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What makes cyclodextrin glycosyltransferase a transglycosylase

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Chapter 2

Thermoanaerobacterium thermosulfurigenes Cyclodextrin Glycosyltransferase: Mechanism and Kinetics of Inhibition by Acarbose and Cyclodextrins

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SUMMARY

Cyclodextrin glycosyltransferase (CGTase) uses an α-retaining double displacement mechanism to catalyze three distinct transglycosylation reactions. To investigate these reactions as catalyzed by the CGTase from *Thermoanaerobacterium thermosulfurigenes* the enzyme was overproduced (8 mg/L culture) using *Bacillus subtilis* as a host. Detailed analysis revealed that the three reactions proceed via different kinetic mechanisms. The cyclization reaction (cyclodextrin formation from starch) is an one-substrate reaction, whereas the other two transglycosylation reactions are two-substrate reactions, which obey substituted enzyme mechanism kinetics (disproportionation reaction) or ternary complex mechanism kinetics (coupling reaction).

Analysis of the effects of acarbose and cyclodextrins on the disproportionation reaction revealed that cyclodextrins are competitive inhibitors, whereas acarbose is a mixed type of inhibitor. Our results showed that one molecule of acarbose binds either in the active site of the free enzyme, or at a secondary site of the enzyme-substrate complex. The mixed inhibition thus indicates the existence of a secondary sugar binding site near the active site of *T. thermosulfurigenes* CGTase.

INTRODUCTION

The α-amylase family, or glycoside hydrolase family 13 [25;81], is a large family of starch processing enzymes, which form a wide variety of oligosaccharide products [23;24], via an α-retaining double displacement mechanism involving a covalent glycosyl-enzyme intermediate [7;11;16].

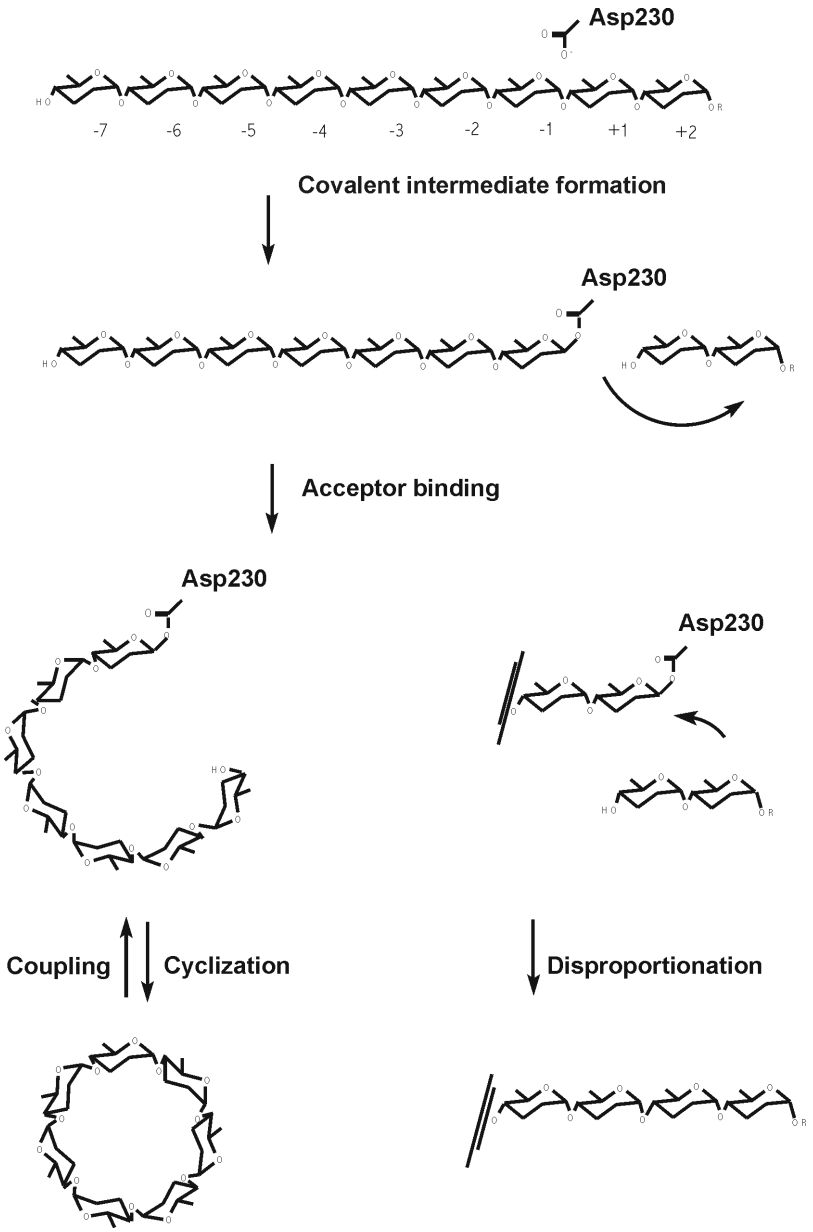

Figure 1. Schematic re-presentation of the reactions catalyzed by CGTase. In the first step of the reaction (bond cleavage) a covalently linked oligosaccharide intermediate is formed. In the second reaction step this oligosaccharide is transferred to an acceptor molecule.

Cyclodextrin glycosyltransferase (CGTase) is an unique member of this family, that forms circular α-(1,4)-linked oligosaccharides from starch (cyclodextrins). Cyclodextrins are composed of 6, 7 or 8 glucose residues, α-, β- and γ-cyclodextrin, respectively. CGTase consists of five domains (A-E) [32;69]. Domains A and B constitute the catalytic core, domain E is a raw starch binding domain [33;34], whereas the functions of the C and D domains are less well understood. CGTase binds its substrate across several sugar binding subsites (labeled –7 to +2 [40]) and cleaves it between the sugars bound at subsites –1 and +1 to form a covalently linked glycosyl-enzyme inter-mediate [11;16] (Fig. 1). Cyclodextrins are subsequently formed via an intra-molecular transglycosylation reaction with the non-reducing end of this intermediate (cyclization) (Fig. 1). CGTase may also transfer the covalently bound oligosaccharide to a second sugar (disproportionation) or to water (hydrolysis) (Fig. 1). A fourth reaction catalyzed by CGTase is the coupling reaction, in which a cyclodextrin molecule is cleaved and transferred to a second sugar. It is expected that an analysis of CGTase enzyme-starch interactions will provide detailed insights in the mechanisms and specificity of action of starch-acting enzymes. Moreover, CGTases are used in industry to produce cyclodextrins, which are used for their ability to form inclusion complexes with many hydrophobic molecules [105]. At present, the hydrolysis and coupling reactions of CGTase limit the cyclodextrin yield in the production process of cyclodextrins [106;107].

Acarbose, a pseudotetrasaccharide compound, is a strong inhibitor of many α-amylase family enzymes [108-114]. Three-dimensional structures of CGTases and α-amylases complexed with acarbose have revealed that acarbose binds in the active site [43;65]. However, in these structures acarbose often appears as a longer oligosaccharide derivative (Fig. 2), indicating that acarbose is slowly processed [61].

Here we report an analysis of the transglycosylation reactions catalyzed by the CGTase from *Thermoanaerobacterium thermosulfurigenes* strain EM1 (*Tabium*). The inhibitory effects of acarbose and cyclodextrins on the disproportionation reaction show that the inhibition mechanisms are different for the two compounds. Acarbose binds in the active site and at a secondary site to inhibit the disproportionation reaction, whereas only cyclodextrin binding in the active site results in inhibition.

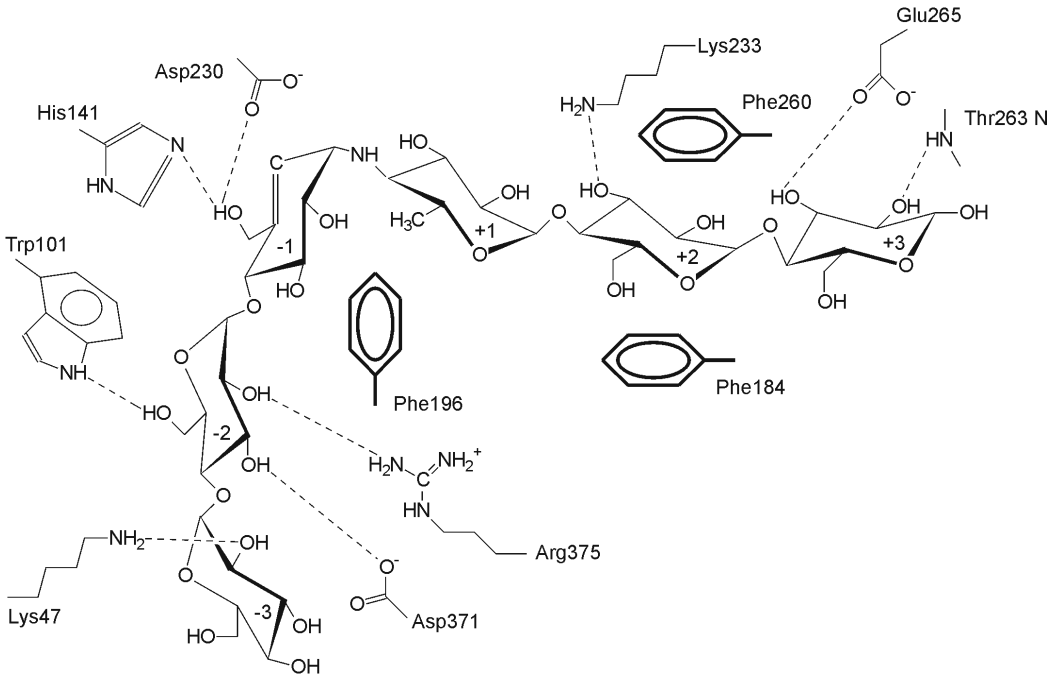

Figure 2. Schematic overview of the interactions between *T. thermosulfurigenes* CGTase and a maltohexaose inhibitor bound from subsites –3 to +3 [58]. The interactions at subsite +3 are weak (3.6 Å). For clarity, not all interactions at subsites –1 and +1 are shown.

MATERIALS AND METHODS

Construction of the expression vector - The *cgt* gene of *Tabium* was amplified from plasmid pCT2 [115] using an N-terminal primer (p1), that introduced an *Nco*I restriction site, and a primer p2 that removed the internal *Nco*I and *Hind*III restriction sites. The 375 bp product was used as primer in a second PCR reaction together with a C-terminal primer (p3) that introduced a *Hind*III restriction site. The 2100 bp product was cut with *Nco*I and *Hind*III and ligated in the pDP66k- vector [33], to obtain pCScgt-tt, which is an *Escherichia coli-Bacillus subtilis* shuttle vector. The following primers were used: p1, 5'-GGGGAACCATGGCTCCTGATACAGTAAG-'3 (*Nco*I); p2, 5'-CAGTAAAGCTGCCAAAGTATGGATTTGTTCTCTTAAAATCACGAGCCCAATAACCGTGGTACGATG-'3; p3, 5'-CCGGCAAGCTTATTATTTTAATCTATTG-'3 (*Hind*III). Restriction sites are underlined.

Production and purification of CGTase - CGTase protein was produced using a 4 L fermentor containing 3 l medium (10 g/L NaCl, 5 g/L yeast extract, 20 g/L tryptone and 10 g/L casamino acids) and *B. subtilis* DB104A as expression host [116], and purified from the culture supernatant as described

[68]. Plasmid carrying strains were grown at 37° C in the presence of 6 μg kanamycin/ml. Transformation of *B. subtilis* was done according to Bron [117].

Enzyme assays - All enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 60º C and repeated (at least) three times. The rates obtained are initial rates.

Cyclodextrin forming activities were determined by incubating 0.2 μg/ml enzyme with 2.5% (w/v) partially hydrolyzed potato starch (AVEBE, Foxhol, The Netherlands). Cyclodextrin concentrations were measured with phenolphthalein (β-cyclodextrin) [118] or with HPLC (α-, β- and γ-cyclodextrin) equipped with an Econosphere NH2 5U column (250 by 4.6 mm; Alltech, Breda, The Netherlands) eluted with acetonitrile/water (60/40 (v/v), 1 ml/min) [74].

Coupling activities were measured as described [75], using 0.05-0.2 μg/ml enzyme, α-, β- and γ-cyclodextrin (up to 10, 2.0 and 10 mM, respectively) as donor substrates and methyl-α-D-glucopyranoside (MαDG) as acceptor substrate (0-200 mM). The linear products formed were converted to glucose with amyloglucosidase (Sigma, Zwijndrecht, The Netherlands) and the amount of glucose formed was determined with the GOD-PAP reagent of Roche (Almere, The Netherlands).

Disproportionation activity was measured with 0.05 μg enzyme/ml and 0.075-1.5 mM 4-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene (EPS; Megazyme, County Wicklow, Ireland) as donor substrate. Glucose (2-100 mM), maltose (1-20 mM), maltotriose (0.25-10 mM) or maltotetraose (0.25-10 mM) were used as acceptor substrate. Reactions were performed in a glass cuvette (1 ml, light pathway of 1 cm) and activity was measured by liberating p-nitrophenol from the cleaved EPS, using an excess of thermostable α-glucosidase (Megazyme). Under these conditions the molar extinction coefficient of p-nitrophenol is 4.4 $mM^{-1}cm^{-1}$ at 401 nm.

Inhibition by acarbose and cyclodextrins was determined for the disproportionation reaction, with maltose as acceptor substrate at a fixed concentration of 25 mM (~5 times K_M). The acarbose concentrations were between 0.05 and 0.4 μM and those of α-, β- and γ-cyclodextrin were between 5-20 mM, 2.5-12 mM and 2.5-10 mM, respectively. The acarbose and cyclodextrin concentrations used did not significantly affect the action of the α-glucosidase used in the assay. Although cyclodextrins are the substrate of the coupling reactions and they were degraded during the disproportionation assay, the cyclodextrin concentrations did not change significantly during the assay

time (data not shown). Acarbose was a gift from J.H. Branolte (Bayer Nederland).

Kinetic analysis – The experimental data were analyzed using the SigmaPlot software(Jandel Scientific), with the following initial rate equations [119]:

(Eq. 1) $v=V\cdot a\cdot b/(K_{mb}\cdot a+K_{ma}\cdot b+a\cdot b)$ substituted-enzyme mechanism
(Eq. 2) $v=V\cdot a\cdot b/(K^*_{ma}\cdot K_{mb}+K_{mb}\cdot a+K_{ma}\cdot b+a\cdot b)$ ternary complex mechanism
(Eq. 3) $v=V\cdot a/\{K_m\cdot(1+i/K_{ic})+a\cdot(1+i/K_{iu})\}$ mixed inhibition
(Eq. 4) $v=V\cdot a/(K_m\cdot(1+i/K_{ic})+a)$ competitive inhibition

In these equations v is the reaction rate, V is the maximum reaction rate, a, b and i are the substrate and inhibitor concentrations, K_m and K^*_m are the concentrations at half maxium rate for the substrates in the absence and presence of a second substrate, respectively. K_{ic} and K_{iu} are the competitive and uncompetitive inhibition constants. Although K^*_{mb} is not shown in Eq. 2, it can be determined since $K_{ma}/K_{mb}=K^*_{ma}/K^*_{mb}$ [119]. The ternary complex mechanism equation applies to the compulsory and random type of this mechanism [119]. The substituted-enzyme and ternary complex mechanisms are two substrate reactions. In the substituted-enzyme mechanism the donor substrate is processed before the acceptor substrate binds to the enzyme, whereas in the ternary complex mechanism both substrates bind to the enzyme before the reaction starts [119].

RESULTS AND DISCUSSION

Enhanced production of CGTase protein - Previously, *Tabium* CGTase was produced using an *E. coli* PC1990 (pCT2) expression system, which yielded about 0.3 mg of purified protein per liter culture in a batch fermentor [58]. By constructing an *E. coli-B. subtilis* shuttle vector (pCScgt-tt) and using *B. subtilis* DB104A as expression host, resulting in extracellular production of CGTase, we succeeded in increasing the production level 27-fold to 8 mg per liter culture.

Cyclization reactions – All reactions start with substrate cleavage and covalent intermediate formation (Fig. 1). In the cyclization reaction this intermediate is circularized to form a cyclodextrin. *Tabium* CGTase forms α-cyclodextrin at the highest rate, although the β-cyclodextrin forming activity is also high (Table 1). K_m values for starch are not reported, since at the low

starch substrate concentrations needed, the amount of cyclodextrin formed is too low for reliable activity measurements. The K_m value, however, is clearly below 0.2 mg/ml of starch. Although *Tabium* CGTase produces more β- than α-cyclodextrinss after prolonged incubation [53;120] our results indicate that the initial α-cyclodextrin forming activity is higher than that of β-cyclodextrin formation (Table 1). Thus, the cyclization, coupling and disproportionation reactions affect the final ratio of α- and β-cyclodextrin formed upon prolonged incubation.

Table 1. Cyclization activities of *T. thermosulfurigenes* CGTase at 60° C.

	α-cyclodextrin	β-cyclodextrin	γ-cyclodextrin
k_{cat} (s^{-1})	375 ± 13	294 ± 4	61 ± 9

The disproportionation reaction follows a substituted-enzyme mechanism – In the disproportionation reaction an EPS (4-nitrophenyl-α-D-maltoheptaoside-4-6-O-ethylidene) molecule is cleaved and the reducing part is transferred to a sugar acceptor to form a linear product. Lineweaver-Burk plots of the initial velocity (v) at various EPS and maltose substrate concentrations resulted in parallel lines (Fig. 3), revealing a substituted-enzyme mechanism, as observed also for the disproportionation reactions catalyzed by *Bacillus circulans* and alkalophilic *Bacillus* CGTases [121;122]. The kinetic parameters were calculated with Eq. 1, revealing a high turnover rate and a low K_m value for the EPS substrate (Table 2). Such a substituted-enzyme mechanism makes sense,

Table 2. Kinetic parameters of the disproportionation reaction catalyzed by *T. thermosulfurigenes* CGTase at 60° C.

k_{cat} (s^{-1})	$K_{m,EPS}$ (mM)	$k_{cat}/K_{m,EPS}$ ($s^{-1}mM^{-1}$)	$K_{m,maltose}$ (mM)
1200 ± 37	0.21 ± 0.02	5714 ± 572	4.6 ± 0.3

as CGTase cleaves and reforms α-(1,4)-glycosidic bonds, which necessitates departure of the cleaved-off part of the donor substrate from the acceptor subsites before a new acceptor substrate can bind again at the acceptor subsites. X-ray structures [11;73] have indeed shown that uncleaved donor substrates bind at the donor and acceptor subsites simultaneously, indicating that an acceptor substrate can only bind after the leaving group has left the active site.

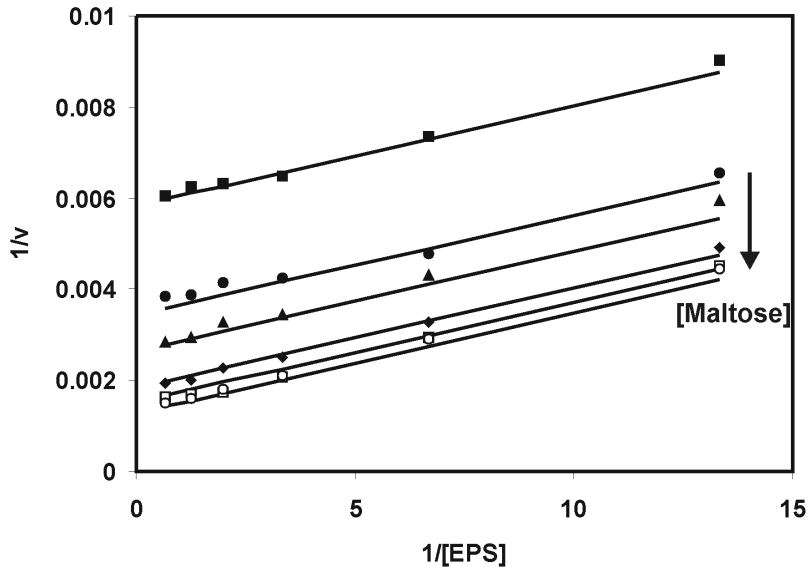

Figure 3. Lineweaver-Burk plots of the disproportionation reaction. The reciprocal velocities are plotted against 1/[EPS] at fixed maltose concentrations (1 (■), 2 (●), 3 (▲), 6 (♦), 10 (□) and 20 (○) mM). The calculated fit for the complete experimental data set (using Eq. 1) is represented by the lines and the symbols represent the experimental data.

Tabium CGTase has three acceptor subsites – Mutagenesis and crystallographic experiments have shown that CGTases contain at least two acceptor subsites [40;53;73;77;123;124]. The structure of a *Tabium* CGTase-hexasaccharide inhibitor complex (Fig. 2) suggested the presence of a third acceptor subsite, albeit with only two weak interactions at subsite +3 (3.6 Å). Moreover, these interactions might be an artefact of the crystallization as the +3 glucose moiety had a closer contact (3.4 Å) with a symmetry related CGTase molecule [58]. Also a mutant *B. circulans* CGTase suggested the presence of a +3 acceptor subsite, as an E264A mutant at subsite +3 (Glu265 in *Tabium*) had reduced disproportionation activity [124]. To investigate this in more detail for *Tabium* CGTase we determined the K_m values for acceptor substrates of increasing length (glucose, maltose, maltotriose and maltotetraose) using the disproportionation reaction. The donor substrate concentration was fixed at 2 mM (~10 times $K_{m,EPS}$). The acceptor substrate used had no large effect on the k_{cat} value (Table 3), but K_m values decreased from glucose to maltotriose, but not further for maltotetraose (Table 3). The lower K_m values for the longer acceptor substrates indicate that the longer acceptor substrates have more binding interactions. Thus, *Tabium* CGTase binds three

glucose units of the acceptor substrate, demonstrating the presence of three functional acceptor subsites.

Table 3. Kinetic parameters of the disproportionation reaction for four acceptor substrates at 60° C. The EPS concentration was fixed at 2 mM.

Acceptor	K_m (mM)	k_{cat} (s^{-1})
Glucose	11.7 ± 1.4	1019 ± 58
Maltose	4.6 ± 0.4	1200 ± 37
Maltotriose	1.2 ± 0.2	1115 ± 46
Maltotetraose	1.1 ± 0.1	1132 ± 41

The coupling reaction proceeds via a ternary complex – In the coupling reaction a cyclodextrin is cleaved and transferred to an acceptor sugar to yield a linear product. Lineweaver-Burk plots of the initial velocity (v) at various α-, β- and γ-cyclodextrin (donor) and MαDG (acceptor) substrate concentrations yielded straight lines of which the slope and the vertical axis intercept increased with decreasing MαDG concentrations, as shown for α-cyclodextrin (Fig. 4). Formation of a ternary complex can account for these results [119].

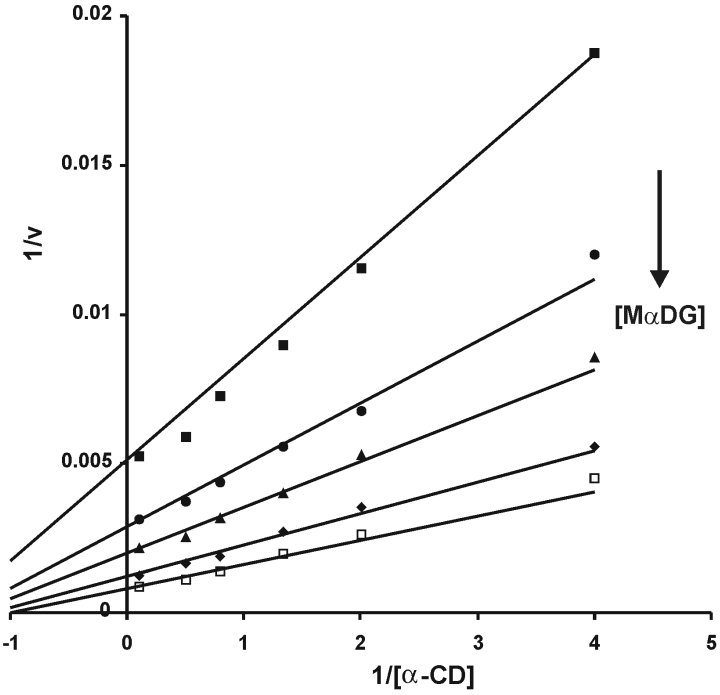

Figure 4. Lineweaver-Burk plot of the coupling reaction. The reciprocal velocities are plotted against 1/[α-CD] at fixed MαDG concentrations (3 (■), 6 (●), 10 (▲), 25 (♦) and 100 (□) mM). Lines represent the calculated fit for the complete experimental data set (using Eq. 2) and symbols represent the experimental data.

Whether the donor and acceptor substrates bind in a random or compulsory order to the enzyme can not be determined from these initial rate measurements, as both mechanisms of ternary complex formation have identical rate equations [119]. While the K_m values for α-, β- and γ-cyclodextrin are between 1 and 1.5 mM, they are much higher for the acceptor substrate MαDG (20-83 mM, depending on the type of cyclodextrin used as acceptor substrate) (Table 4). Interestingly, after binding of the first substrate (either cyclodextrin of MαDG) the K_m value for the second substrate decreased, as indicated by the lower K^*_m values (Table 4), suggesting cooperativity.

When a ternary complex is formed both substrates bind in the active site before the reaction starts. However, X-ray analysis has shown that intact cyclodextrin molecules bind across the donor and acceptor subsites [41;73], preventing acceptor binding at subsites +1/+2. Nevertheless, our kinetic results indicate the formation of a ternary complex during the coupling reaction of *Tabium* CGTase. This was also observed for the coupling reaction of *B.*

Table 4. Kinetic parameters of the coupling reactions catalyzed by *T. thermosulfurigenes* strain EM1 CGTase at 60° C. CD, cyclodextrin.

parameter	α-CD coupling	β-CD coupling	γ-CD coupling
$K_{m,CD}$ (mM)	1.1 ± 0.1	1.0 ± 0.1	1.5 ± 0.1
$K_{m,M\alpha DG}$ (mM)	20 ± 2	83 ± 14	45 ± 3
$K^*_{m,CD}$ (mM)[a]	0.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
$K^*_{m,M\alpha DG}$ (mM)[a]	11 ± 2	42 ± 11	30 ± 6
k_{cat} (s^{-1})	1586 ± 48	509 ± 44	588 ± 17

[a] K^*_m is the K_m value when the other substrate is already bound.

circulans CGTase [122]. To explain this, it has been suggest that the acceptor substrate binds close to subsite +1 and that it rapidly moves into subsite +1 after cyclodextrin-ring cleavage [122] (the cleaved cyclodextrin is now covalently linked to Asp230). The covalently bound oligosaccharide intermediate subsequently changes from a circular to a linear conformation [42]. If this linearization step is slow compared to the movement of the acceptor, the displacement of the acceptor will not appear in the kinetic analysis. Therefore, although the kinetic mechanism points to ternary complex formation, we deem a substituted enzyme mechanism, as observed for the disproportionation reaction, more likely. Although, subsite +3 seems to be a likely candidate to bind the acceptor substrate before cleavage of the

cyclodextrin, as the +1 and +2 subsites are blocked the cyclodextrin, this is unlikely as an E264A mutation at subsite +3 in *B. circulans* CGTase (Glu265 in *Tabium* CGTase) had no effect on the coupling reaction [124]. Thus, the data indicates the presence of sugar binding site in the vicinity of the catalytic site that has not (yet) been identified.

Inhibition by acarbose – Because *Tabium* CGTase has a very low K_m value for starch substrates, which make it impossible to perform reliable kinetic studies of the cyclization reaction, we used the disproportionation reaction with EPS to study the inhibitory effects of acarbose. A Lineweaver-Burk plot of the reciprocal velocity (1/v) against 1/[EPS] yielded straight lines of which the slope and the vertical axis intercept increased with increasing acarbose concentrations (Fig. 5), revealing a mixed type of inhibition.

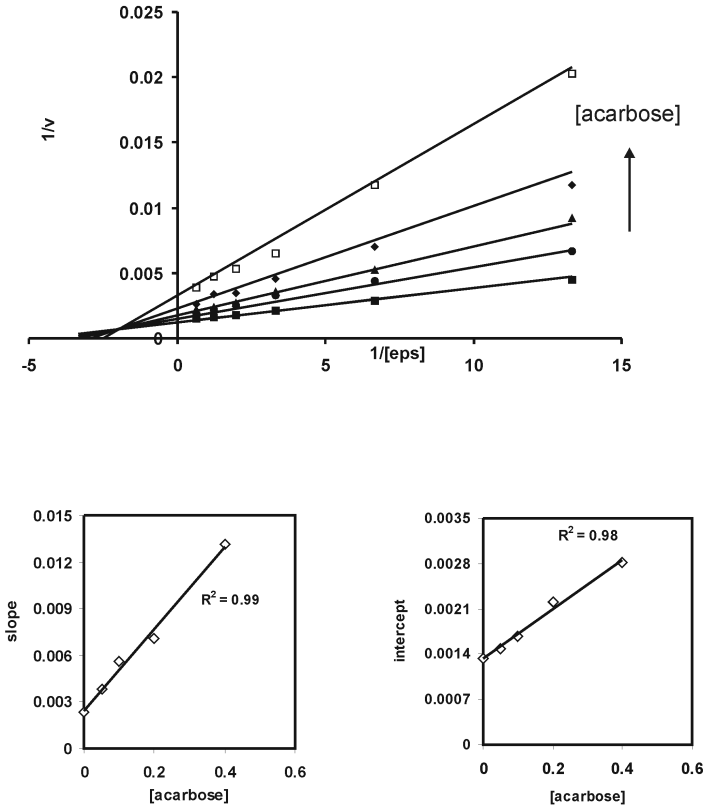

Figure 5. Lineweaver-Burk plot of the disproportionation reaction with acarbose as inhibitor. (A) The reciprocal velocities plotted against 1/[EPS] at different acarbose concentrations (0 (■), 0.05 (●), 0.1 (▲), 0.2 (♦) and 0.5 (□) µM). The lines represent the calculated fit for the complete experimental data set (using Eq. 3) and the symbols represent the experimental data. (B and C) Secondary plots showing the dependence of the slope and vertical axis intercept of the Lineweaver-Burk plot on the acarbose concentration.

Table 5. Inhibition constants of acarbose and cyclodextrins in the disproportionation reaction at 60° C.

Inhibitor	$K_{i,competitive}$	$K_{i,uncompetitive}$
Acarbose (μM)	0.10 ± 0.01	0.25 ± 0.04
α-cyclodextrin (mM)	3.4 ± 0.6	-
β-cyclodextrin (mM)	0.7 ± 0.1	-
γ-cyclodextrin (mM)	1.0 ± 0.1	-

Secondary plots of the slopes and vertical axis intercepts from the Lineweaver-Burk plot against the acarbose concentrations resulted in straight lines (Fig. 5), indicating that only one molecule of acarbose binds to free enzyme or to the enzyme-substrate complex, respectively [110]. A second order (parabolic) line would indicate the binding of a second acarbose molecule. The kinetic parameters (calculated with Eq. 3) show that acarbose is a powerful inhibitor, as indicated by the low K_i values (Table 5). Thus, the inhibition is caused by a single acarbose molecule, which binds either to the CGTase active site (competitive inhibition) or to a CGTase-EPS complex, in which acarbose is bound to a secondary site that is only accessible after EPS binding (uncompetitive inhibition). A schematic model of this mechanism is shown in Fig 6.

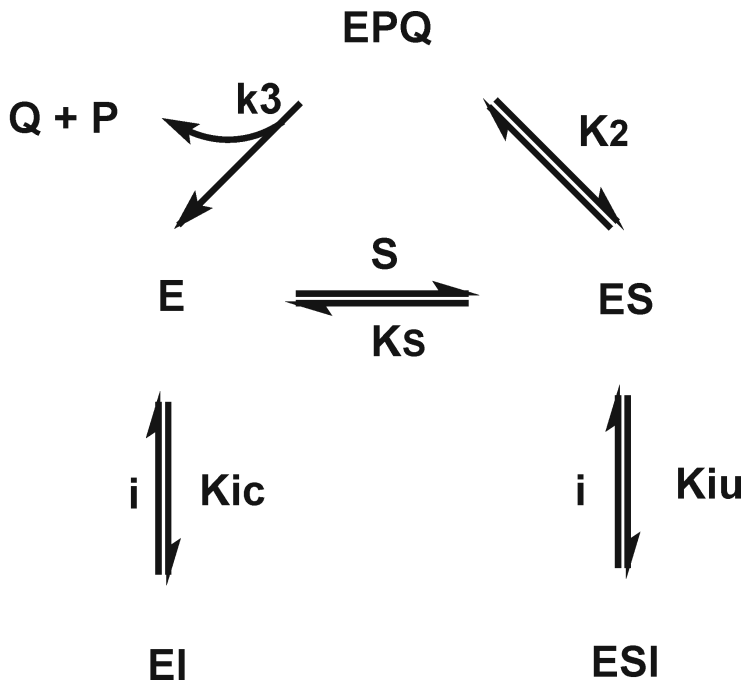

Figure 6. A schematic model of inhibition by acarbose on the disproportionation reaction (mixed-type). E, S, i, P and Q are the enzyme, substrate, inhibitor and the products respectively. K_S, K_{ic}, K_{iu}, K_2 are the dissociation constants and k_3 is a rate constant.

The precise location of this secondary acarbose binding site requires further investigation. Although X-ray structures have revealed the binding of sugar ligands to the surface of CGTase at various positions at the non-catalytic domains [32;43], binding of acarbose to those sites is unlikely to effect the disproportionation reaction, as they are far from the active site, in particular because the substrate used, the maltoheptaose compound EPS, is relatively short. Thus, the data reveals the presence of a secondary sugar binding site is in the vicinity of the active site.

Cyclodextrins are competitive inhibitors – Whereas cyclodextrins are the products of the cyclization reaction and the substrates for the coupling reaction, they act as inhibitors in the disproportionation reaction, competing with the EPS substrate, as previously observed for *B. circulans* CGTase [76]. Lineweaver-Burk plots of the initial disproportionation rates (v) at various EPS and α-, β- and γ-cyclodextrin (inhibitor) concentrations yielded straight lines that intersect at a single point on the vertical axis, as shown for α-cyclodextrin (Fig. 7), indicating competitive inhibition. The inhibition constants (Table 5), calculated using Eq. 4, are ~10^4-fold higher than those of acarbose (Table 5), showing that cyclodextrins are much less efficient inhibitors than acarbose.

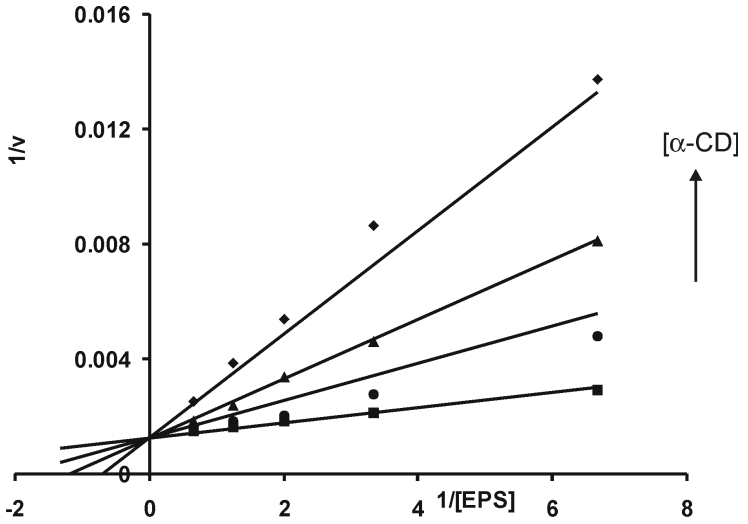

Figure 7. Lineweaver-Burk plot of the disproportionation reaction with α-CD as inhibitor. The reciprocal velocities are plotted against 1/[EPS] for different α-CD concentrations (0 (■), 5 (●), 10 (▲) and 20 (♦) mM). The calculated fit for the complete experimental data set (using Eq. 4) is represented by the lines and the symbols represent the experimental data.

X-ray analysis has shown that cyclodextrins bind in the active site as well as to the non-catalytic domains of CGTase [41]. The observation of competitive inhibition demonstrates, however, that only cyclodextrin binding in the active site inhibits the disproportionation reaction. This indicates that cyclodextrin binding to the non-catalytic domains either does not occur under the assay conditions, or that it has no effect on the disproportionation reaction. The latter explanation seems more likely, since the EPS substrate is too short to reach the non-catalytic domains. Thus, cyclodextrins give competitive inhibition in CGTase catalyzed disproportionation reaction involving a short substrate.

Acarbose and cyclodextrins bind both in the active site of CGTase; the difference between them is, however, that acarbose can also bind at a second site to inhibit the disproportionation reaction. To explain this difference we suggest that a cyclodextrin molecule is too bulky to bind at the secondary site, or that this secondary site has a much lower K_m value for acarbose than for cyclodextrins.

Conclusions – Characterization of the transglycosylation reactions catalyzed by *Tabium* CGTase revealed that they proceed via different kinetic mechanisms, similar to *B. circulans* CGTase [122]. Whereas the cyclization reaction is a single-substrate reaction, the disproportionation and coupling reactions are two-substrate reactions that have substituted-enzyme and ternary complex mechanism kinetics, respectively. The characterization also revealed that *Tabium* CGTase contains three functional acceptor sugar-binding subsites. Inhibition studies showed that cyclodextrins inhibit the disproportionation reaction in a competitive manner, whereas acarbose exerts a mixed-type of inhibition. The ternary complex mechanism kinetics (of the coupling reaction) as well as the mixed-type of inhibition (of the disproportionation reaction) indicate the presence of a secondary sugar binding site in the vicinity of the active site of *Tabium* CGTase.

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