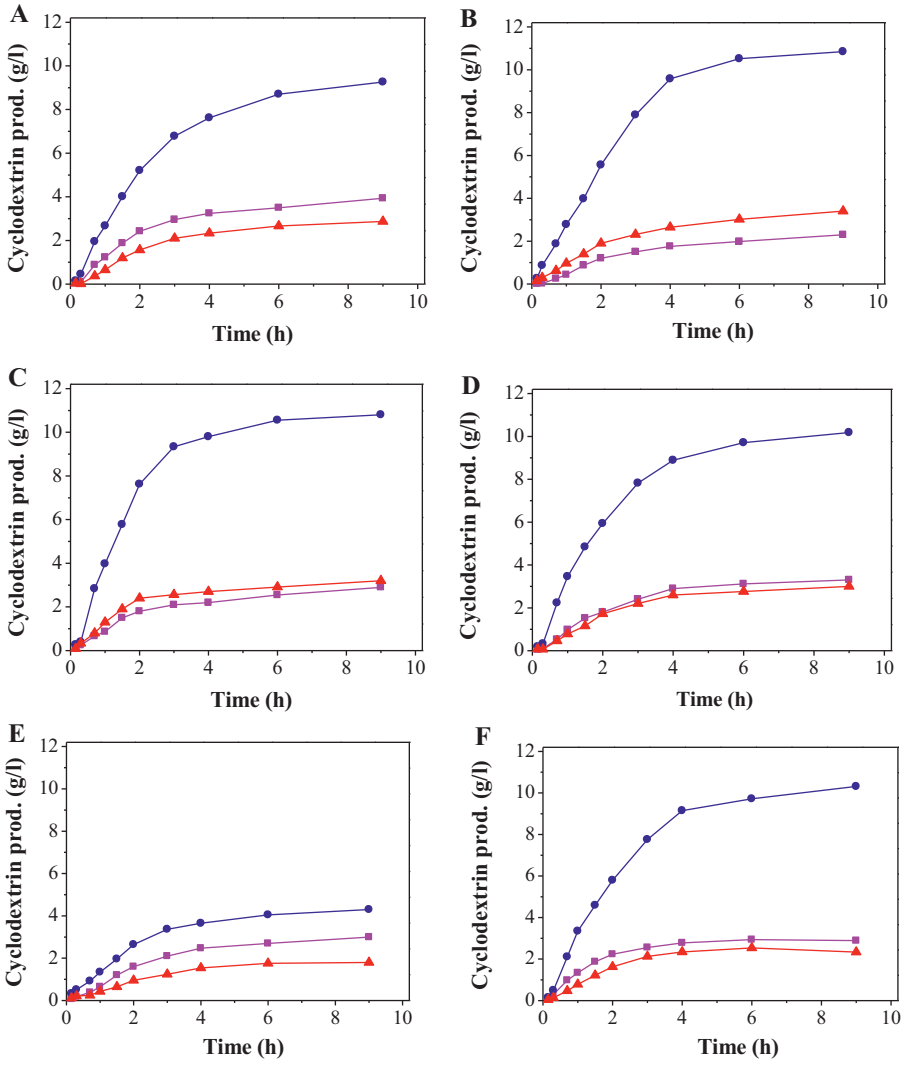

Fig. 3. Cyclodextrins formed during incubation of a (mutant) CGTase from *B. circulans* strain STB01 (1 unit/ml total cyclization activity) with 5% (w/v, wet basis) maltodextrin (DE 3) solution at pH 6.0 and 50 °C for 9 h. Each value represents the mean of three independent measurements. ■, α-cyclodextrin; ●, β-cyclodextrin; ▲, γ-cyclodextrin. (A) wild-type CGTase; (B) mutant A31R; (C) mutant A31P; (D) mutant A31T; (E) mutant A31S; and (F) mutant A31G.

Table 3
Starch conversion of the wild-type and mutant CGTases from *B. circulans* STB01.[a]

CGTase	Conversion of starch into cyclodextrins (%)	Products (g/l)			Ratios	
		α	β	γ	β:α	β:γ
Wild type	35.0	3.9 ± 0.2 (24)	9.3 ± 0.1 (58)	2.9 ± 0.1 (18)	2.4	3.2
A31R	37.8	2.3 ± 0.1 (13)	11.7 ± 0.2 (67)	3.4 ± 0.1 (20)	5.1	3.4
A31P	36.7	2.9 ± 0.1 (17)	10.8 ± 0.2 (64)	3.2 ± 0.1 (19)	3.7	3.4
A31T	35.9	3.3 ± 0.1 (20)	10.2 ± 0.1 (62)	3.0 ± 0.2 (18)	3.1	3.4
A31S	19.7	3.0 ± 0.1 (33)	4.3 ± 0.1 (47)	1.8 ± 0.1 (20)	1.4	2.4
A31G	37.8	4.3 ± 0.2 (25)	10.1 ± 0.1 (58)	3.0 ± 0.1 (17)	2.3	3.4

[a] CGTase proteins (1 units/ml total cyclization activity) were incubated with 5% (w/v, wet basis) maltodextrin (DE 5) solution at pH 6.0 and 50 °C for 9 h. Each value represents the mean of three independent measurements. Numbers between brackets indicate the percentage of each cyclodextrin present in the product mixture.

2.1-, 1.5-, and 1.3-fold of that of the wild-type enzyme, respectively. Both their conversion efficiencies and preferences for the production of β-cyclodextrin followed the order A31T < A31P < A31R. For the mutant A31R, after 9 h incubation, the amount of β-cyclodextrin in the reaction mixture was approximately 26% higher than that of the wild-type enzyme, with a concomitant approximately 41% decrease in the amount of α-cyclodextrin when compared to the wild-type CGTase.

4. Discussion

Biochemical studies have revealed three different calcium ion binding sites in the CGTase from *B. circulans* STB01. One of calcium ions binds at the active site, and the other two are outside of the active center. Many of the amino acids within these calcium binding regions are conserved in CGTases. The calcium binding region we chose to study is located in domain A1 and contains 6 residues. This calcium binding site (CaI) is connected to residues that form a stretch of loops and short alpha helices that resemble a distorted U-shape. Ala31 is very near to a calcium-interacting residue (Asn 29) (Fig. 2), on the top of the U-shape. Sequence comparisons (Fig. 1) suggest that the identity of residue 31 influences cyclodextrin product discrimination, and led us to formulate the hypothesis that mutagenesis at this residue would produce enzymes with altered cyclodextrin product specificity. Our results support this hypothesis, demonstrating that cyclodextrin product profile changes do occur upon mutation of Ala31.

Generally, mutations that alter cyclodextrin-forming activities may alter product inhibition, and may also affect the other activities of the enzyme: coupling, disproportionation and hydrolysis. The production of cyclodextrins results from a combination of these CGTase activities. With prolonged incubation time, the undesired reactions (coupling, disproportionation and hydrolysis) become more significant. Therefore, one might think that changes in product inhibition and coupling activities might be partially responsible for the change of proportion of cyclodextrins in this study. However, the conversion of the substrate to cyclodextrins is unchanged by the mutations we studied, suggesting that the final ratio of cyclodextrins is directly related to cyclodextrin-forming activities of CGTase.

The replacement of Ala31 by a hydrophilic threonine residue, a conformationally restrictive proline residue, or a large cationic arginine residue all led to increase in β- and γ-cyclodextrin-forming activities at the expense of the formation of α-cyclodextrin. The calcium binding site upon which we focused is approximately 20 Å from the site at which the cyclization reaction occurs (Fig. 4), so it is unlikely that these mutations affect the cyclization process directly. It seems more likely that these mutations alter the conformation, or the conformational rigidity of the loops and helices that intervene between CaI and the active center. This change in conformation or flexibility increases the enzyme's ability to form larger cyclodextrins, shifting the product specificity toward β- and γ-cyclodextrins. It should be noted that proline and arginine are not found in the CGTases listed in Fig. 1; they are unnatural substitutions. A threonine substitution is found in the α-CGTase of *Paenibacillus macerans* and the β/γ-CGTase of *Bacillus sp.* G-825-6. However, in each case, the threonine occurs in combination with other changes from the *B. circulans* sequence, making the contribution of the threonine impossible to predict.

In contrast, the A31S mutation has an affect that is dramatically different than its close analog A31T. Its lower cyclodextrin-forming activity suggests that A31S adopts an alternate conformation or possesses a distinct activity that disfavors the production of all cyclodextrins, and skews the product distribution toward α-cyclodextrin. This conformation is apparently not a preferred

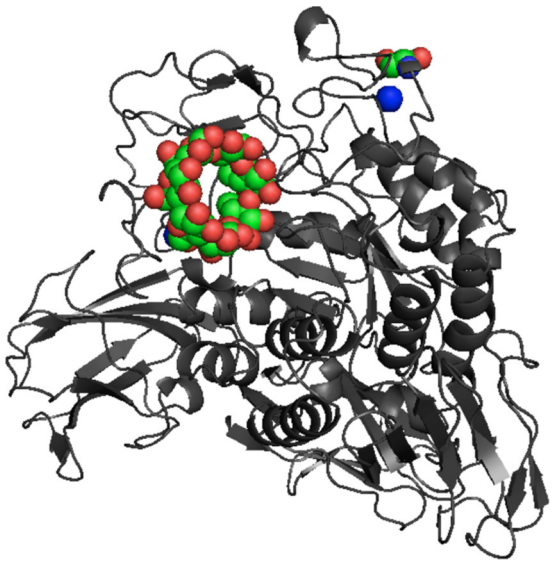

Fig. 4. Rendering of the X-ray crystal structure of the CGTase from *B. circulans* with a bound molecule of β-cyclodextrin. The structure was obtained from the RCSB Protein Data Bank (PDB accession code 3CGT). The protein is displayed as a gray cartoon, with the calcium ion of calcium binding site I shown as a large blue sphere. Residue Ala31 is rendered and the bound molecule of β-cyclodextrin are also rendered as spheres; carbon is rendered in green, oxygen in red, and nitrogen in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

conformation for A31T. This alternate conformation/activity is apparently also not preferred by the mutant A31G, which displays activity that is essentially identical to the activity of the wild-type enzyme. This evidence suggests that it is the presence of the hydroxyl group the serine at position 31, and not the absence of a methyl group, that makes this alternate conformation/activity accessible. This interpretation requires that the methyl group of the threonine at position 31 adopts a conformation similar to the methyl group of the wild-type alanine and prevents the threonine hydroxyl group from forming whatever interaction causes the serine to compromise cyclization activity. The nature of this interaction is not clear, but it may involve nearby Asn 29 and affect the geometry or stability of the calcium binding site. In contrast to the arginine, proline and threonine cases, the serine and glycine mutants have direct precedents in our sequence comparison. Our results with the serine mutant are consistent with the α-cyclodextrin preference of serine-containing CGTases. Our glycine mutant, however, does not noticeably increase the proportion of γ-cyclodextrin in the product mixture, as the sequence comparison suggests it might. This inability to accurately predict the effect of a single mutation on the cyclodextrin product ratio supports our approach of targeting residues that show different preferences, but including a wide range of residues in the mutagenesis strategy.

In summary, residue 31, which occupies a position on the top of U-shaped calcium binding assembly, plays an important role in cyclodextrin product specificity. Four mutations increased β-cyclodextrin forming activity in the order A31R > A31P > A31T > A31G, and the A31S mutation increased α-cyclodextrin specificity. Furthermore, under conditions resembling an industrial production processes, the A31R, A31P and A31T mutations displayed increased β-cyclodextrin product specificity. The A31S mutation decreased the total conversion of starch into cyclodextrins, but displayed enhanced α-cyclodextrin specificity. The enhancement of β-cyclodextrin specificity might be a result of stabilizing CaI, while the enhancement of α-cyclodextrin specificity might result from altering the conformation of the calcium

binding site. These experiments provided evidence that CaI, though somewhat removed from the site at which chemistry occurs, might play an important role in cyclodextrin product specificity of CGTase. They also suggest that the mutation A31R may enhance the industrial utility of the CGTase from *B. circulans* STB01 in β-cyclodextrin production.

Acknowledgments

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