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Site-Directed Mutations in Alanine 223 and Glycine 255 in the Acceptor Site of γ-Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus clarkii* 7364 Affect Cyclodextrin Production

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A cyclodextrin glucanotransferase (CGTase) from *Bacillus clarkii* 7364 converts starch into γ-cyclodextrin (γ-CD) with high specificity. Comparison of the deduced amino acid sequence of this CGTase with those of other typical CGTases revealed that several amino acids are deleted or substituted with others at several subsites. Of these amino acids, Ala223 at subsite +2 and Gly255 at subsite +3 in the acceptor site of the enzyme were replaced by several amino acids through site-directed mutagenesis. The replacement of Ala223 by lysine, arginine and histidine strongly enhanced the γ-CD–forming activity in the neutral pH range. On the other hand, all mutants obtained on replacing Gly255 with the above amino acids showed significant decreases in the γ-CD–forming activity. Taking into account both the kinetic parameters and pK_a values of the side chains of the three basic amino acids, the protonation state of the amino groups in their side chains at subsite +2 seems to enhance the hydrogen bonding interaction between these basic amino acids and the glucose residues of linear oligosaccharides. The enhancement of the interaction may play an important role by helping the substrate reach subsite +1, hence increasing the γ-CD–forming activity and k_{cat} value.

Key words: acceptor site, cyclodextrin, γ-CD–forming activity, γ-CGTase, site-directed mutagenesis.

Abbreviations: CD, cyclodextrin; CGTase, cyclodextrin glucanotransferase; α-, β- and γ-CGTase, CGTase that produces α-, β- and γ-CD from starch as a major product, respectively.

Cyclodextrin (CD) has a closed ring structure consisting of D-glucose residues linked through α-(1,4)-linkages. The most common CDs consist of six, seven and eight glucose residues, and are named α-, β- and γ-CD, respectively. The structure of CDs has a hydrophilic outer surface and a relatively hydrophobic internal cavity. CDs are able to form complexes with various molecules, therefore there have been extensive applications of CDs in the food, cosmetic, agricultural and pharmaceutical industries (*1*). CDs are formed by cyclodextrin glucanotransferase [CGTase; EC 2.4.1.19] from starch and related α-(1,4)–linked glucose polymers through the transglucosylation reaction (*2*). These CGTases are classified as α-, β- and γ-CGTases according to the major CD product. Since the separation of one type CD from a mixture of CDs is costly and time-consuming, there is a need for a CGTase that predominantly produces one type of CD (*3*, *4*). Both α- and β-CD can be enzymatically produced on an industrial scale by using the CGTases from *Bacillus macerans* (*5*) and *Bacillus* sp. strain No. 38-2 (*6*). These CGTases specifically convert starch into α- or β-CD as the major product but still give mixtures of CDs in different ratios. Although there is a strong demand for γ-CD that can trap larger molecules that cannot be trapped by α- or β-CD, the industrial production of γ-CD is not yet practical due to the low yield of this substance even with use of the current CGTases (*2*, *7*). Several attempts have been made to change the properties of CGTases and to enhance the γ-CD yield, but no satisfactory results have been obtained so far (*8*, *9*).

We recently reported a novel CGTase from an alkalophilic bacterium, *Bacillus clarkii* 7364, which converts pre-gelatinized potato starch into γ-CD with high specificity (*10*). Comparison of the deduced amino acid sequence of our CGTase with those of other typical CGTases revealed that several amino acids forming subsites in the substrate binding groove (*2*, *10–14*) are deleted or substituted with other amino acids at subsites +3, +2, –3 and –7 (Table 1). Ala223 (*B. clarkii* 7364 CGTase numbering) at subsite +2 and Gly255 at subsite +3 are conserved in both β/γ- and γ-CGTase, whereas these amino acids are replaced by lysine and glutamic acid in both α- and β-CGTase, respectively. Lysine at subsite +2 and glutamic acid at subsite +3 play important roles in the structural integrity of the active site and the disproportionation reaction, respectively (*15*). Since the amino acids at subsites +2 and +3 in both β/γ- and γ-CGTase are hydrophobic, there might be few, if any, hydrogen-bonding interactions between these amino acids and glucose residues, and the enzyme reaction might be affected.

In this study, we focused on the hydrophobic amino acids of our γ-CGTase from *B. clarkii* 7364 at subsites +2 and

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Table 1. **Comparison of the amino acid residues around the active center in the four types of CGTase.**

Residue No.	Residue in α-CGTase	Residue in β-CGTase	Residue in β/γ- and γ-CGTase[a]	Residue in γ-CGTase[b]	Function in CGTase
Subsite +3					Acceptor
255	E	E	G	G	Disp.[c]
Subsite +2					Acceptor
174	F	F	F	F	Cycl.[c]
223	K	K	A	A	Disp.[c]
250	F	F	F	F	Cycl.[c] and disp.[c]
Subsite +1					Acceptor
185	L	L	L	L	Disp.[c]
221	A	A	A	A	–
224	H	H	H	H	General activity
Subsite –1					Catalytic
The catalytic residues (D220, E248 and D318) are completely conserved.					
Subsite –2					Donor
95	H	H	H	H	General activity
98	W	W	W	W	–
365	R	R	R	R	–
Subsite –3					Donor
88–92	NYSGVNNT	NYSGVNNT	P-GGF–A	P-EGF–S	Cycl.[c] and spec.[c]
361	D	D	D	D	Cycl.[c]
187	D	D	D	D	Cycl.[c]
47	K	R	T	T	Cycl.[c]
Subsite –4 and –5					
No side chain contacts					
Subsite –6				Donor	
158	Y	Y	Y	Y	–
170–171	GG	GG	GG	GG	–
184	N	N	N	N	Cycl.[c]
Subsite –7					Donor
142–143	SSTDPSFA	SSDQPSFA	D------I	D------I	Spec.[c]
Central					
186	Y	Y	Y	Y	Cycl.[c]

Reproduced from Uitdehaag, J.C.M. *et al*. (*12*), and Takada *et al*. (*10*), with some modifications. Dashes denote deletions. The underlined residues and sequences are very specific in CGTases that produce γ-CD primarily. α-, β-, β/γ-, γ-[a], and γ-CGTase[b] indicate the CGTases from *B. macerans* (*5*), *B. circulans* strain 251 (*12*), *Bacillus* sp. 290-3 (*3*), *Bacillus* sp. G-825-6 (*14*), and *B. clarkii* 7364 (*10*), respectively. [c]Disp., disproportionation; Cycl., cyclization; Spec., specificity.

+3 in the acceptor site and also investigated the effects of replacement of these amino acids by several others through site-directed mutagenesis. The replacement of Ala223 at subsite +2 by basic amino acids strongly enhanced the γ-CD–forming activity in the neutral pH range.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—*Bacillus clarkii* 7364 was used for the production of wild-type CGTase (*10*). *Escherichia coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet^r)] (Stratagene) was used for site-directed mutagenesis. *Escherichia coli* BL21(DE3) [F$^-$ *opmT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)] (Novagen) was used for the production of mutant CGTase proteins. Plasmid pETGW was constructed by ligating the gene coding the mature wild-type CGTase into plasmid pET12a (Novagen) according to the supplier's manual and was then used for site-directed mutagenesis.

Site-Directed Mutagenesis—Site-directed mutagenesis was conducted using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the supplier's manual. The sequences of the mutagenic primers are shown in Table 2. All mutations were confirmed by the chain termination method (*16*) with an automated DNA sequencer (Beckman Coulter, CEQ2000).

Production and Purification of CGTase—The wild-type CGTase from *B. clarkii* 7364 was produced and purified as described previously (*10*). The mutant CGTase was purified as follows. *E. coli* BL21(DE3) cells containing a recombinant plasmid were cultured aerobically in 1 liter LB broth at 30°C in a 5 liter baffled flask with shaking at 90 rpm in the presence of ampicillin (50 μg/ml). Isopropyl-β-D-thiogalatopyranoside was added to a final concentration of 0.25 mM at the log phase of bacterial growth and then the culture was continued. After 16 h incubation, the culture broth was centrifuged and the cell-free supernatant was then concentrated to 100 ml using an ultrafiltration membrane, PM-10 (Amicon). The concentrate was purified by the same method as used for the wild-type CGTase. The protein concentration of the purified enzyme was determined by the method of Lowry *et al*. with crystalline bovine serum albumin as a standard (*17*). The purified enzyme was stored at 4°C until use.

Enzyme Activity Assay—All enzyme activity assays were performed in 25 mM HEPES-NaOH buffer (pH 7.5) and 25 mM Gly-NaCl-NaOH buffer (pH 10.0) at 40°C. The γ- and β-CD–forming activities were measured by incubating the enzyme solution with 1.35% (w/v) soluble starch (Nacalai Tesque) in the above buffers for 10 min. The amounts of γ- and β-CD were photometrically determined with bromocresol green (*10*, *18*) and phenolphthalein (*19*), respectively. One unit of each activity was defined as the amount of enzyme that produced 1 μmol of the corresponding CD per min. Starch-degrading activity was measured by the blue value method of Fuwa (*20*) with some modifications using 0.1% (w/v) amylose EX-III (SEIKAGAKU CORPORATION) in the above buffers. One unit of the activity was defined as the amount of enzyme that gave a 10% decrease in the absorbance at 700 nm per min. Each enzyme solution was diluted up to 0.02 unit for measuring γ- and β-CD–forming activities, and 0.4 unit for measuring starch-degrading activity.

Kinetic Studies—The kinetic parameters were determined by measuring γ-CD–forming activity as described above, but the substrate solution was prepared with the following concentrations: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1, 0.14 and 0.2% (w/v). The kinetic parameters were determined using the Michaelis-Menten equation with IGOR Pro software (WaveMetics).

Determination of the Proportions of CDs Produced—The proportions of CDs produced were determined on a HPLC column, Aminex HPX-42A (ϕ8 × 300 mm, Bio-Rad Laboratories), as described previously (*10*).

Structual Modeling of γ-CGTase—The theoretical structure of the γ-CGTase was obtained by homology modeling from the SWISS-MODEL protein-modeling server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) with the crystal structures of CGTases (PDB accession codes 1CIU, 1A47, 1CYG, 1PAM and 1I75) as templates. The maltononaose (G9) structure was derived from the complex structure of *Bacillus circulans* 251 CGTase with G9 (PDB accession code 1CXK). The proposed complex structure of our γ-CGTase with G9 was modeled by means of superpositioning of the above CGTase structures, followed by least-square fitting of the Cα atoms.

Table 2. **Primers used for site-directed mutagenesis.**

	Sequence (5′ to 3′ direction)
A223D for	cgcgtggatgcggttgaccacatgcctttgggc
A223D rev	gcccaaaggcatgtggtcaaccgcatccacgcg
A223E for	cgcgtggatgcggttgaacacatgcctttgggc
A223E rev	gcccaaaggcatgttcaaccgcatccacgcg
A223H for	cgcgtggatgcggttcaccacatgcctttgggc
A223H rev	gcccaaaggcatgtggtgaaccgcatccacgcg
A223K for	cgcgtggatgcggttaaacacatgcctttgggc
A223K rev	gcccaaaggcatgtgtttaaccgcatccacgcg
A223R for	cgcgtggatgcggttcgacacatgcctttgggc
A223R rev	gcccaaaggcatgtgtcgaaccgcatccacgcg
A223S for	cgcgtggatgcggtttcacacatgcctttgggc
A223S rev	gcccaaaggcatgtgtgaaaccgcatccacgcg
G255D for	tacaggagcacaagacagcaatcattacc
G255D rev	ggtaatgattgctgtcttgtgctcctgta
G255E for	tacaggagcacaagagagcaatcattacc
G255E rev	ggtaatgattgctctcttgtgctcctgta
G255K for	tacaggagcacaaaaaagcaatcattacc
G255K rev	ggtaatgattgctttttgtgctcctgta
G255R for	tacaggagcacaacgcagcaatcattacc
G255R rev	ggtaatgattgctgcgttgtgctcctgta
G255S for	tacaggagcacaaagcagcaatcattacc
G255S rev	ggtaatgattgctgctttgtgctcctgta

Underlining denotes the coding sequence of the mutated amino acids.

RESULTS

Specific Activities of the Wild-Type CGTase and Its Mutants—Ala223 at subsite +2 and Gly255 at subsite +3 were replaced by aspartic acid and glutamic acid (acidic amino acids), lysine, arginine and histidine (basic amino acids), and serine (hydroxyamino acid), and all mutant CGTases were successfully produced and purified to homogeneity. There was little difference in the expression levels between the mutant CGTases and the recombinant wild-type CGTase expressed by the same method, as the mutant CGTases were expressed and 30–40 mg of mutant CGTase protein per liter was produced. Assays of various enzyme activities of the (mutant) CGTases were performed (Table 3). The A223R mutant showed 4-fold (pH 7.5) and

Table 3. **Starch-degrading, β-CD and γ-CD forming activities of the wild-type CGTase and its mutants.**

Enzyme	Starch-degrading activity (units/mg)		β-CD–forming activity (units/mg)		γ-CD–forming activity (units/mg)	
	pH 7.5	pH 10.0	pH 7.5	pH 10.0	pH 7.5	pH 10.0
Wild type	323	959	0.11	0.50	0.47	5.41
A223D	9	41	ND	ND	ND	0.06
A223E	112	529	0.02	0.05	0.08	0.96
A223H	547	1,143	0.16	0.49	0.99	3.71
A223K	663	981	0.14	0.41	1.45	4.45
A223R	812	1,548	0.20	0.93	1.86	8.38
A223S	171	603	0.02	0.12	0.17	1.83
G255D	108	328	0.02	0.12	0.17	1.72
G255E	95	335	0.02	0.09	0.15	0.96
G255K	170	517	0.04	0.22	0.25	1.72
G255R	206	553	0.03	0.19	0.31	2.13
G255S	167	426	0.03	0.22	0.34	2.36

ND, not detected.

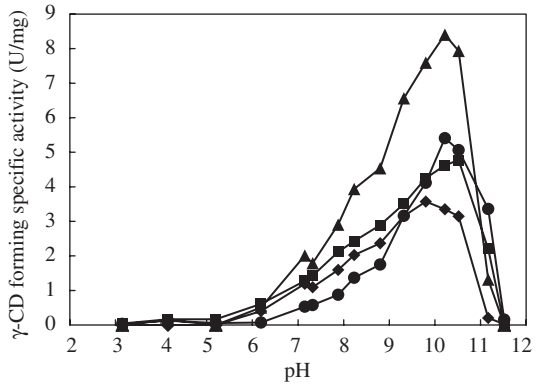

Fig. 1. **Effects of pH on the γ-CD forming activity of the wild-type CGTase and its mutants.** The activity was measured under the standard assay conditions given under "MATERIALS AND METHODS" except at various pHs. The buffers used were 1/4× McIlvaine buffer (pH 3–7), 25 mM HEPES-NaOH buffer (pH 7.3–8.2), 25 mM Gly-NaCl-NaOH buffer (pH 8.8–10.2), and 25 mM Na_2HPO_4-NaOH buffer (pH 10.5–11.5). The pH activity profiles of wild type (solid circles), A223H (solid diamonds), A223K (solid squares), and A223R (solid triangles) are presented.

1.5-fold (pH 10.0) increases in γ-CD–forming activity. The A223H and A223K mutants showed slight decreases in γ-CD–forming activity at pH 10.0, but they showed 2-fold and 3-fold increases at pH 7.5, respectively. The replacement of Ala223 with basic amino acids resulted in significant increases in γ-CD–forming activity, especially at pH 7.5. Although the starch-degrading activity and β-CD–forming activity of the A223H, A223K and A223R mutants also showed significant increases compared with those of the wild type CGTase, the rates of the increases in these activities were relatively low at pH 7.5 and the same at pH 10.0 compared with that of γ-CD–forming activity. The A223D, A223E and A223S mutants, obtained by replacing Ala223 with acidic amino acids and hydroxyamino acid, and all mutants obtained by replacing Gly255 at subsite +3 with acidic amino acids, basic amino acids and hydroxyamino acid showed significant decreases in γ-CD–forming activity at both pH 7.5 and 10.0. The γ-CD–forming activities of the wild type CGTase and its three mutants, A223H, A223K and A223R, were measured at various pH values ranging from 3 to 11.5 (Fig. 1). The pH activity profiles of the A223H and A223K mutants showed higher activity at neutral pHs (pH 6–9) than that of the wild type CGTase, whereas that of the A223R mutant showed higher activity in a broader pH range (pH 6–10.5). Although the pH profiles of γ-CD–forming activity changed, especially at neutral pH, the optimum pH did not change, remaining around 10.0.

Kinetic Parameters of the Wild-Type CGTase and Its Mutants—The kinetic parameters of the wild type CGTase and its three mutants, A223H, A223K and A223R, were determined by measuring γ-CD–forming activities at pH 7.5 and 10.0 (Table 4). At pH 10.0, the K_m values of the A223H and A223K mutants were higher than that of the wild type CGTase, while the K_m value of the A223R mutant was lower than that of the wild type CGTase. The k_{cat} values of both the A223H and A223K mutants at pH 10.0 were slightly higher than that of the wild-type CGTase, whereas the catalytic efficiencies (k_{cat}/K_m) of both mutants were significantly reduced. The values of both k_{cat} and k_{cat}/K_m of the A223R mutant at pH 10.0 were approximately 2-fold higher than those of the wild type CGTase. The K_m values of the wild type CGTase and the A223R mutant at pH 7.5 were higher than those values at pH 10.0. The K_m values of both the A223H and A223K mutants at pH 7.5 were lower than those values at pH 10.0 and approached those of the wild type CGTase. The values of both k_{cat} and k_{cat}/K_m of the three mutants, A223H, A223K and A223R, at pH 7.5 were significantly increased compared with those of the wild type CGTase. In the case of the A223R mutant, the values of k_{cat} and k_{cat}/K_m were 4.1-fold and 5.0-fold higher than those of the wild type CGTase, respectively.

Table 4. **Kinetic parameters of the γ-CD–forming activity of the wild-type CGTase and its mutants.**

Enzyme		K_m (%)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} $\%^{-1}$)
Wild type	pH 7.5	0.041 ± 0.011	0.77 ± 0.21	19.0 ± 2.3
	pH 10.0	0.033 ± 0.005	8.27 ± 0.15	256.0 ± 33.0
A223H	pH 7.5	0.050 ± 0.002	1.99 ± 0.28	40.0 ± 4.5
	pH 10.0	0.061 ± 0.013	8.73 ± 1.62	143.9 ± 20.4
A223K	pH 7.5	0.036 ± 0.000	2.79 ± 0.01	77.3 ± 1.0
	pH 10.0	0.046 ± 0.017	9.37 ± 1.71	172.5 ± 18.1
A223R	pH 7.5	0.033 ± 0.001	3.21 ± 0.37	96.8 ± 8.9
	pH 10.0	0.026 ± 0.011	14.11 ± 1.37	586.7 ± 18.0

Table 5. **HPLC analysis of CDs produced in reaction mixtures containing 1% soluble starch and the wild-type CGTase and its mutants at pH 7.5 and 10.0, and at 50°C after 48 h incubation.**

Enzyme	pH 7.5			pH 10.0		
	α-CD	β-CD	γ-CD	α-CD	β-CD	γ-CD
	(HPLC area %)					
Wild type	ND	0.8	10.9	ND	3.5	27.5
A223D	ND	ND	0.1	ND	ND	Trace
A223E	ND	ND	3.3	ND	ND	2.0
A223H	ND	1.5	12.8	ND	1.7	8.8
A223K	ND	1.3	21.3	ND	0.8	23.8
A223R	ND	0.8	20.8	ND	1.3	19.2
A223S	ND	ND	5.4	ND	0.2	17.4
G255D	ND	ND	3.9	ND	ND	1.3
G255E	ND	ND	2.7	ND	ND	0.7
G255K	ND	0.1	5.7	ND	ND	0.7
G255R	ND	ND	6.3	ND	ND	0.7
G255S	ND	ND	5.6	ND	ND	2.0

ND, not detected.

Analysis of the Proportions of CDs Produced by the Wild-Type CGTase and Its Mutants—The production of CDs in reaction mixtures containing 1% soluble starch and a (mutant) CGTase (0.15 mg/g dry starch) incubated at pH 7.5 and 10.0, and at 50°C was analyzed (Table 5). The wild type CGTase produced 10.9% γ-CD from 1% soluble starch at pH 7.5 and 50°C after 48 h incubation. The three mutants, A223H, A223K and A223R, produced approximately 1.2- to 2-fold greater amounts of γ-CD. At this time, the mutants showed almost the same productivity of β-CD as that of the wild type CGTase. Other mutants produced lower amounts of γ-CD (0.1–6.3%) than that

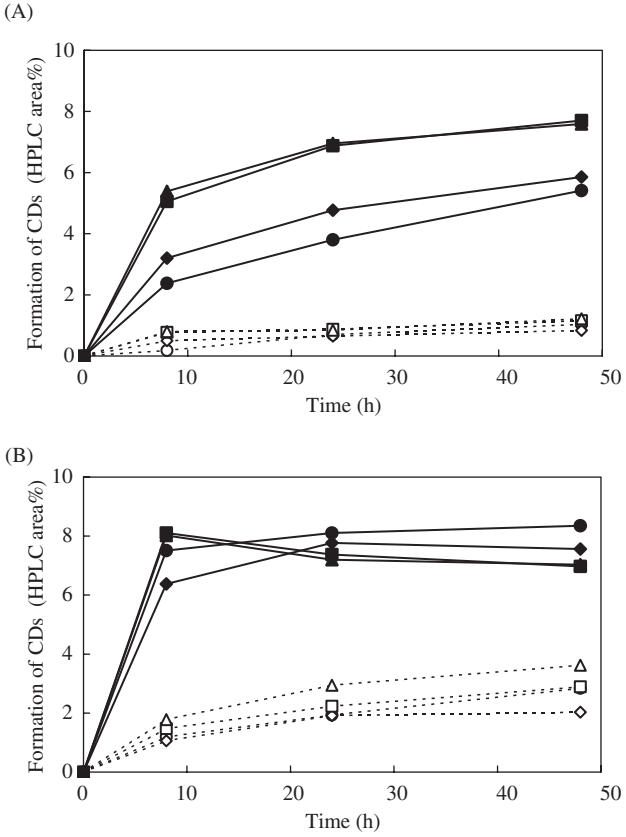

Fig. 2 **Courses of CD production in reaction mixtures containing 10% soluble starch and the wild-type CGTase and its mutants at pH 7.5 (A) and 10.0 (B), and 50°C.** The enzymes used were the wild type (circles), A223H (diamonds), A223K (squares), and A223R (triangles). The production of β- and γ-CD is shown by open and closed symbols, respectively.

produced by the wild type CGTase. The wild type CGTase produced 27.5% γ-CD from 1% soluble starch at pH 10.0 and 50°C after 48 h incubation. The three mutants, A223H, A223K and A223R, showed lower production of CDs than that by the wild type CGTase, producing 8.8, 23.8 and 19.2% of γ-CD, respectively. Other mutants except for the A223S mutant produced only small amounts of γ-CD (0.0–2.0%). The wild type CGTase and its three mutants, A223H, A223K and A223R, which produced greater amounts of γ-CD from 1% soluble starch at pH 7.5, were used for reactions with 10% soluble starch at pH 7.5 and 10.0 (Fig. 2). From 10% soluble starch as well, the A223H, A223K and A223R mutants produced greater amounts of γ-CD at pH 7.5 and 50°C than did the wild type CGTase. Both the A223K and A223R mutants produced approximately 8% γ-CD after 48 h incubation. At this time, the mutants showed almost the same production of β-CD as that by the wild type CGTase. All CGTases produced the maximum amount of 8% γ-CD at pH 10.0 and 50°C. The production of γ-CD by the wild type CGTase and the A223H mutant reached the maximum after 24 h incubation, whereas the maximum production by the A223K and A223R mutants was achieved after only 8 h incubation. After the maximum production of γ-CD had reached a plateau, only the γ-CD formation increased.

DISCUSSION

In this study, the effects of replacement of hydrophobic amino acids in the γ-CGTase from *B. clarkii* 7364 at subsites +2 and +3 were investigated. Our results showed that the replacement of Ala223 at subsite +2 by basic amino acids caused increases in the γ-CD–forming activity and the k_{cat} values, and decreases in the K_m values, especially in the neutral pH range. To assess the interaction between a (mutant) CGTase and linear or cyclic oligosaccharides, the coupling and disproportionation reactions are analyzed; however, both activities were too weak to detect in the initial stages of the reactions (data not shown). The pK_a values of three basic amino acids, histidine, lysine and arginine, are 6.0, 10.5 and 12.5, respectively. Although the experimental pK_a values for His223, Lys223 and Arg223 in each mutant were not measurable at pH 7.5, the amino group in the side chain of His223 is thought to slightly conjugate the protonated form ($-NH_3^+$), and those of Lys223 and Arg223 are thought to predominantly conjugate the protonated form. At pH 10.0, only the amino group in the side chain of Arg223 is thought to predominantly conjugate the protonated form. The results suggest that the high proportion of the protonation state of the amino groups in the side chains of these amino acids positioned at 223 corresponds to the high level of γ-CD–forming activity. It has been reported that the amino group in the side chain of lysine at subsite +2 undergo a hydrogen bonding interaction with the glucose residue of a linear oligosaccharide and little interaction with those of CDs (*12*, *21*). The protonation state seems to enhance the hydrogen bonding between these basic amino acids at subsite +2 and the glucose residues of linear oligosaccharides. The enhancement of the interaction at subsite +2 may play an important role by helping the substrate reach subsite +1, hence increasing the γ-CD–forming activity and k_{cat} value. It has been also reported that the hydrophobic interaction between the aliphatic side chain of Lys232 at subsite +2 and Trp258 that neighbors the acid/base catalyst Glu259 in *B. circulans* 251 CGTase plays an important role in the structural integrity of the active site (*12*). The replacement of Ala223 by these basic amino acids at subsite +2 might enhance the hydrophobic interaction with Trp248 that neighbors the acid/base catalyst Glu249 in *B. clarkii* 7364 CGTase. This might contribute to the structural integrity of the active site and the increases in k_{cat} values as well. We constructed a theoretical structure of γ-CGTase by homology modeling using the crystal structures of five CGTases (PDB accession codes 1CIU, 1A47, 1CYG, 1PAM and 1I75) as templates, and simulated the mutational effects of the A223H, A223K and A223R mutations (Fig. 3). The constructed models of the wild-type CGTase and these mutants indicate that Ala223 at subsite +2 in the wild-type CGTase is located apart from the glucose residues of linear oligosaccharides whereas the amino groups in the side chains of His223, Lys223 and Arg223 are located close to the glucose O3 atom of the +2 glucose residue. The distance between these amino acid residues positioned at 223 and the glucose residue of a linear oligosaccharide seems to correlate with the kinetic parameter results.

In contrast, both the A223D and A223E mutants, obtained by replacing Ala223 with acidic amino acids,

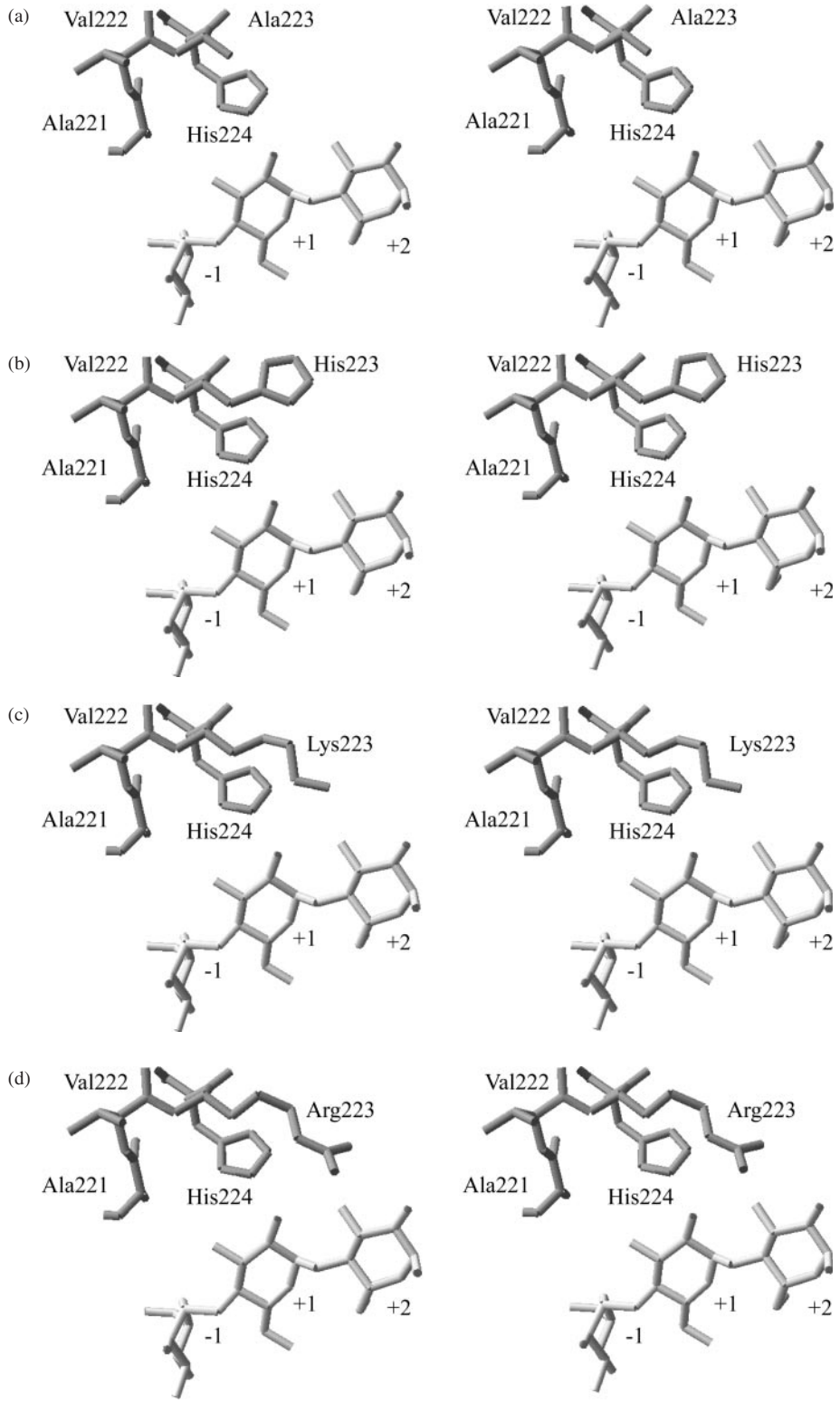

Fig. 3 **Stereoviews of the structural conformation around the amino acid residues positioned at 223 of the wild-type CGTase (dark gray) (a), A223H (dark gray) (b), A223K (dark gray) (c), and A223R (dark gray) (d) complexed with maltononaose (light gray).** The glucose residues are numbered +2, +1, and –1 as their corresponding subsite numbering.

showed slight γ-CD–forming activity at both pH 7.5 and 10.0. This might be caused by the repulsion between the carboxyl group in the side chains of these amino acids and the substrate. In general, glutamic acid at subsite +3 is conserved in both α- and β-CGTase, and it has been reported that the β-CD–forming activity of the E264A mutant was hardly affected in the case of CGTase from *B. circulans* strain 251 (*15*). However, all mutants obtained by replacing Gly255 at subsite +3 with various amino acids, including glutamic acid, showed significant decreases in various activities at both pH 7.5 and 10.0. According to the constructed model of wild-type *B. clarkii* 7634 CGTase, the region around Gly255 forms a helix (data not shown). Compared with the corresponding region of *B. circulans*

251 CGTase, the helix bends more tightly and the conformation around Gly255 is much closer. Therefore, it seems that mutations at the site of Gly255 introduce steric hindrance around subsite +3 and the mutants show significant decreases in their activities.

The analysis of production of CDs from 1% soluble starch by the wild-type CGTase and its mutants at pH 7.5 showed that higher γ-CD–forming activity leads to greater γ-CD production. In the case of the A223H, A223K and A223R mutants, the γ-CD production increased but there was little change in the β-CD production. This fact suggests that the replacement of Ala223 by basic amino acids probably has little effect on the donor site involved in γ-CD specificity, and probably has an effect on the acceptor site only. Both the A223K and A223R mutants showed approximately 1.4-fold γ-CD production from 10% soluble starch at pH 7.5 and 50°C after 48 h incubation compared to the amounts produced by the wild type CGTase, the maximum production being about 8% γ-CD. At pH 10.0, the rates of γ-CD formation by the wild-type CGTase, and the A223H, A223K and A223R mutants differed, but the γ-CD production by all these CGTases reached a plateau at 8%. In the case of the wild type CGTase and A223H mutant, even when the reaction was prolonged and/or excessive amounts of CGTase were used at pH 7.5, the γ-CD production still reached a plateau at 8% (data not shown). The plateau in γ-CD production might be caused by two phenomena (*22–25*). One is product inhibition. The binding of produced CD to the active site and the maltose binding site of CGTase inhibits the binding of the substrate to these sites. The other is a coupling reaction. At the start of the reaction, the CGTase reaction is almost a cyclization reaction. However, as the reaction proceeds, the coupling reaction with produced CD and low molecular weight saccharides is not negligible. The coupling reaction causes the formation of smaller CDs. Our results showed that only the β-CD formation increased after the maximum production of γ-CD had reached a plateau. Although the maximum amounts of γ-CD produced from 10% soluble starch by the wild-type CGTase and its mutants were not different from each other, this might be due to little interaction of alanine and three basic amino acids positioned at 223 with CD. The maximum amounts of γ-CD did not change but the rates of γ-CD formation were efficiently accelerated without any change in CD production specificity.

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