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The Remote Substrate Binding Subsite −6 in Cyclodextrin-glycosyltransferase Controls the Transferase Activity of the Enzyme via an Induced-fit Mechanism*

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Cyclodextrin-glycosyltransferase (CGTase) catalyzes the formation of α-, β-, and γ-cyclodextrins (cyclic α-(1,4)-linked oligosaccharides of 6, 7, or 8 glucose residues, respectively) from starch. Nine substrate binding subsites were observed in an x-ray structure of the CGTase from *Bacillus circulans* strain 251 complexed with a maltononaose substrate. Subsite −6 is conserved in CGTases, suggesting its importance for the reactions catalyzed by the enzyme. To investigate this in detail, we made six mutant CGTases (Y167F, G179L, G180L, N193G, N193L, and G179L/G180L). All subsite −6 mutants had decreased k$_{cat}$ values for β-cyclodextrin formation, as well as for the disproportionation and coupling reactions, but not for hydrolysis. Especially G179L, G180L, and G179L/G180L affected the transglycosylation activities, most prominently for the coupling reactions. The results demonstrate that (i) subsite −6 is important for all three CGTase-catalyzed transglycosylation reactions, (ii) Gly-180 is conserved because of its importance for the circularization of the linear substrates, (iii) it is possible to independently change cyclization and coupling activities, and (iv) substrate interactions at subsite −6 activate the enzyme in catalysis via an induced-fit mechanism. This article provides for the first time definite biochemical evidence for such an induced-fit mechanism in the α-amylase family.

Cyclodextrin-glycosyltransferase (CGTase[1]; EC 2.4.1.19) produces circular α-(1,4)-linked oligosaccharides (cyclodextrins) from starch. The major products are α-, β- and γ-cyclodextrin (with 6, 7, or 8 glucose residues), but larger cyclodextrins are also formed (1–3). CGTase belongs to glycoside hydrolase family 13, or α-amylase family (4), which is an extensively studied enzyme family (5, 6). All members contain a catalytic (β/α)$_8$-barrel domain (6) and use an α-retaining double displacement mechanism (7). Although the catalytic residues

and the architecture of the catalytic site are conserved within this family, its members may catalyze a variety of reactions, including hydrolysis of α-(1,4)- and α-(1,6)-glycosidic linkages (*e.g.* α-amylases and isoamylases, respectively), as well as the formation of α-(1,4)- and α-(1,6)-glycosidic bonds (*e.g.* amylomaltases and branching enzymes, respectively) (5).

High resolution x-ray structures are known for the CGTases from *Bacillus circulans* strain 8 (8) and strain 251 (BC251) (9), *Thermoanaerobacterium thermosulfurigenes* strain EM1 (10), *Bacillus stearothermophilus* (11), and alkalophilic *Bacillus* sp. 1011 (12). The structures of CGTases are organized in five domains (*A–E*). The N-terminal part consists of the catalytic (β/α)$_8$-barrel fold (domain A) with a loop of ~60 residues protruding at the third β-strand (domain B). Domains A and B together form the substrate binding groove and contain the catalytic site residues (8, 13). Domains C and E are involved in starch binding (14), whereas the function of domain D remains to be elucidated. The substrate binding groove of the BC251 CGTase consists of at least nine sugar binding subsites (13), labeled −7 to +2, with bond cleavage occurring between subsites −1 and +1. Fig. 1 gives an overview of the interactions between the enzyme and a maltononaose substrate and shows the binding mode of this maltononaose in the active site of CGTase.

CGTase uses an α-retaining double displacement mechanism to catalyze four different reactions, cyclization, coupling, disproportionation, and hydrolysis. The cyclization (and disproportionation) reactions start with the binding of a linear malto-oligosaccharide substrate, followed by cleavage of the α-(1,4)-glycosidic bond between the residues bound at subsites +1 and −1, resulting in an intermediate that is covalently linked to Asp-229 (15, 16). Subsequently, the non-reducing end moves from subsite −7 (for β-cyclization) into subsite +1. This step is called circularization, which is followed by intramolecular bond formation. Circularization is most likely the rate-determining step in the cyclization reaction (17, 18). In the disproportionation reaction the non-reducing end of a second sugar molecule is used as acceptor. CGTase also catalyzes the reverse reaction of cyclization, which is called the coupling reaction. In this reaction a cyclodextrin ring is opened, and the resulting covalently bound, linear oligosaccharide is transferred to a second sugar molecule, the acceptor. Besides these three transglycosylation reactions, CGTase catalyzes the hydrolysis of α-(1,4)-glycosidic bonds in starch. Interestingly, the hydrolysis activity of CGTase is much lower than its transglycosylation activities, making the enzyme an efficient transferase (3). Of

[1] The abbreviations used are: CGTase, cyclodextrin-glycosyltransferase; CD, cyclodextrin; BC251, *B. circulans* strain 251; MαDG, methyl-α-D-glucopyranoside; EPS, 4-nitrophenyl-α-D-maltoheptaoside-4–6-*O*-ethylidene; G5-pNP, 4-nitrophenyl-α-D-maltopentaoside; HPLC, high pressure liquid chromatography.

TABLE I

Data collection statistics and quality of the B. circulans strain 251 CGTase mutants G179L and N193G

	G179L	N193G
Data collection		
Spacegroup	$P2_12_12_1$	$P2_12_12_1$
Cell axes a, b, c (Å)	114.7, 109.5, 65.8	120.7, 111.2, 65.9
Resolution range (Å)	50.0–1.94	50.0–2.43
No. of unique reflections	60480	31710
Completeness (%)[a]	97.5 (96.6)	92.1 (70.2)
Refinement statistics		
No. of amino acids	686 (all)	686 (all)
No. of Ca^{2+} ions	2	2
No. of 2-methyl-2,4-pentanediol molecules	1	
No. of water molecules	1026	420
Active site ligand	Maltotetraose	Acarbose
Maltose binding site ligand	Maltotriose	Maltotriose
Maltose binding site ligand	Maltose	Maltotriose
Maltose binding site ligand	Maltose	Maltose
Ligand	Glucose	
Average B-factor	22.2	24.5
Final R-factor (%)	15.2	15.3
Final free R-factor (%)	18.6	19.9
Root mean square deviation from ideal geometry		
Bond lengths	0.005	0.006
Angles	1.28	1.25
Dihedrals	24.3	24.5
Improper	0.71	0.73

[a] Highest resolution shell in parentheses.

all reaction types, the disproportionation reaction is most efficiently catalyzed by CGTase (17, 19).

The high transferase activity of CGTase was investigated recently by comparing x-ray structures of CGTase representing different stages of its reaction cycle (20). From these studies it appeared that the protein backbone of CGTase can undergo small but significant conformational changes after binding of substrate sugars at the acceptor subsites +1 and +2 and at the donor subsites −3 and −6. The conformation of Asn-139 and His-140 is changed only if sugars are simultaneously bound at these subsites (21). This enables His-140 to make a hydrogen bond to the O-6 atom of the −1 sugar (see Fig. 1*A*) and helps to distort the −1 sugar toward transition state planarity (16, 20, 21). It was suggested that in this way, these distant sugar binding subsites communicate the presence of long oligosaccharide substrates and acceptors and ensure that they are preferentially processed.

Support for this mechanism has come from site-directed mutagenesis experiments of the residues in the acceptor subsites and of His-140 (22–24). Subsite −6 has not been studied so far, but its position far from the catalytic site (see Fig. 1*B*) makes it unlikely that the mutants interfere directly with the catalytic process. Instead, they may affect substrate binding or the proposed induced-fit mechanism, providing an excellent opportunity to test whether distant subsites play a role in regulating transglycosylation activity.

At present there are no mutagenesis data concerning subsite −6. Because this subsite is identical in all known CGTases, subsite −6 must be important for the function and the unique characteristics of CGTase. We constructed mutants that block subsite −6 (G179L, G180L, and G179L/G180L) or that abolish interactions at subsite −6 (Y167F, N193G, and N193L). Here we report a kinetic analysis of these mutants. The results obtained show that subsite −6 has an important function in all three transglycosylation reactions. They provide new insights in the catalytic mechanism employed by CGTase.

EXPERIMENTAL PROCEDURES

Structure Determination—Crystals of mutant BC251 CGTases were grown from 60% (v/v) 2-methyl-2,4-pentanediol, 100 mM HEPES (pH 7.5) and 5% (w/v) maltose (9). Soaking of N193G crystals with acarbose

was carried out as described earlier (13). For G179L data were collected to 1.94 Å at 120 K on an in-house MacScience DIP2030H image plate (Nonius, Delft, The Netherlands) using CuKa radiation from a Nonius FR591 rotating-anode generator with Franks' mirrors. Processing was done with DENZO and SCALEPACK (25). The structure of CGTase liganded with maltotetraose (PDB code 1CXF), with all waters and sugars removed, was used as a starting model. Refinement was done with the Crystallography & Nuclear Magnetic Resonance System (26) in a standard way. The compression of the longest cell axis of G179L compared with that of N193G (Table I) is because of a locally changed crystal packing at the maltose binding site near Trp-616 and Trp-662. This has improved the crystal quality, as shown by the increased resolution of the data at the in-house source and the low overall B-factor of structure and the low R-factors. For N193G data were collected to 2.43 Å at room temperature on a MacScience DIP2020 imaging plate mounted on an Elliot GX21 rotating-anode generator producing CuKa radiation. Data were reduced and scaled using the program XDS (27) and programs from the Groningen BIOMOL software package. Sugar ligands were manually placed in sigmaA-weighted $2F_o − F_c$ and $F_o − F_c$ electron density maps with the program O (28). The atomic coordinates and the structure factors of the structures have been deposited in the Protein Data Bank (code IKCL for G179L and IKCK for N193G; www.rcsb.org).

Bacterial Strains, Plasmids, and Growth Conditions—*Escherichia coli* strain MC1061 (hsdR mcrB araD139 Δ (araABC-leu)7679 Δ*lac*X74 *gal*U *gal*K *rps*L *thi*) (29) was used for recombinant DNA manipulations. CGTase (mutant) proteins were produced with the α-amylase and protease-negative *Bacillus subtilis* strain DB104A (*amy his npr*R2 *npr*E18 *apr*A3) (30). Plasmid pDP66k− (14), with the *cgt* gene of *B. circulans* strain 251, was used for site-directed mutagenesis and enzyme production. Plasmid-carrying strains were grown on LB medium at 37 °C in the presence of kanamycin, 50 or 5 μg/ml for *E. coli* or *B. subtilis*, respectively. Transformation of *B. subtilis* was done according to Bron (31).

DNA Manipulations—Mutant CGTases were constructed via a double PCR method using Pwo-DNA polymerase (Roche Molecular Biochemicals) as described previously (3). The PCR product was cut with *Pvu*II and *Sal*I and exchanged for the corresponding fragment of pDP66k−. The following oligonucleotides were used to introduce the mutations: 5′-CTCGGGGGGATTCACGAACGATACGCAAAACCTG-3′ (Y167F), 5′-CTACAAAGGCCTGTACGATCTCGCAGATCTGAACCATA AC-3′ (N193G), 5′-CTACAAACTCCTGTACGATCTCGCAGATCTGAA CCATAAC-3′ (N193L), 5′-GTTCCACCATAACCTAGGCACGGACTTT-TCCACG-3′ (G179L), 5′-GTTCCACCATAACGGGGTTAACGGACTTTT-CCACG-3′ (G180L), and 5′-GTTCCACCATAACCTGTTAACGGACTT-TTCCACG-3′ (G179L/G180L). Successful mutagenesis resulted in the appearance of the underlined restriction sites as follows: *Bln*I for

	k_{cat} α-CD	k_{cat} β-CD	k_{cat} γ-CD	k_{cat} δ-CD	k_{cat} Hydrolysis
			s^{-1}		
Wild-type	17.0 ± 1.0	338 ± 2	61 ± 2	36.2 ± 0.8	4.0 ± 0.2
G179L	8.8 ± 0.3	269 ± 3	52 ± 2	24.9 ± 0.5	3.9 ± 0.2
G180L	25.5 ± 0.4	77 ± 1	24 ± 1	11.1 ± 0.3	3.9 ± 0.2
G179L/G180L	21.4 ± 0.4	81 ± 1	20 ± 1	12.0 ± 0.4	4.0 ± 0.2
Y167F	19.5 ± 0.8	278 ± 9	50 ± 3	29.3 ± 1.5	3.5 ± 0.3
N193G	25.8 ± 0.9	166 ± 5	64 ± 5	31.1 ± 1.3	4.3 ± 0.3
N193L	19.3 ± 1.2	316 ± 8	53 ± 4	33.1 ± 1.1	4.0 ± 0.3

G179L, *Hinc*II for G180L and G179L/G180L, and *Bgl*II for N193G and N193L. Mutation Y167F removed an *Xmn*I restriction site. All mutations were confirmed by DNA sequencing of the complete *Pvu*II/*Sal*I fragment obtained with PCR.

DNA Sequencing—Cycle sequencing (32) was performed on double stranded DNA using the Thermo Sequence fluorescent primer cycle sequence kit (Amersham Biosciences, Inc.). Sequence reactions were run on the Amersham Biosciences, Inc. ALF-Express sequencing machine at the BioMedical Technology Center (Groningen, The Netherlands).

Enzyme Assays and Enzyme Purification—CGTase proteins were produced and purified as described before (3). All enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 50 °C.

Cyclization activities were determined by incubating 0.1–0.5 μg/ml enzyme with 2.5% (w/v) Paselli SA2 starch (partially hydrolyzed potato starch with an average degree of polymerization of 50; AVEBE, Foxhol, The Netherlands), as described by Penninga *et al.* (14).

Cyclodextrin product specificity under industrial process conditions was measured by incubating 10% (w/v) Paselli WA4 starch (pregelatinized drum-dried starch with a high degree of polymerization; AVEBE) with 2 units/ml of enzyme activity (1 unit is μmol min⁻¹ β-cyclodextrin-forming activity per mg of protein). Samples were taken at regular intervals, boiled for 10 min, and analyzed by HPLC, as described below.

Coupling activities were measured as described by Nakamura *et al.* (24), with some modifications (33), with α-, β-, and γ-cyclodextrin as donor substrates and methyl-α-D-glucopyranoside (MαDG) as acceptor substrate, using 0.1–0.5 μg/ml enzyme. Values of k_{cat} and K_m were determined by measuring rates at 5 donor and 5 acceptor substrate concentrations (25 conditions) ranging from 0.2 to 5 times the K_m values.

Disproportionation activity was determined as described by Nakamura *et al.* (19), with some modifications (33), using 0.1–0.5 μg/ml enzyme, 4-nitrophenyl-α-D-maltoheptaoside-4–6-*O*-ethylidene (EPS; Roche Molecular Biochemicals), or 4-nitrophenyl-α-D-maltopentaoside (G5-pNP; Megazyme, County Wicklow, Ireland) as donor substrate and maltose as acceptor substrate. With the EPS substrate, values of k_{cat} and K_m were determined by measuring rates at 6 donor and 5 acceptor substrate concentrations (30 conditions) ranging from 0.2 to 5 times the K_m values. With the G5-pNP substrate, values of k_{cat} and K_m were determined by measuring rates at 12 different donor concentrations at fixed maltose concentration (10 mM).

Hydrolyzing activity was determined as described before (3) by measuring the increase in reducing power upon incubation of 5 μg of enzyme with 1% (w/v) soluble starch (Lamers & Pleuger, Wijnegen, Belgium).

HPLC Analysis—Products formed were analyzed by HPLC, using an Econosphere NH₂ 5 u column (250 × 4.6 mm) (Alltech Nederland bv; Breda, The Netherlands) linked to a refractive index detector. A mobile phase of acetonitrile/water (60:40) (v/v) at a flow rate of 1 ml/min was used.

Analysis of the Experimental Data—The results obtained for the coupling and disproportionating reactions were analyzed using SigmaPlot (Jandel Scientific). The coupling reaction followed the random order ternary complex mechanism (17). The disproportionating reaction proceeded via the substituted enzyme mechanism (or ping-pong mechanism) (19).

RESULTS

Structures—The G179L structure had a maltotetraose ligand bound from subsites +2 to −2 with the glucose at subsite −1 in its β-anomeric configuration. Because the G179L crystals were not soaked with sugars, the maltotetraose sugar must be the remainder of α-cyclodextrin used for the purification of the enzyme. This mutant has indeed a very low coupling activity

(see Table V). The G179L structure also had a glucose molecule bound at the surface near Gln-287, Arg-290, Arg-294, Asp-295, and Glu330, about 8 Å from subsite +2. A sugar at this position was not seen before. Its functional relevance for the enzyme is not known. The φ/ψ angles of the mutated residue 179 were 66/−151 compared with 97/−162 in the wild-type enzyme (PDB code 1CDG). The protein backbone conformation was hardly affected by this mutation, however. The N193G structure had an acarbose molecule bound from subsites +2 to −2. The φ/ψ angles of the mutated residue were hardly changed, −72/150 compared with −60/145 in wild-type (PDB code 1CDG), and the protein backbone conformation was not significantly altered. In both structures the Asn-139/His-140 conformation is identical to that of the unliganded wild-type CGTase (34), as expected for structures that have no sugar bound at the −6 subsite.

Cyclization Activities of Wild-type and Mutant CGTases—The cyclization activities of the (mutant) CGTases are summarized in Table II. Substrate affinity values are not reported, because at the low substrate concentrations needed the amount of cyclodextrin formed is too low for reliable activity measurements. Low starch concentrations are needed, because BC251 CGTase has a high affinity for starch (<0.5 mg/ml) (17). All subsite −6 mutants have reduced β-cyclodextrin-forming activities, most pronounced for mutations that introduce a leucine at position 180 (G180L and G179L/G180L) or remove a side chain at position 193 (N193G) (Table II). Gly-180 is most important for β-, γ-, and δ-cyclodextrin formation, whereas the G179L mutation especially affects α-cyclization. Mutant N193L is only slightly affected in its cyclization activities. The N193G mutation, in contrast, specifically decreases β-cyclodextrin formation. Thus, subsite −6 plays an important role in the cyclization reactions catalyzed by CGTase.

Subsite −6 Mutations Affect the Disproportionation Reaction—All mutants show reduced disproportionating activities with the maltoheptaose EPS (Table III), most prominently for G179L, G180L, and G179L/G180L. With the shorter G5-pNP substrate, which cannot reach subsite −6, the mutants G179L, G179L/G180L, and N193G had decreased disproportionation activity, whereas the Y167F, G180L, and N193L mutants had wild-type activity (Table IV). Furthermore, the wild-type CGTase had a lower disproportionation activity with the shorter G5-pNP substrate than with EPS, indicating that substrate interactions at subsite −6 are important in this reaction. Mutation of Gly-179 and Gly-180 resulted in 4- to 5-fold increased $K_{m,EPS}$ values (Table III), demonstrating that introducing leucines at positions 179 and 180 negatively affects binding of the maltoheptaose compound EPS. This indicates that the wild-type enzyme has interactions with EPS at subsite −6. Indeed, product analysis of the disproportionation reaction showed that EPS is able to reach subsite −6 (data not shown). Mutation of Tyr-167 and Asn-193, in contrast, has no significant effect on the $K_{m,EPS}$ value (Table III). The specificity constants ($k_{cat}/K_{m,EPS}$) (Table III) also show that Gly-179 and Gly-180 are especially important for the disproportionation activity of CGTase. The apparent affinities for the acceptor

TABLE III
Kinetic parameters of the disproportionating reaction with EPS catalyzed by wild-type and mutant cyclodextrin glycosyltransferases from B. circulans strain 251

	$K_{M,\text{EPS}}$	$K_{M,\text{maltose}}$	$k_{\text{cat,disp.}}$[a]	$k_{\text{cat}}/K_{M,\text{EPS}}$	$k_{\text{cat,}\beta\text{-cycl.}}/k_{\text{cat,disp.}}$
	m_M		s^{-1}	$s^{-1}\ m_M^{-1}$	
Wild-type	0.22 ± 0.02	0.83 ± 0.05	1213 ± 22	5514 ± 511	0.28
G179L	1.06 ± 0.05	0.80 ± 0.04	597 ± 15	563 ± 30	0.45
G180L	0.92 ± 0.08	0.87 ± 0.09	493 ± 21	539 ± 52	0.16
G179L/G180L	1.04 ± 0.06	0.35 ± 0.02	317 ± 8	305 ± 19	0.26
Y167F	0.21 ± 0.03	0.91 ± 0.10	990 ± 23	4714 ± 682	0.28
N193G	0.20 ± 0.02	1.04 ± 0.08	774 ± 22	3870 ± 402	0.21
N193L	0.21 ± 0.02	0.86 ± 0.07	1076 ± 35	5124 ± 516	0.29

[a] disp., disproportionation; cycl., cyclization.

TABLE IV
Kinetic parameters of the disproportionating reaction with G5-pNP catalyzed by wild-type and mutant cyclodextrin glycosyltransferases from B. circulans strain 251

	$K_{M,\text{G5-pNP}}$	$k_{\text{cat,disp.}}$	$k_{\text{cat}}/K_{M,\text{G5-pNP}}$
	m_M	s^{-1}	$s^{-1}\ m_M^{-1}$
Wild-type	1.1 ± 0.1	761 ± 32	692 ± 69
G179L	2.3 ± 0.2	360 ± 18	257 ± 26
G180L	1.2 ± 0.1	788 ± 35	657 ± 62
G179L/G180L	2.6 ± 0.3	246 ± 15	95 ± 12
Y167F	1.2 ± 0.1	790 ± 20	658 ± 57
N193G	1.1 ± 0.1	327 ± 9	297 ± 28
N193L	1.1 ± 0.1	774 ± 41	704 ± 74

substrate ($K_{m,\text{maltose}}$) are not significantly changed, except for the double mutant G179L/G180L, which has a 2-fold lower $K_{m,\text{maltose}}$ value (Table III).

In All Mutants Coupling Activity Is Decreased—All subsite −6 mutants have reduced coupling activities (Table V). The G179L mutant has a 4-fold decreased k_{cat} value for β-cyclodextrin coupling. Unexpectedly, the coupling activities with α- and γ-cyclodextrin are fully abolished (Table V). Furthermore, the coupling activities of the G179L/G180L mutant are also virtually absent for each of the three cyclodextrins (Table V). In contrast, the Y167F, G180L, N193G, and N193L mutants retained significant coupling activity with all cyclodextrins tested (data not shown for α- and γ-cyclodextrin). The G180L and N193G mutants strongly reduced the coupling activity with β-cyclodextrin, whereas the effect was small for Y167F and N193L. Thus, subsite −6 is very important in the coupling reactions. The mutations at subsite −6 also have an effect on acceptor binding as shown by the apparent affinity constants for the acceptor substrate (Table V). Especially the G180L mutation drastically increases the $K_{m,\text{M}\alpha\text{DG}}$ values, whereas the effect is smaller for mutant N193G. This is unlikely to be a direct effect of the mutation, because Gly-180 and Asn-193 are positioned a large distance from the acceptor site (Fig. 1). This shows that subsite −6 influences the acceptor binding subsites.

Hydrolyzing Activities—The hydrolyzing activity of CGTase results in the formation of linear products from starch. Although the substrate used (starch) is able to reach subsite −6, the mutations at this subsite have no significant effect on the hydrolyzing activity (Table II), showing that it is possible to selectively alter one of the CGTase activities without affecting another activity.

Cyclodextrin Product Ratios of (Mutant) CGTases—In this production assay the α:β:γ ratio of formed cyclodextrins changes in time as the combined result of all four reactions described above. Fig. 2 shows this time dependence of cyclodextrin production for the wild-type and mutant enzymes. After 6 h, 30–37% of the starch has been converted into cyclodextrins (Table VI), with only small amounts (<3%) converted into linear products (not shown). Initially wild-type CGTase produces mainly β- and γ-cyclodextrin, whereas smaller amounts

of α-cyclodextrin are formed. Compared with wild-type, the G180L, G179L/G180L, and N193G CGTases produce larger amounts of α- and γ-cyclodextrin in the first minutes of the reaction (Fig. 2). After 6 h all mutants have produced more α-cyclodextrin, with the exception of G179L, which produced significantly less α-cyclodextrin (see Fig. 2 and Table VI). The wild-type and the G179L and G179L/G180L mutants formed more α- than γ-cyclodextrin, whereas the other mutants formed more γ- than α-cyclodextrin after 6 h of incubation (Fig. 2). The data thus show that subsite −6 is involved in cyclodextrin product specificity. Although the mutant CGTases have lower or minimized coupling activities, they do not produce significantly more cyclodextrins than the wild-type enzyme.

DISCUSSION

Substrate Binding Sites in CGTases—The substrate binding groove of BC251 CGTase consists of at least nine sugar binding subsites, ranging from +2 to −7 (Fig. 1A) (13, 35). Although subsites +1, −1, and −2 are conserved in CGTases and most α-amylases (6, 21), subsites +2, −3, −4, −5, −6, and −7 are typical for CGTases (21, 23). Mutagenesis studies have shown that subsite +2 is important for acceptor binding in all three transglycosylation reactions (22, 23) and that subsites −3 and −7 are important for CGTase product specificity (33, 36–38). The function of subsite −6 is unknown, and no subsite −6 mutants have yet been described. X-ray structures of the BC251 CGTase complexed with linear maltononasaccharide ligands showed strong interactions of this subsite with the oligosaccharide (Fig. 1A) (13, 21). These interactions are provided by the side chains of Tyr-167 and Asn-193 and the backbone nitrogen and oxygen atoms of Ala-144, Gly-179, Gly-180, and Asp-196 (Fig. 1A). The conservation of the residues in this subsite (21, 39) suggests that they are important for the functionality of the enzyme (21).

Circularization—One of the striking effects of the mutations is their influence on the cyclodextrin product ratio. An explanation for this is suggested from circularization pathway calculations, which revealed that subsite −6 stabilizes intermediary stages of the circularization process by successively binding the 6th, 7th, and 8th (γ-cyclodextrin formation) glucose residue during the movement of the non-reducing end of the substrate toward the +1 acceptor subsite (18). Thus, mutations in subsite −6 are expected to interfere with the cyclization reaction. Indeed, mutations in subsite −6 especially affect β-, γ-, and δ-cyclization. No negative effect is seen for the α-cyclization activity, which only involves binding of six glucose residues. An exception, however, is mutant G179L, of which the α-cyclization activity has been reduced to 50% of the wild-type activity.

Function of Subsite −6 in the Cyclization Reactions—The rate-limiting step in the β-cyclization reaction is most likely the 23-Å movement of the non-reducing end of the substrate from subsite −7 to subsite +1 (circularization) (16, 18). The lower β-cyclization activities of the subsite −6 mutant CGTases thus indicate that substrate binding, or the circularization process

TABLE V

Kinetic parameters of the coupling reactions catalyzed by wild-type and mutant cyclodextrin glycosyltransferases from B. circulans strain 251

|	$K_{M,CD}$	$K_{M,M\alpha DG}$	$K'_{M,CD}$	$K'_{M,M\alpha DG}$	k_{cat}
			mM		s^{-1}
α-CD					
Wild-type	2.2 ± 0.3	0.45 ± 0.05	5.3 ± 1.2	1.1 ± 0.3	240 ± 7
G179L	ND[a]	ND	ND	ND	<0.1
G180L	5.4 ± 0.3	10 ± 1.3	2.8 ± 0.2	5.2 ± 1.1	196 ± 11
G179L/G180L	ND	ND	ND	ND	<0.1
β-CD					
Wild-type	0.32 ± 0.02	18.1 ± 1.4	0.15 ± 0.04	8.5 ± 2.2	368 ± 10
G179L	0.57 ± 0.05	5.0 ± 1	1.25 ± 0.20	11 ± 3	95 ± 4
G180L	0.59 ± 0.07	245 ± 18	0.28 ± 0.03	116 ± 14	233 ± 10
G179L/G180L	ND	ND	ND	ND	<0.1
Y167F	0.29 ± 0.03	16.2 ± 1.8	0.23 ± 0.03	12.7 ± 2.5	326 ± 14
N193G	0.39 ± 0.05	52.2 ± 6	0.41 ± 0.05	54.9 ± 12	104 ± 6
N193L	0.38 ± 0.05	16.7 ± 1	0.22 ± 0.02	9.7 ± 1.7	331 ± 16
γ-CD					
Wild-type	0.13 ± 0.02	16.6 ± 3.0	0.12 ± 0.03	15.7 ± 6.1	188 ± 11.3
G179L	ND	ND	ND	ND	<0.1
G180L	0.12 ± 0.02	38 ± 3	0.46 ± 0.05	146 ± 9	83 ± 5
G179L/G180L	ND	ND	ND	ND	<0.1

[a] ND, not detectable.
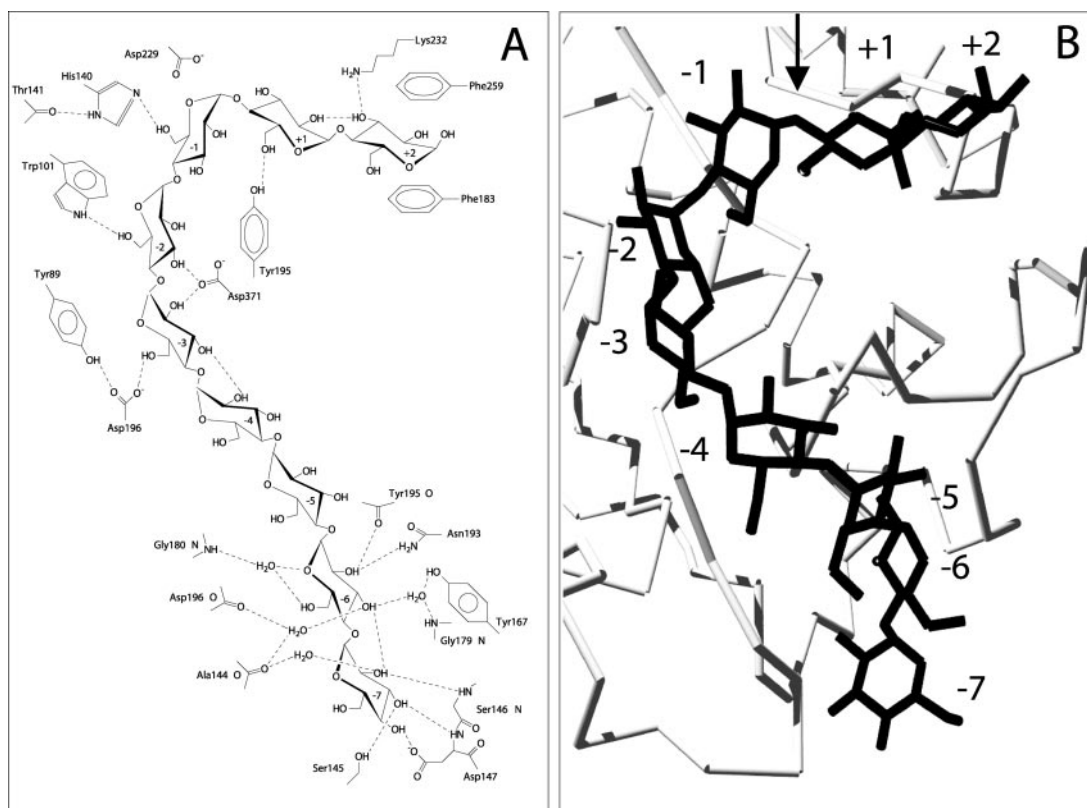
FIG. 1. *A*, schematic overview of the interactions between the *B. circulans* strain 251 CGTase and a maltononaose substrate. For clarity not all interactions at subsites +1, −1, and −2 are shown (21). *B*, maltononaose (*black*) conformation in the active site of CGTase. The *arrow* indicates the cleavage site. *B* was made using Swiss-PDB-Viewer (41).

or both, are affected. Because subsite −6 provides several strong interactions with linear substrates, it has been suggested that this subsite selects for substrates of sufficient length for cyclodextrin formation (13). In addition, it was suggested that Gly-179 and Gly-180 are conserved in CGTases, because the absence of side chains is a requirement for substrate binding at subsite −6 (21). The increased K_m values (Table III) for the maltoheptaose compound used in the disproportionation reaction (EPS) show that linear substrate binding is indeed hindered by mutations in Gly-179 and Gly-180. For G179L this is most likely caused by the presence of the leucine side chain, because the protein backbone conformation was hardly affected by this mutation. The especially strongly de-

creased α-cyclization activity of the G179L mutant (Table II) can be explained by the assistance of subsite −7 in binding of the longer sugar chains required for β-, γ-, and δ-cyclization. This assistance of subsite −7 cannot occur in the α-cyclization reaction.

It has been derived that the ratio of $k_{cat,\ \beta\text{-cyclization}}/k_{cat,\ disproportionation}$ can be used as an indicator for cyclization efficiency (18). For the mutants discussed here, this ratio is decreased most drastically by mutation G180L (Table III), indicating that this mutation especially hampers the circularization process. Thus, a side chain at position 180 interferes strongly with the circularization process, explaining the conservation of Gly-180 in CGTases.
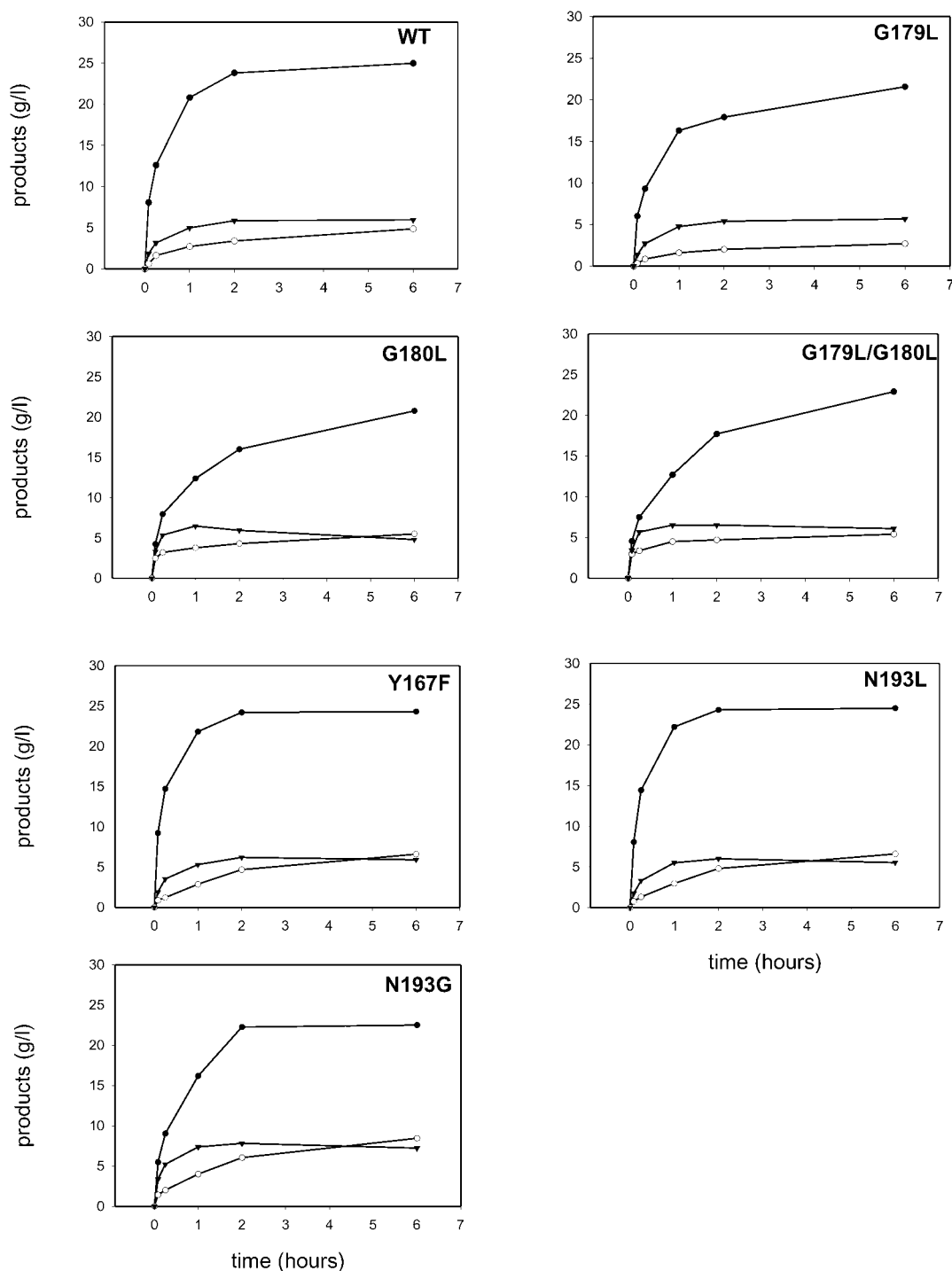
FIG. 2. **Production of cyclodextrins (g/liter) from 10% (w/v) pregelatinized starch by the action of (mutant)** *B. circulans* **strain 251 CGTases.** ○, ●, and ▼ indicate α-, β-, and γ-cyclodextrin, respectively. *WT*, wild-type.

Substrate Binding at Subsite −6 Activates the Enzyme in Catalysis—Substrate binding at subsite −6 has been suggested to stimulate processing of longer oligosaccharides (21). The mutants give clear evidence for this. The much lower k_{cat} and $k_{cat}/K_{m,EPS}$ values for disproportionation of the Gly-179 and Gly-180 mutants (Table III) indicate that substrate interactions at subsite −6 are important for the catalytic efficiency of the enzyme. Because subsite −6 is far away (>16 Å) from the catalytic nucleophile (Asp-229) (Fig. 1*B*) it is unlikely that the mutations directly affect bond cleavage. Indeed, the mutants Y167F, G180L, and N193L have wild-type activity with the shorter maltopentaose substrate (Table IV) but not with EPS

(Table III). The decreased disproportionation activities of G179L and N193G with the maltopentaose substrate are most likely caused by changes in structural flexibility, because the G179L and N193G structures showed no significant difference compared with the wild-type CGTase. Furthermore, the wild-type enzyme has a higher k_{cat} value with the longer EPS substrate than with the shorter G5-pNP substrate (see Table III and Table IV). Together this supports the presence of an induced-fit mechanism that is operated by substrate binding at subsite −6, as suggested by x-ray structure comparisons (21). This induced-fit mechanism can explain the high transglycosylation activity of CGTase with longer sugar chains (20) and the

TABLE VI
Cyclodextrin production from 10% (w/v) pregelatinized starch after 6 h of incubation with wild-type and mutant cyclodextrin glycosyltransferases from B. circulans strain 251

	Conversion of starch into CDs	α-CD	β-CD	γ-CD
	g/liter	*mass %*		
Wild-type	36 ± 1	13.6 ± 0.4	69.7 ± 1.1	16.7 ± 0.7
G179L	30 ± 1	9.0 ± 0.5	72.0 ± 1.3	19.0 ± 0.5
G180L	31 ± 1	17.7 ± 0.4	66.8 ± 0.9	15.5 ± 0.4
G179L/G180L	34 ± 1	15.7 ± 0.3	66.6 ± 1.1	17.7 ± 0.8
Y167F	36 ± 2	18.5 ± 0.3	64.9 ± 1.6	16.6 ± 0.6
N193G	37 ± 2	22.1 ± 0.5	59.0 ± 0.8	19.0 ± 1.0
N193L	35 ± 1	18.6 ± 0.2	65.9 ± 0.4	15.5 ± 0.3

conservation of subsite −6 in all known CGTases.

Function of Subsite −6 in the Coupling Reaction—Subsite −6 has no interactions with a cyclodextrin molecule bound in the active site of CGTase (21, 40). Unexpectedly, mutants in this subsite showed decreased coupling activities, demonstrating that subsite −6 is important for the coupling reaction. Especially mutation G179L drastically reduces the coupling activities, whereas its effect on the cyclization reaction is much smaller. Mutation G180L, in contrast, especially decreases the cyclization activities. In the coupling reaction binding of the cleaved cyclodextrin molecule to subsite −6 is necessary for efficient transfer of the covalent intermediate to the acceptor as shown by the decreased coupling activities. The strongly reduced acceptor affinities of G180L and N193G indicate that acceptor binding at subsite +1 is hampered in these mutants, although these subsite −6 residues are far from the acceptor subsite +1 (Fig. 1*B*). Therefore, we propose that attachment of the opened cyclodextrin molecule to subsite −6, together with acceptor binding at subsite +1, activates the enzyme in the coupling reaction. This proposal thus extends and strengthens a previous hypothesis based on structural results only (20).

Conclusions—This study shows that subsite −6 of CGTase is of great importance in all three transglycosylation reactions catalyzed by the enzyme but not in the hydrolysis reaction (Table II). The data provide for the first time definite biochemical support for a hypothesis based on x-ray crystallographic evidence (21) that substrate binding at subsite −6 activates an induced-fit mechanism. Such an induced-fit mechanism favors the processing of longer oligosaccharides. In addition, our results explain the conservation of Gly-180, because a larger residue interferes with the cyclization reaction. In addition, we provide clear evidence that it is possible to independently change the cyclization and coupling reactions.

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