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 2511 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 kcat 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); 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 (βα)_2-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. amylo maltases 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), Thermoaerobacterium thermosulfurigenes strain EM1 (10), Bacillus steatorrhophilus (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 (βα)_2-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 maltoligosaccharide 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
all reaction types, the disproportionation reaction is most efficiently catalyzed by CGTase (17, 19).

The transglycosidation 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. The appearance of the underlined restriction sites as follows:

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| Restriction Site | Sequence          |
|------------------|-------------------|
| Bln              | CCACG-3           |
| Pvu              | AATCTGC-3         |
| Bgl              | GGTGG-3           |
| Eag              | GAGCT-3           |
| EcoRI            | GCGAAG-3          |
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The conformation of Asn-139 and its position far from the catalytic site (see Fig. 1A) 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. 1B) 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). Seeding 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 Frank’s mirrors. Processing was done with DENZO and SCALEPACK (25). The structure of CGTase liganded with maltotetraose (PDB code 1CFH), 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 G193L 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 GX11 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 F̄̄′ − F and F̄̄′ − F 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 (Ade Strmc B araD139 Δ [araABC-leu]7679 lacX74 galU galK rpsL thi-1) (29) was used for recombinant DNA manipulations. CGTase (mutant) proteins were produced with the α-amylase and transglycosidase-negative Bacillus subtilis strain DB104A (amy his supR2 aprE18 aprA3) (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 BglII and SalI and exchanged for the corresponding fragment of pDP66k –. The following oligonucleotides were used to introduce the mutations: 5′-CTCCGGGGGGATTCCACAGCAAGGATAGCGAAAAACCCTG3′ (Y167F), 5′-CTACAAAGACGGTCTGACATCTCGACAGCTGAA CCAATAAC-3′ (N193L), 5′-GTCACCTAAACTGAGCTGCTGCATGAA AAGATCTTTAGCGACTTTTTCACAG-3′ (G179L), 5′-GTTCCACATCTGAAGCAGACATCTTTTCACAG-3′ (G180L), and 5′-GTTCCACATCTGAAGCAGACATCTTTTCACAG-3′ (G179L/G180L). Successful mutagenesis resulted in the appearance of the underlined restriction sites as follows: Bln for
Substrate Binding Subsite \textit{\textendash}6 Controls Transferase Activity

### TABLE II

|          | \( k_{\text{cat}} \) \( \alpha\text{-CD} \) | \( k_{\text{cat}} \) \( \beta\text{-CD} \) | \( k_{\text{cat}} \) \( \gamma\text{-CD} \) | \( k_{\text{cat}} \) \( \delta\text{-CD} \) | \( k_{\text{cat}} \) Hydrolysis |
|----------|--------------------------------------------|--------------------------------------------|--------------------------------------------|--------------------------------------------|---------------------------------|
| Wild-type| 17.0 \( \pm \) 1.0                        | 328 \( \pm \) 2                           | 61 \( \pm \) 2                              | 36.2 \( \pm \) 0.8                         | 4.0 \( \pm \) 0.2               |
| G179L    | 8.8 \( \pm \) 0.3                         | 269 \( \pm \) 3                           | 52 \( \pm \) 2                              | 24.9 \( \pm \) 0.5                         | 3.9 \( \pm \) 0.2               |
| G180L    | 25.5 \( \pm \) 0.4                        | 77 \( \pm \) 1                            | 24 \( \pm \) 1                              | 11.1 \( \pm \) 0.3                         | 3.9 \( \pm \) 0.2               |
| G179L/G180L | 21.4 \( \pm \) 0.4               | 81 \( \pm \) 1                            | 20 \( \pm \) 1                              | 12.0 \( \pm \) 0.4                         | 4.0 \( \pm \) 0.2               |
| Y167F    | 19.5 \( \pm \) 0.8                        | 278 \( \pm \) 9                           | 50 \( \pm \) 3                              | 29.3 \( \pm \) 1.5                         | 3.5 \( \pm \) 0.3               |
| N193G    | 25.8 \( \pm \) 0.9                        | 166 \( \pm \) 5                           | 64 \( \pm \) 5                              | 31.1 \( \pm \) 1.3                         | 4.3 \( \pm \) 0.3               |
| N193L    | 19.3 \( \pm \) 1.2                        | 516 \( \pm \) 8                           | 53 \( \pm \) 4                              | 31.1 \( \pm \) 1.1                         | 4.0 \( \pm \) 0.3               |

G179L, Hin\text{cII} for G180L and G179L/G180L, and Bgl\text{II} for N193G and N193L. Mutation Y167F removed an XmnI restriction site. All mutations were confirmed by DNA sequencing of the complete PstI/SulI 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 mg/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 = \( \mu \)mol 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-\( \alpha\)- or -\( \beta\)-glucopyranoside (M\text{Gp}) as acceptor substrate, using 0.1–0.5 mg/ml enzyme. Values of \( k_{\text{cat}} \) 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 mg/ml enzyme, 4-nitrophenyl-\( \alpha\)-\( \delta\)-maltoheptaoside-4-6-0-ethylidene (EPS; Roche Molecular Biochemicals), or 4-nitrophenyl-\( \alpha\)-\( \delta\)-maltoheptaoside (G5-pNP; Megazyme, County Wicklow, Ireland) as donor substrate and maltose as acceptor substrate. With the EPS substrate, values of \( k_{\text{cat}} \) 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 \( k_{\text{cat}} \) and \( K_m \) were determined by measuring rates at 12 different donor concentrations in fixed maltose concentration (10 mM).

Hydrolyzing activity was determined as described before (3) by measuring the increase in reducing power upon incubation of 5 \( \mu \)g of enzyme with 1% (w/v) soluble starch (Lamers & Pleuger, Wijnegen, Belgium). HPLC Analysis—Products formed were analyzed by HPLC, using an Econosphere NH\(_4\), 5 \( \mu \)m column (250 \times 4.6 mm) (Alltech Nederland bv; Breda, The Netherlands) linked to a refractive index detector. A mobile phase of acetonitrile/water (60:40) (w/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 Glu-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 \( \phi/\psi \) 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 \( \phi/\psi \) 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 affinities 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 substrate −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 \( K_m \) values. The results of 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 \( K_m \) value (Table III). The specificity constants \( k_{\text{cat}}/K_m \) (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
null
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 Km 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 decreased 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 kcat, α-cyclization/kcat, 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.

**Table V**

|           | Km,CD | Km,M,α-DG | Km,CD | Km,M,β-DG | kcat | 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 | 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 ± 0.2 | 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.
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 $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ 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. 1B) 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 $k_{\text{cat}}$ 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

**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.
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. The strongly reduced acceptor affinities of G180L and N193G indicate that subsite −6 is necessary for the 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. 1B). Therefore, we propose that attachment of the closed 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 definitive 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.

REFERENCES

1. French, D., Pulley, A. O., Effenberger, J. A., Rougier, M. A., and Abdullah, M. (1965) Arch. Biochem. Biophys. 111, 153−160
2. Terada, Y., Yanase, M., Takata, H., Takaha, T., and Okada, S. (1997) J. Biol. Chem. 272, 15729−15733
3. Penninga, D., Strokopytov, B., Rozeboom, H. C., Dijkstra, B. W., Bergsma, J., and Dijkhuizen, L. (1995) Biochemistry 34, 3368−3376
4. Henriët, B., and Davies, G. (1987) Curr. Opin. Struct. Biol. 7, 637−644
5. Janecsek, S. (1997) Prog. Biophys. Mol. Biol. 25, 67−97
6. Svensson, B. (1994) Plant Mol. Biol. 25, 141−157
7. McCarter, J. D., and Withers, S. G. (1994) Curr. Opin. Struct. Biol. 4, 885−892
8. Klein, C., and Schulz, G. E. (1991) J. Mol. Biol. 217, 737−750
9. Lawson, C. L., van Montfort, R., Strokopytov, B., Rozeboom, H. J., Kalk, K. H., de Vries, G. E., Penninga, D., Dijkhuizen, L., and Dijkstra, B. W. (1994) J. Mol. Biol. 236, 590−600
10. Knegt, R. M., Wind, R. D., Rozeboom, H. J., Kalk, K. H., Buitechlaar, B. M., Dijkhuizen, L., and Dijkstra, B. W. (1996) J. Mol. Biol. 256, 611−622
11. Kubota, M., Matsuura, Y., Sakai, S., and Katsube, Y. (1991) Denpun Kagaku 38, 141−146
12. Harata, K., Haga, K., Nakamura, A., Aoyagi, M., and Yamane, K. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. 52, 1136−1145
13. Strokopytov, B., Knegt, R. M., Penninga, D., Rozeboom, H. J., Kalk, K. H., Dijkhuizen, L., and Dijkstra, B. W. (1996) Biochemistry 35, 4241−4249
14. Penninga, D., van der Veen, B. A., van Hijum, S. A. F. T., Rozeboom, H. J., Kalk, K. H., Dijkstra, B. W., and Dijkhuizen, L. (1996) J. Biol. Chem. 271, 3277−3276
15. Mosi, R. S., Utdehaag, J. C. M., Dijkstra, B. W., and Withers, S. G. (1997) Biochemistry 36, 9927−9934
16. Utdehaag, J. C. M., Mosi, R. S., Kalk, K. H., van der Veen, B. A., Dijkhuizen, L., Withers, S. G., and Dijkstra, B. W. (1999) Nat. Struct. Biol. 6, 432−436
17. van der Veen, B. A., van Abeele, G. J., Utdehaag, J. C. M., Dijkstra, B. W., and Dijkhuizen, L. (2000) Eur. J. Biochem. 267, 658−665
18. Utdehaag, J. C. M., van der Veen, B. A., Dijkhuizen, L., Elber, R., and Dijkstra, B. W. (2001) Proteins Struct. Funct. Genet. 43, 327−335
19. Nakamura, A., Haga, K., and Yamane, K. (1994) FEBS Lett. 337, 66−70
20. Utdehaag, J. C. M., van Abeele, G. J., van der Veen, B. A., Dijkhuizen, L., and Dijkstra, B. W. (2000) Biochemistry 39, 7772−7780
21. Utdehaag, J. C. M., Kalk, K. H., van der Veen, B. A., Dijkhuizen, L., and Dijkstra, B. W. (1999) J. Biol. Chem. 274, 34668−34676
22. van der Veen, B. A., Leemhuis, H., Kralj, S., Utdehaag, J. C. M., Dijkstra, B. W., and Dijkhuizen, L. (2001) J. Biol. Chem. 276, 44557−44562
23. Nakamura, A., Haga, K., and Yamane, K. (1994) Biochemistry 33, 9929−9936
24. Nakamura, A., Haga, K., and Yamane, K. (1995) Biochemistry 34, 6624−6631
25. Gwosdowski, Z. (1995) in Data Collection and Processing (Sawyer, L., Isacca, N., and Bailey, S., eds), pp. 56−62, SERC Laboratory, Daresbury, United Kingdom
26. Brugger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Glos, P., Grosse-Kunstleve, R. W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 965−971
27. Kalchev, W. (1989) J. Appl. Crystallogr. 21, 916−924
28. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110−119
29. Meissner, P. S., Sisk, W. P., and Berman, M. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4171−4175
30. Smith, H. de Jong, A., Bron, S., and Venema, G. (1986) Gene 70, 351−361
31. Bron, S. (1990) in Modern Microbiological Methods for Bacillus (Harwood, C.R., and Cutting, S.M., eds), pp. 146−147, John Wiley & Sons, Inc., New York
32. Murray, V. (1989) Nucleic Acids Res. 17, 8889
33. van der Veen, B. A., Utdehaag, J. C. M., Penninga, D., van Abeele, G. J., Smith, L. M., Dijkstra, B. W., and Dijkhuizen, L. (2000) J. Mol. Biol. 296, 1027−1038
34. Knegt, R. M., Strokopytov, B., Penninga, D., Faber, O. G., Rozeboom, H. J., Kalk, K. H., Dijkhuizen, L., and Dijkstra, B. W. (1995) J. Mol. Biol. 256, 2926−2928
35. Bender, H. (1990) Carbohydr. Res. 206, 257−267
36. Wind, R. D., Utdehaag, J. C. M., Buitechlaar, R. M., Dijkstra, B. W., and Dijkhuizen, L. (1998) J. Biol. Chem. 273, 5771−5779
37. Kim, Y. H., Bae, K. H., Kim, T. J., Park, K. H., Lee, H. S., and Byun, S. M. (1997) Biochem. Mol. Biol. Int. 41, 227−234
38. Parsiegla, G., Schmidt, A. K., and Schulz, G. E. (1998) Eur. J. Biochem. 253, 710−717
39. van der Veen, B. A., Utdehaag, J. C. M., Dijkstra, B. W., and Dijkhuizen, L. (2000) Biochim. Biophys. Acta 1543, 336−340
40. Schmidt, A. K., Cottar, S., Driguez, H., and Schulz, G. E. (1998) Biochemistry 37, 5809−5815
41. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714−2723
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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