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The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms

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Cyclodextrin glycosyltransferase (CGTase) catalyzes three transglycosylation reactions via a double displacement mechanism involving a covalent enzyme–intermediate complex (substituted-enzyme intermediate). Characterization of the three transglycosylation reactions, however, revealed that they differ in their kinetic mechanisms. Disproportionation (cleavage of an α-glycosidic bond of a linear malto-oligosaccharide and transfer of one part to an acceptor substrate) proceeds according to a ping-pong mechanism. Cyclization (cleavage of an α-glycosidic bond in amylose or starch and subsequent formation of a cyclodextrin) is a single-substrate reaction with an affinity for the high molecular mass substrate used, which was too high to allow elucidation of the kinetic mechanism. Michaelis–Menten kinetics, however, have been observed using shorter amylose chains. Coupling (cleavage of an α-glycosidic bond in a cyclodextrin ring and transfer of the resulting linear malto-oligosaccharide to an acceptor substrate) proceeds according to a random ternary complex mechanism.

In view of the different kinetic mechanisms observed for the various reactions, which can be related to differences in substrate binding, it should be possible to mutagenize CGTase in such a manner that a single reaction is affected most strongly. Construction of CGTase mutants that synthesize linear oligosaccharides instead of cyclodextrins thus appears feasible. Furthermore, the rate of interconversion of linear and circular conformations of oligosaccharides in the cyclization and coupling reactions was found to determine the reaction rate. In the cyclization reaction this conversion rate, together with initial binding of the high molecular mass substrate, may determine the product specificity of the enzyme. These new insights will allow rational design of CGTase mutant enzymes synthesizing cyclodextrins of specific sizes.

Keywords: cyclodextrin glycosyltransferase; kinetic mechanism; transglycosylation; substrate binding; maltose binding site.

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a starch degrading enzyme belonging to the important α-amylase family (family 13) of glycosyl hydrolases [1]. All bacterial CGTases studied convert starch into a mixture of cyclodextrins mostly consisting of 6, 7 or 8 α(1,4)-linked glucose residues (α-, β- or γ-cyclodextrins, respectively) [2,3]. These cyclodextrins are cyclic molecules with a hydrophilic outside and a hydrophobic cavity that enables them to form specific inclusion complexes with small hydrophobic molecules [4]. Cyclodextrins are increasingly used in industrial and research applications [5]. At present, organic solvents are used for selective crystallization of α-, β- and γ-cyclodextrins. To avoid these expensive procedures, and to produce cyclodextrins for applications involving human consumption, the development of mutant CGTase enzymes that produce only one particular form of cyclodextrin is desirable.

The formation of cyclodextrins by CGTase proceeds through an intramolecular transglycosylation reaction. However, two other transglycosylation reactions are also catalyzed: coupling is the reverse reaction, in which a cyclodextrin ring is cleaved and transferred to a linear acceptor substrate, and disproportionation is the major transferase reaction, in which a linear malto-oligosaccharide is cleaved and transferred to a linear acceptor substrate. In addition, the enzyme has a weak hydrolyzing activity (Fig. 1) [6]. In the industrial production process for cyclodextrins all CGTase-catalyzed reactions participate in starch degradation and thus affect the overall efficiency and outcome of the process. Rational engineering of specific CGTases thus requires detailed knowledge of the kinetic mechanisms which drive the different reactions.

Extensive crystallographic and mutagenesis studies on members of the α-amylase family have shown that three

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Abbreviations: CGTase, cyclodextrin glycosyltransferase; MαDG, methyl α-D-glucopyranoside; EPS, 4-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene; MBS, maltose binding site.

Enzyme: cyclodextrin glycosyltransferase (EC 2.4.1.19).

**Note*: these authors contributed equally to this work,

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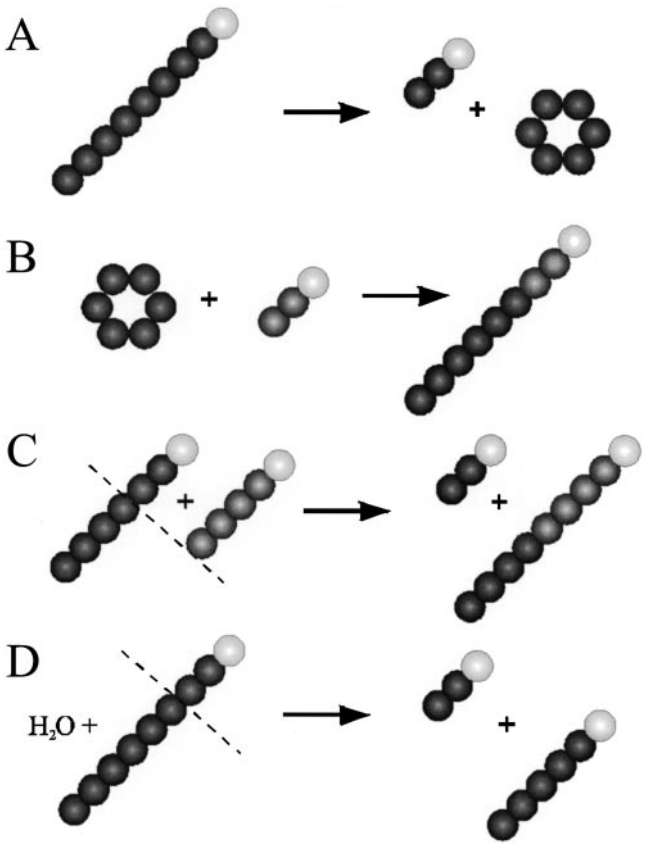

Fig. 1. Schematic representation of the CGTase-catalyzed transglycosylation reactions. (A) Cyclization; (B) coupling; (C) disproportionation; and (D) hydrolysis. The shaded circles represent glucose residues; the white circles indicate the reducing end glucoses.

invariant carboxylic amino acids (Asp229, Glu257 and Asp328, CGTase numbering) constitute the catalytic residues [7–9]. Catalysis in this family proceeds with retention of the substrate's anomeric α-configuration via a double displacement reaction involving a covalent enzyme–intermediate complex [10]. The kinetic mechanism is therefore expected to be a substituted-enzyme mechanism [11], usually a ping-pong mechanism in case of a two substrate reaction (Fig. 2A). For CGTase this mechanism can easily be imagined with the aid of a maltononaose substrate bound in the active site (Fig. 3), as derived from the X-ray structure of the enzyme complexed with a maltononaose inhibitor [12]. From this complex, either a cyclization or a disproportionation reaction can occur. In both cases the oligosaccharide chain is cleaved between subsites −1 and +1, and the glucose residue at subsite −1 is covalently linked to Asp229 [10,13]. Subsequently the residues bound at subsites +1 and +2 have to make space for the nonreducing end of the covalently linked maltoheptaose (cyclization), or an incoming acceptor substrate (disproportionation).

Here we present a detailed characterization of all three CGTase-catalyzed transglycosylation reactions and show that they proceed via different kinetic mechanisms. The differences in kinetic mechanisms reflect differences in the binding modes of the various substrates and products of the reactions. As a consequence it may be possible to manipulate each of the reactions separately by subtly changing the active site architecture by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Enzyme assays

The previously purified *Bacillus circulans* strain 251 wild-type CGTase enzyme described by Penninga *et al*. [14] was used. The various CGTase activities were measured by incubating appropriately diluted enzyme (0.1–0.2 units of specific activity for the specific reactions) at 50 °C with substrate solutions in 10 mM sodium citrate (pH 6.0).

Cyclization activity

This was determined using up to 5% Paselli SA2 (partially hydrolyzed potato starch with an average degree of polymerization of 50; Avebe, Foxhol, the Netherlands), as a substrate. Paselli SA2 was incubated in 10 mM citrate buffer (pH 6.0) for 10 min at 50 °C before the reaction was started with appropriately diluted CGTase. At regular time intervals (0.25–0.5 min), 100-μL samples were taken and added to 900 μL detection reagent: phenolphthalein [15] or bromocresol green [16] for the detection of β- or γ-cyclodextrin, respectively. α-Cyclodextrin formation was analyzed by HPLC using a 25-cm Econosphere-NH_2 5μ column (Alltech Associates Inc., City, USA) eluted with acetonitrile/water (60 : 40, v/v) at a flow rate of 1 mL·min^{-1}. One unit of activity is defined as the amount of enzyme able to produce 1 μmol of cyclodextrin per min.

Coupling activity

This determined as described previously [17] with the following modifications: α-cyclodextrin (Sigma C-4642), β-cyclodextrin (Fluka) and γ-cyclodextrin (Fluka) at concentrations of up to 20 mM, 5 mM and 1 mM, respectively, were used as donor in the reaction; up to 100 mM methyl α-D-glucopyranoside (MαDG, Fluka) was used as acceptor substrate. Cyclodextrin and MαDG were incubated in 10 mM citrate buffer (pH 6.0) for 10 min at 50 °C before the reaction was started with appropriately diluted CGTase. At regular time intervals (0.5 min), 100-μL samples were added to 20 μL 1.2 M HCl (4 °C) followed by incubation at 60 °C for 10 min to inactivate the CGTase. Subsequently, samples were neutralized with 20 μL 1.2 M NaOH and subjected to a 30-min incubation on ice with 60 μL (0.25 U) amyloglucosidase (E.C. 3.2.1.3, Sigma) in 167 mM NaAc (pH 4.5) to convert the products (linear oligosaccharides) to single glucose residues. The glucose concentration was determined with the glucose/glucose oxidase/peroxidase method (Boehringer Mannheim). One unit of activity is defined as the amount of enzyme coupling 1 μmol of cyclodextrin to MαDG per min.

Disproportionation activity

This measured using a modification of the method of Nakamura *et al*. [18]. The reaction mixture contained as a donor substrate up to 6 mM 4-nitrophenyl-α-D-maltoheptaoside-4–6-*O*-ethylidene (EPS), a maltoheptasaccharide blocked at the nonreducing end and with a *para*-nitrophenyl group at its reducing end (Boehringer Mannheim) and as acceptor substrate up to 10 mM maltose (Fluka) in 10 mM citrate buffer (pH 6.0). The mixtures were incubated for 10 min at 50 °C before the reaction was started with appropriately diluted CGTase. At regular time intervals (0.5 min), 100-μL samples were added to 20 μL 1.2 M HCl (4 °C) followed by incubation at 60 °C for 10 min to

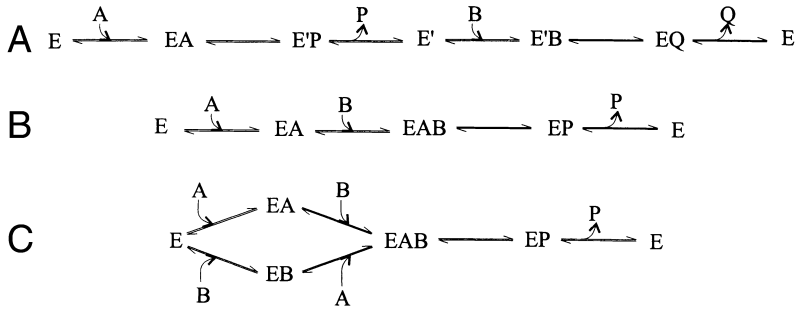

Fig. 2. Reaction mechanisms of two-substrate reactions. (A) Ping-pong (substituted-enzyme) mechanism, reaction rates at given concentrations A and B can be fitted according to Eqn (1); (B) compulsory order ternary complex mechanism, reaction rates at given concentrations A and B can be fitted according to Eqn (2); (C) random order ternary complex mechanism, reaction rates at given concentrations A and B can be fitted according to Eqn (3); A and B represent donor and acceptor substrates, respectively; E is the enzyme, and E′ is the substituted-enzyme intermediate (ping-pong mechanism).

inactivate the CGTase. Subsequently, the samples were neutralized with 20 μL 1.2 M NaOH and incubated for 60 min at 37 °C with 60 μL (1 U) α-glucosidase (E.C.3.2.1.20, Boehringer Mannheim) in 833 mM potassium phosphate (pH 7.0) to liberate *para*-nitrophenol from the product of the disproportionation reaction, nonblocked linear oligosaccharide. The pH of the samples was raised to above 8 by adding 1 mL 1 M sodium carbonate and the absorbance at 401 nm measured (ε_{401} = 18.4 mM^{-1}). One unit of activity is defined as the amount of enzyme converting 1 μmol of EPS per min.

Kinetic activity

Kinetic analysis of the results obtained with the various enzyme assays was performed using SIGMAPLOT (Jandel Scientific). The following equations [11] were used to fit the experimental data to determine which of the kinetic mechanisms depicted in Fig. 2 applies to the transglycosylation reactions catalyzed by CGTase:

Substituted-enzyme mechanism:

Ping-pong (Fig. 2A):

$$v = V{\cdot}a{\cdot}b/(K_{mB}{\cdot}a + K_{mA}{\cdot}b + a{\cdot}b) \quad (1)$$

Ternary complex mechanisms:

Compulsory-order (Fig. 2B):

$$v = V{\cdot}a{\cdot}b/(K'_{mA}{\cdot}K_{mB} + K_{mB}{\cdot}a + a{\cdot}b) \quad (2)$$

Random-order (Fig. 2C):

$$v = V{\cdot}a{\cdot}b/(K'_{mA}{\cdot}K_{mB} + K_{mB}{\cdot}a + K_{mA}{\cdot}b + a{\cdot}b) \quad (3)$$

In these equations: v is the reaction rate; V is the maximal reaction rate; a and b are the donor and acceptor substrate concentrations, respectively; A and B are the donor and acceptor substrates, respectively; and K_m and K'_m are the affinity constants for the substrates in the absence and presence of the second substrate, respectively. In Eqn (3), K'_{mB} is lost in the derivation of the equation, but it can easily be determined, because $K_{mA}/K_{mB} = K'_{mA}/K'_{mB}$ [11].

Protein concentrations were determined with the Bradford method using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA, USA).

RESULTS

The cyclization reaction requires a single substrate, from which two products are formed

Previously [14], evidence had been obtained that the cyclization reaction involves Hill kinetics when using the starch derivative Paselli SA2 (average degree of polymerization of 50) as a substrate. The current experiments, however, showed that in these studies activities at the lower substrate concentrations (< 0.05% Paselli SA2, as required to determine affinity constants) were underestimated. When incubating 0.015% Paselli SA2 with 0.6 μg of enzyme and samples taken at 30-s intervals, the reaction appeared to be linear in time, with a velocity of 30 $U{\cdot}mg^{-1}$ (β-cyclization). When using less enzyme (0.15 μg) and shorter time intervals (12 s), the apparent reaction rate shifted to 230 $U{\cdot}mg^{-1}$. In these latter experiments, however, the changes in phenolphthalein absorbance at the

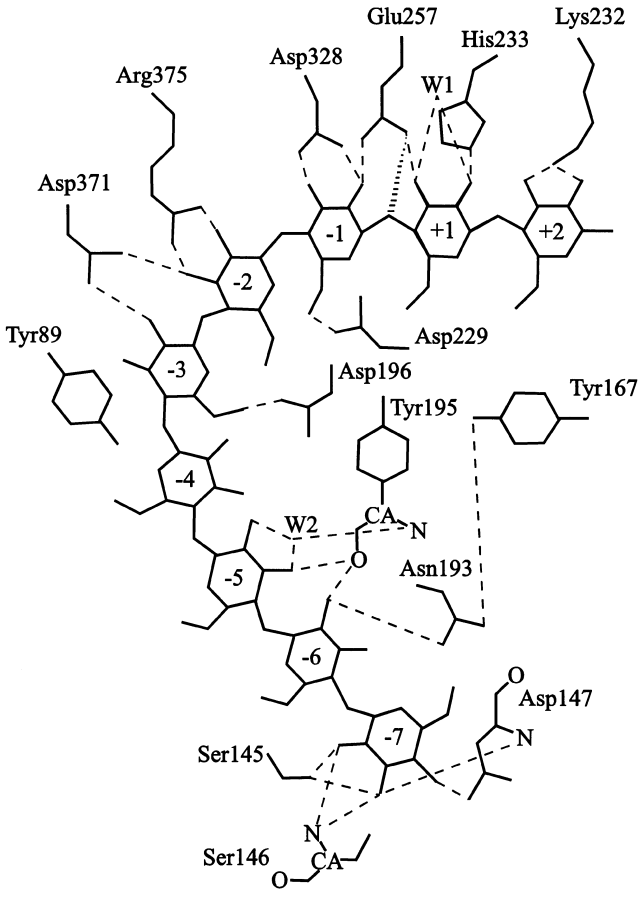

Fig. 3. Schematic representation of interactions between the *B. circulans* strain 251 CGTase and a maltononaose substrate bound at the active site. The maltononaose substrate was derived from the X-ray crystallographic structure of an acarbose derived maltononaose inhibitor bound in the CGTase active site [12]. Hydrogen-bonding interactions are indicated by dashed lines.

Table 1. Cyclization activities of CGTase from *B. circulans* strain 251.

Parameter	α-Cyclization	β-Cyclization	γ-Cyclization
V_{max} (U·mg^{-1})	20 ± 2.0	276 ± 4.4	53 ± 3.1
k_{cat} (s^{-1})	25 ± 2.5	345 ± 5.5	66 ± 3.6

lower substrate concentrations became too small (because only minute amounts of cyclodextrins are formed) to allow a reliable determination of the reaction rates. Hence no affinity constant for Paselli SA2 could be determined. The turnover rates (k_{cat}) for formation of α-, β- and γ-cyclodextrin at high substrate concentrations were determined as 25, 345 and 66 (s^{-1}), respectively (Table 1).

The coupling reaction is the reverse of the cyclization reaction

The coupling reaction is the reverse of the cyclization reaction and involves two substrates, which are combined to produce one product. One of the cyclodextrins is used as the donor molecule and MαDG is the acceptor molecule. The Lineweaver–Burk plots of 1/*v* versus 1/[cyclodextrin] at different acceptor concentrations (Fig. 4) did not result in parallel lines. The data could best be fitted according to Eqn (3), revealing a random ternary complex mechanism for the coupling reaction. Thus, both substrates can bind simultaneously and in random order to the active site cleft of CGTase (Fig. 2C). This leads to two apparent affinity constants for cyclodextrins and acceptor, one in the absence (K_m) and one in the presence (K'_m) of the other substrate (Table 2). Affinities for α-cyclodextrin are much lower than those for β- and γ-cyclodextrins, while the affinities for the acceptor are much higher in the α-cyclodextrin coupling reaction than in the β- and γ-cyclodextrin coupling reactions. In the presence of acceptor the affinity for α-cyclodextrin decreased, while the affinity for β-cyclodextrin increased, and for γ-cyclodextrin the affinity was hardly affected by the presence of acceptor (Table 2).

The disproportionation reaction is a two substrate reaction, yielding two products

EPS is used as the donor molecule and maltose as the acceptor molecule. The results could best be fitted according to Eqn (1) and the Lineweaver–Burk plots of 1/*v* versus 1/[maltose] at different EPS concentrations (Fig. 5A) and of 1/*v* versus 1/[EPS] at different maltose concentrations (Fig. 5B) resulted in parallel lines. This indicates that the disproportionation reaction is catalyzed according to a ping-pong mechanism, as expected for a reaction involving a substituted-enzyme intermediate. Calculation of the kinetic parameters (Table 3) revealed a very high turnover rate (k_{cat}) for the disproportionation reaction, with high affinities for both EPS and maltose. Despite the two-substrate character of the disproportionation reaction, its k_{cat} value is threefold higher than that for the cyclization reaction.

DISCUSSION

There are three glycosyltransferase reactions catalyzed by CGTase

The three glycosyltransferase reactions catalyzed by CGTase were analyzed in detail in the present study. Cyclization,

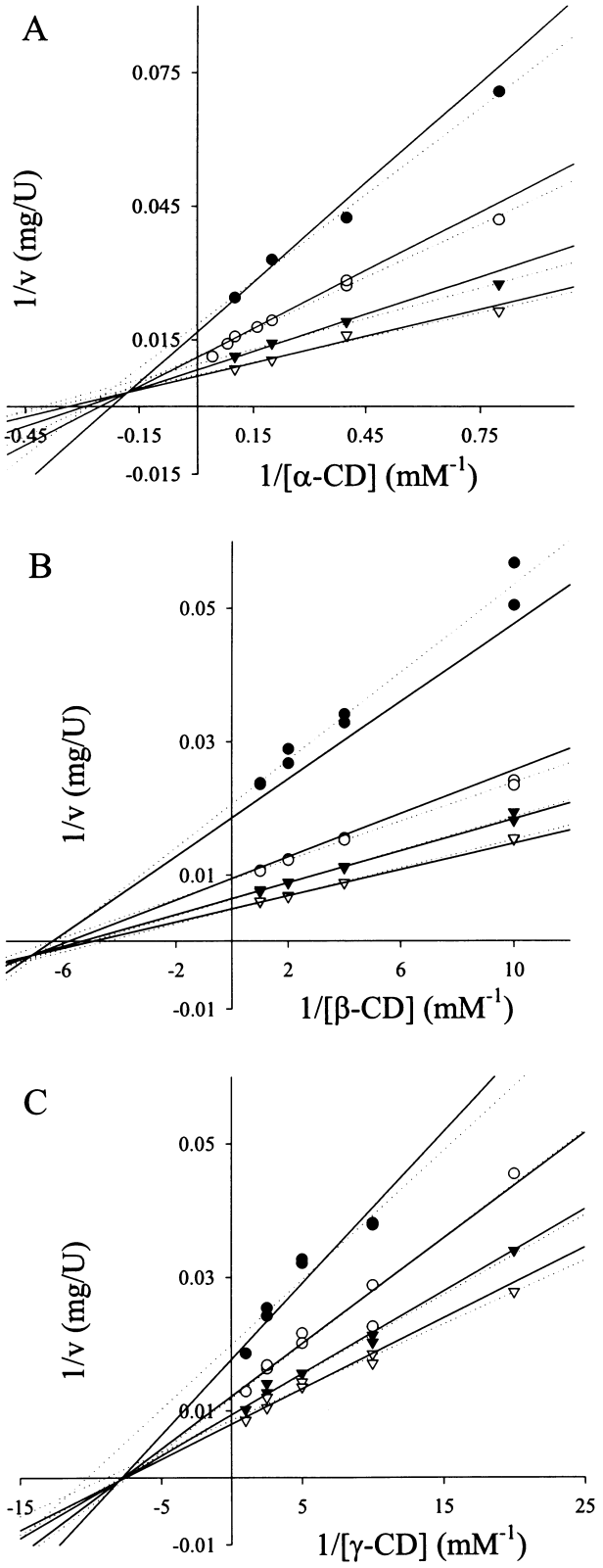

Fig. 4. Lineweaver–Burk plots of the coupling reaction. The reciprocal of the specific activity is plotted against the reciprocal of the α- (A), β- (B), and γ-cyclodextrin (C) concentration at fixed MαDG concentrations (0.2 mM (●), 0.4 mM (○), 0.8 mM (▼), and 1.6 mM (▽) for α-cyclodextrin; 4 mM (●), 10 mM (○), 20 mM (▼), and 40 mM (▽) for β-cyclodextrin; and 10 mM (●), 20 mM (○), 40 mM (▼), and 80 mM (▽) for γ-cyclodextrin). Linear regression results in the dotted lines. The calculated fit with the SIGMAPLOT program using Eqn (3) is represented by solid lines.

Table 2. Kinetic properties of the coupling reaction of CGTase from *B. circulans* strain 251. CD, cyclodextin.

	α-Cyclodextrin coupling	β-Cyclodextrin coupling	γ-Cyclodextrin coupling
K_m^{CD} (mM)	2.2 ± 0.3	0.32 ± 0.02	0.13 ± 0.02
$K_m^{M\alpha DG}$ (mM)	0.45 ± 0.05	18.1 ± 1.4	16.6 ± 3.0
K'^{CD}_m (mM)	5.3 ± 1.2	0.15 ± 0.04	0.12 ± 0.03
$K'^{M\alpha DG}_m$ (mM)	1.09 ± 0.26	8.5 ± 2.2	15.7 ± 6.1
V_{max} (U·mg^{-1})	192 ± 5.7	294 ± 7.6	150 ± 9.0
k_{cat} (s^{-1})	240 ± 7.1	368 ± 9.5	188 ± 11.3

coupling and disproportionation all involve the same catalytic residues (Asp 328, Glu 257 and Asp 229), and the chemical mechanism of catalysis by CGTase is a double-displacement reaction involving a covalent enzyme–intermediate complex [10]. Nevertheless, the transglycosylation reactions were found to proceed via different kinetic mechanisms.

The kinetic mechanism of the disproportionation reaction

The disproportionation reaction was found to be the only CGTase-catalyzed reaction proceeding according to a ping-pong mechanism (Fig. 2A). From the structural information showing the binding mode of a linear maltononaose in the active site (Fig. 3) [12] it is clear that the binding sites of the linear donor (EPS) and acceptor (maltose) substrates used overlap at least partially. The cleaved-off part of the donor substrate (occupying subsites +1 and +2; Fig. 3) has to dissociate from the active site before the acceptor substrate can bind at these acceptor subsites (Fig. 6A).

The kinetic mechanism of the coupling reaction

Whereas Nakamura *et al*. [18] hypothesized that the ping-pong mechanism would apply to all CGTase-catalyzed reactions, the coupling reaction is operated by a random ternary complex mechanism (Fig. 2C). This indicates that both the donor (cyclodextrin) and the acceptor (monosaccharide) are bound simultaneously to the active-site cleft before the cyclodextrin is processed. As the disproportionation reaction also proceeds via a ping-pong mechanism when a monosaccharide is used as acceptor (results not shown), the deviating kinetic mechanism of the coupling reaction must result from the use of a cyclic donor substrate. Recently the X-ray structure of the *B. circulans* strain 251 CGTase complexed with a γ-cyclodextrin [19] and *B. circulans* strain 8 GTase complexed with a β-cyclodextrin derivative [20] have been elucidated. These structural studies revealed that the bound cyclodextrins lack certain hydrogen bonding interactions at the acceptor site, notably at subsite +2 (Lys232), compared with the hydrogen bonding interactions with the linear substrate [12], as depicted in Fig. 3. However, the bound cyclodextrins leave no room for glucose binding at subsite +1, which is required for catalysis. Therefore, we hypothesize that in the ternary complex the monosaccharide is bound close to subsite +1 and that after cleavage of the cyclodextrin it moves to this subsite. This small displacement of the acceptor molecule would then be faster than the linearization of the covalently linked intermediate, explaining why it is not observed in the kinetic analysis. The

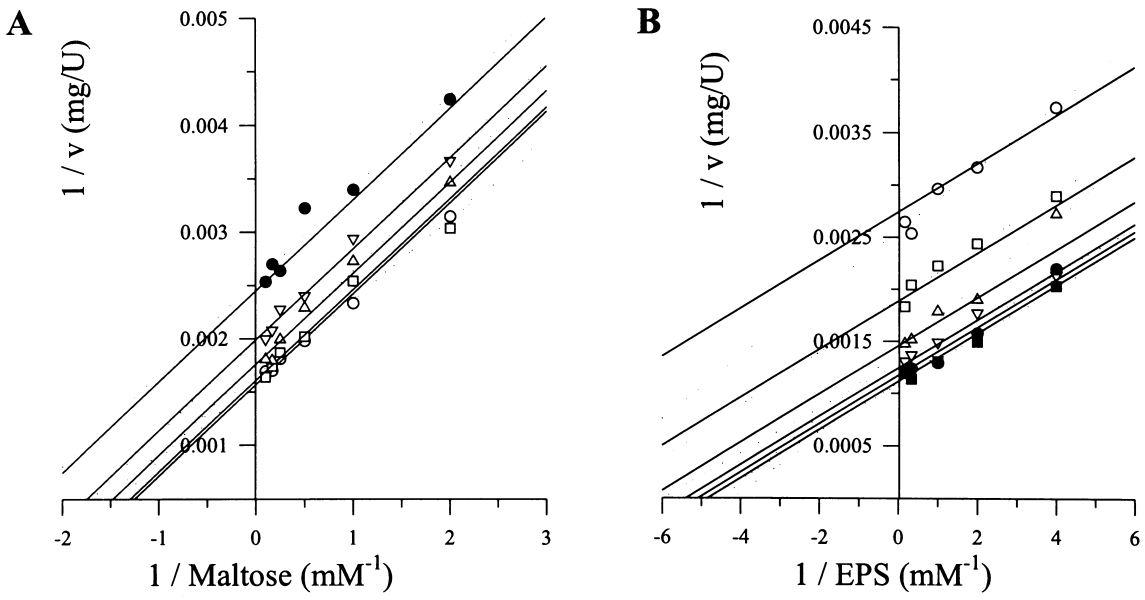

Fig. 5. Lineweaver–Burk and secondary plots of the diproportionation reaction. The reciprocal of the specific activity ($1/v$) is plotted against (A) the reciprocal of the maltose concentration at fixed EPS concentrations: 6 (○), 3 (□), 1(△), 0.5 (▽), and 0.25 (●) mM; and against (B) the reciprocal of the EPS concentration at fixed maltose concentrations:10 (●), 6 (■), 4 (▽), 2 (△), 1 (□), and 0.5 (○) mM. Linear regression results in the dotted lines. The calculated fit with the SIGMAPLOT program using Eqn (1); is represented by solid lines.

Table 3. Kinetic properties of the disproportionation reaction of CGTase from *B. circulans* strain 251.

Parameter	Value
K_m^{EPS} (mM)	0.223 ± 0.015
$K_m^{maltose}$ (mM)	0.827 ± 0.050
V_{max} (U·mg^{-1})	970 ± 17.6
k_{cat} (s^{-1})	1213 ± 22

kinetic mechanism is hence characterized as a ternary complex, whereas the events taking place at the active site resemble those of a ping-pong mechanism (see Fig. 6B).

The ternary complex formed in the coupling reaction

The monosaccharide acceptor must bind sufficiently close to the catalytic residues to result in a random ternary complex mechanism. Likely candidates for such a docking site for the monosaccharide are acceptor subsites +2 and +3. Although the

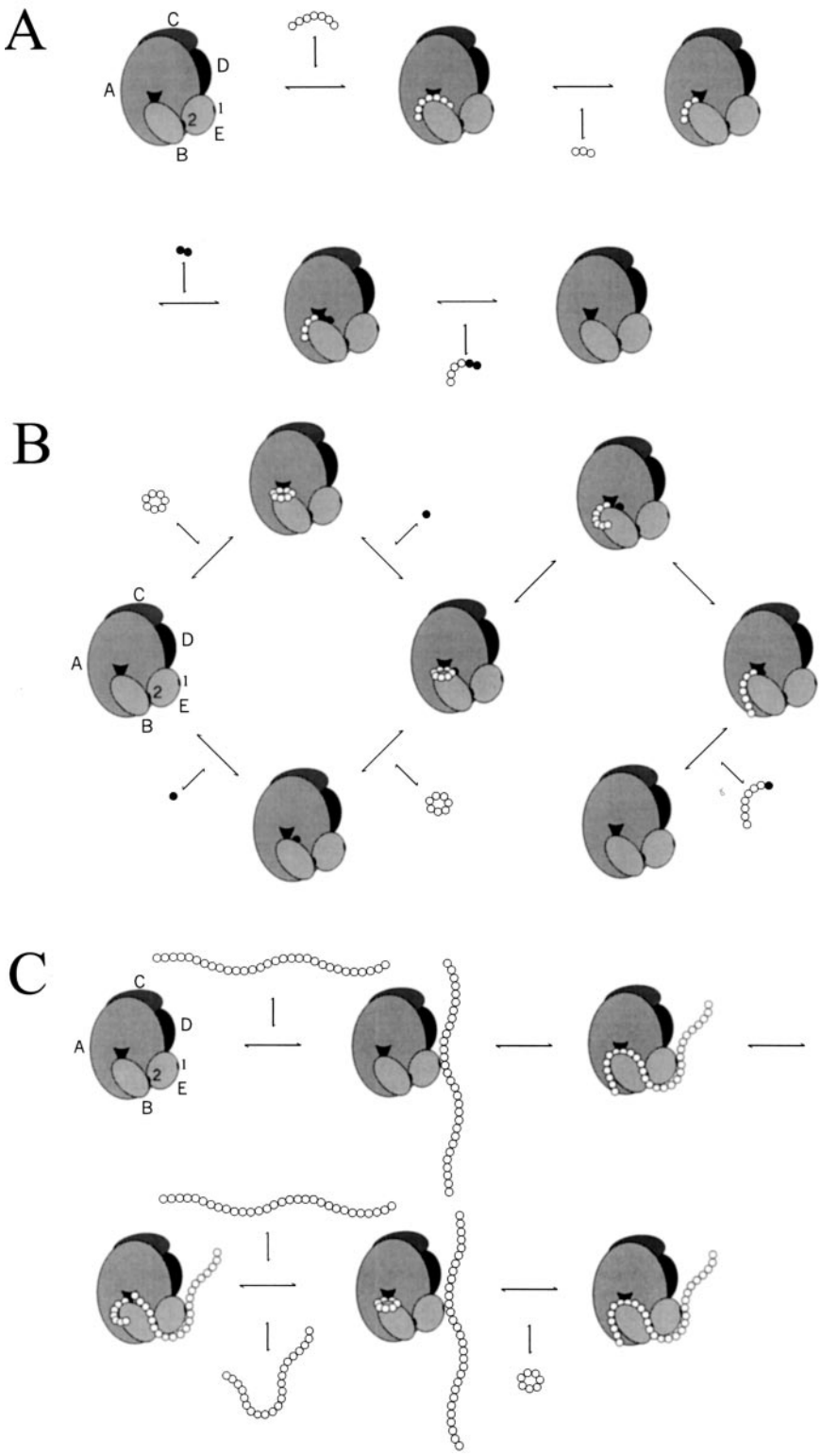

Fig. 6. Proposed model of the events taking place in the CGTase-catalyzed reactions. (A) Disproportionation, (B) coupling, (C) cyclization. The different CGTase domains are indicated (A, B, C, D, and E). 1 and 2 indicate the maltose binding sites on the E-domain. The triangle indicates the cleavage site in the active site. Circles represent glucose residues; acceptor residues are represented in black.

latter subsite has not been observed in structures of *B. circulans* strain 251 CGTase, evidence for its existence comes from the structure of *T. thermosulfurogenes* complexed with a maltohexaose, in which the side chain of Glu265 (Glu264 in *B. circulans* strain 251 CGTase) has a weak hydrogen bonding interaction (> 3.5 Å) with the glucose residue at subsite +3 [21]. The hydrogen-bonding interactions of Lys232 with a glucose residue at subsite +2 are much stronger (< 3 Å) and glucose binding at this subsite is further stabilized by hydrophobic interactions with Phe183 and Phe259 [12]. Binding of the smallest (α-) cyclodextrin may allow acceptor binding at subsite +2 in the ternary complex, while with β- and γ-cyclodextrin this subsite is less accessible, or only subsite +3 is available. This may account for the significantly higher affinity for the acceptor (lower K_m) in the α-cyclodextrin coupling reaction when compared to those in the β- and γ-cyclodextrin coupling reactions.

The mechanism of the cyclization reaction

Although the mechanism of cyclization could not be determined kinetically, from the results obtained with the disproportionation and coupling reactions combined with the fact that CGTase-catalyzed reactions proceed via a covalently linked intermediate, we propose a scheme for the cyclization reaction as depicted in Fig. 6C. In this scheme, the involvement of the maltose binding sites (MBS) located in the E-domain as observed previously [14] is included. The first step is binding of a starch chain at MBS1, after which this binding is extended to the active site via MBS2. Cleavage of the starch chain results in formation of the substituted-enzyme complex, followed by migration of the nonreducing end of the covalently linked intermediate to the acceptor site, resulting in the formation of a cyclodextrin (Fig. 6C). As shown by the ternary complex mechanism for the coupling reaction, cyclic and linear compounds can bind closely to each other in the active site (Fig. 6B). Thus, the starch chain bound to the MBSs does not have to move far to allow ring closure. Upon dissociation of the synthesized cyclodextrin, the same starch chain can immediately migrate further down in to the active site. Alternatively at MBS1 the bound starch chain may be replaced by another chain at any time during the reaction, without affecting the reaction taking place in the active site. Presumably, both phenomena occur leading to faster replacement of processed substrate, and resulting in higher affinities for high molecular mass substrates.

Implications for cyclodextrin product specificity

Apart from the differences in kinetic mechanisms, the CGTase-catalyzed transglycosylation reactions show notable differences in one further property. The reaction rate of disproportionation is threefold to fourfold higher than those of both cyclization and coupling. This may be explained by the involvement of only linear substrates and products in the disproportionation reaction. Consequently, the reaction rates of cyclization and coupling should be determined by the rate at which the conformation changes from linear substrate to circular product and from circular substrate to linear product, respectively. This rate of conformational change presumably differs for the various cyclodextrins, explaining the variation in cyclization and coupling activities between the cyclodextrins. Also the rates of linearization and circularization for the individual cyclodextrins are different. Particularly for α-cyclodextrin, the rate of formation through cyclization is much lower than that of degradation through coupling. Work showing that these differences are indeed linked to the conformational change of the oligosaccharide is currently in progress.

When comparing the cyclization and coupling reactions, another aspect has to be considered. For coupling, the different cyclodextrins can be added separately for measurements of specific activities, while for cyclization one substrate is used from which all cyclodextrins are produced simultanuously; α-, β- and γ-cyclization are therefore competing reactions. The number of glucose residues (6, 7 or 8) binding in the active site cleft past the cleavage site determines which cyclodextrin is formed (α-, β- or γ-cyclodextrin, respectively). The k_{cat} values for α-, and γ-cyclodextrin formation (25 and 66 s^{-1}, respectively), are much lower than the k_{cat} for β-cyclodextrin formation (345 s^{-1}). In addition to differences in circularization rates between oligosaccharides of different lengths, this observation may also be explained by the fact that the active-site cleft of this CGTase is specifically suited for binding 7 residues past the cleavage site (Fig. 3) [12]. Site-directed mutagenesis studies aimed at changing the interactions depicted in Fig. 3 are in progress.

In conclusion, the results clearly indicate different kinetic mechanisms for the CGTase-catalyzed transglycosylation reactions. The differences can be largely explained by the different binding modes of the substrates used in the reactions. This opens possibilities for the rational design of enzymes displaying specific activities. Recently, work based on the new insights presented here, combined with the detailed knowledge of the active site architecture, has led to the construction of CGTase mutants with enhanced α-cyclodextrin production (B. A. van der Veen, G.-J. W. M. van Alebeek, J. C. M. Uitdehaag, B. W. Dijkstra and L. Dijkhuizen, unpublished results). Current work involves rational design of mutants with decreased coupling activities and mutants with decreased transferase activities in favour of hydrolysis.

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