Chemical modification of lysine side chains of cyclodextrin glycosyltransferase from *Thermoanaerobacter* causes a shift from cyclodextrin glycosyltransferase to α-amylase specificity

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Abstract cyclodextrin glycosyltransferases and α-amylases are two groups of enzymes with related secondary structures. However, cyclodextrin glycosyltransferases display transferase activities not present in α-amylases, probably derived from the existence of two more domains and different amino acid sequences. The hydrolytic activity of cyclodextrin glycosyltransferases is generally quite low, except for two cyclodextrin glycosyltransferases from termophiles. In this work, we have carried out the chemical modification (with acetic anhydride) of the amino groups of cyclodextrin glycosyltransferase from *Thermoanaerobacter* to assess their contributions to protein function. The acetylated cyclodextrin glycosyltransferase showed a significant reduction of its cyclization, coupling and disproportionation activities. Surprisingly, the hydrolytic (saccharifying) activity was slightly enhanced. These results suggest the participation of one or more lysine side chains in the interactions contributing to the transferase activity, either in any of the S_n subsites or in the acceptor binding site.

Key words: Cyclodextrin glycosyltransferase; α-Amylase; Cyclization; Coupling; Disproportionation; Hydrolysis; Cyclodextrin; Chemical modification

1. Introduction

Cyclodextrin glycosyltransferases (CGTases: EC 2.4.1.19) catalyse the formation of cyclodextrins (CDs) from starch and related α(1→4) linked glucose polymers via a transglycosylation reaction [1]. Three different CDs are known, α-, β- and γ-CD, that consist of 6, 7 and 8 α(1→4) linked D-glucose units, respectively. Each of these cyclic molecules is a torus (doughnut-shaped), with a hydrophilic shell and a hydrophobic central cavity [2]. CDs are widely used as complexing agents in food, pharmaceutical and cosmetic applications [3].

The CD forming enzymes are classified, depending on their specificity, as α-, β- or γ-CGTases. Theses enzymes are known to catalyze four different reactions: cyclization, coupling (opening of the rings of CDs and transfer to acceptors), disproportionation (transfer of linear malto-oligosaccharides to acceptors) and hydrolysis of starch. CGTases are functionally related to α-amylases [1,4,5]. It has been postulated that the two groups of enzymes are evolutionary related, the transferase activity of CGTase evolving from an ancestral hydrolase [6]. Despite their low overall degree of similarity in amino acid sequence, the secondary structures are very much related to each other.

Two highly thermostable CGTases have been isolated from thermophilic anaerobic microorganisms, belonging to the genus *Thermoanaerobacter* [7,8] and to *Thermoanaerobacterium thermosulfurigenes* EM1 (Tabium) [4,5,9], respectively. These enzymes exhibit similar biochemical characteristics and are able to degrade starch under liquefaction industrial conditions (15–30% starch, pH 5.0–6.0, 105°C, 5 min), thus avoiding the use of α-amylase pretreatment for the gelatinization or primary liquefaction of starch [8]. In this way, the enzymatic synthesis of CDs can be performed using one single enzyme (CGTase).

In general, CGTases posses a weak hydrolyzing activity. However, the Tabium CGTase exhibits an unusually high saccharifying activity (30 U/mg) [4], initially resulting in its misidentification as an α-amylase [9]. Saccharifying activities of CGTases reported in the literature are much lower, i.e. the CGTases from *B. circulans* strain 251 and *B. stearothermophilus* display saccharifying activities of 3.0 and 1.88 U/mg, respectively. The CGTase from *Thermoanaerobacter* is also effective in the hydrolysis of starch [7].

The CGTases from Tabium and *Thermoanaerobacter* have 683 residues, showing a great similarity in their amino acid compositions [5,7]. The optimum temperature for *Thermoanaerobacter* CGTase is 85°C at pH 5.5. This CGTase is able to convert about 30% of a 25% starch slurry into a mixture of CDs, with a ratio α:β:γ of 3:5:2.

The active site of α-amylases (which hydrolyze the α-1,4-glucosidic bonds of starch) and CGTases, located in the A domain, has three carboxylic residues involved in catalysis: Asp-230, Glu-258 and Asp-329 (Tabium CGTase numbering). However, two main structural features differentiate CGTases from α-amylases. First, in all the CGTases structurally characterized, an aromatic residue (Phe or Tyr) in position 196 (Tabium CGTase numbering) seems to play a central role as a cyclization axis during the CD formation [10]. Second, CGTases are larger and contain two additional domains (D and E).

An extraordinary effort is being done on CGTase site-directed mutagenesis, trying to get enzymes with an improved specificity towards a particular cyclodextrin or trying to reduce the coupling activity [4,5,10–12]. This is related to the great demand that exists for a process that could produce α-, β- or γ-CD at economic prices [8]. The isolation of pure CDs

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Abbreviations: CDs, cyclodextrins; CGTases, cyclodextrin glycosyltransferases; Tabium, *Thermoanaerobacterium thermosulfurigenes* EM1

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by crystallization/complexation methodologies (especially using organic solvents) presents significant difficulties.

Although rather less specific than protein engineering, chemical modification of enzymes has proven to be an interesting approach in order to alter the activity, stability, specificity and resistance to denaturants of numerous enzymes [13–15]. Group-specific chemical modification of proteins may be performed simply and rapidly and may give insight into the role of different amino acids of a given enzyme.

In the present work, and continuing our contribution to the study of the molecular features determining CGTase specificity [16], chemical modification of amino groups of CGTase from *Thermoanaerobacter*, that are normally charged at the pH (5.5) of CD production, was carried out using acetic anhydride (removal of charge to give an uncharged acetamide). The effect of this modification on the enzyme specificity was analyzed.

2. Materials and methods

CGTase from *Thermoanaerobacter* (strain 501) was kindly provided by Novo Nordisk. Acetic anhydride, phenolphthalein, bromocresol green, malto-oligosaccharides (G1–G7), α-, β- and γ-CD, methyl α-D-glucopyranoside, potato soluble starch, amyloglucosidase (E.C. 3.2.1.3) from *Aspergillus niger*, hydroxylamine and fluorescamine were purchased from Sigma. GOD-Perid reagent for the determination of glucose, *p*-nitrophenyl α-D-maltoheptaoside-4,6-O-ethylidene (EPS) and α-glucosidase (E.C. 3.2.1.20) from *S. cerevisiae* were from Boehringer, Mannheim. 3,5-dinitrosalicylic acid was from Fluka. Methyl orange was from Aldrich. Ampholites (pH 3–9) and pI markers for isoelectric focusing were from Bio-Rad. Paselli SA2 was donated by Avebe B.A. (Netherlands).

2.1. Enzyme purification

The CGTase was purified by affinity chromatography using a column (1×12 cm) packed with Sepharose-6FF (Pharmacia) covalently coupled to α-cyclodextrin. The gel was activated with α-cyclodextrin as described by Sundberg [17]. The binding capacity of the affinity column is 3.5 mg CGTase/ml gel. The crude enzyme (4 ml, containing 10 mg protein/ml) was loaded onto the column. The column was washed with 200 ml of 10 mM sodium acetate buffer (pH 5.5) at 2.5 ml/min and the bound CGTase was eluted with 50 ml of the same buffer containing α-cyclodextrin (10 mg/ml). Fractions containing the CGTase were pooled and stored at −20°C (in the presence of α-CD).

2.2. Assays of activity

The CGTase activities were measured at 85°C by incubating appropriately diluted enzyme with substrate solutions in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. Reactions were followed during 5–10 min: at time intervals of 1 min aliquots were removed and assayed using the corresponding reagent.

2.2.1. Cyclization activity. The production of CD was detected spectrophotometrically via the formation of inclusion complexes with several organic compounds. Paselli SA2 (partially hydrolyzed potato starch with an average degree of polymeration of 50) was used as substrate at final concentrations of 5% (w/v) for β- and γ-CD, and 2% (w/v) for α-CD. α-CD was determined at 490 nm on the basis of its ability to form a stable, colorless inclusion complex with methyl orange [18]. β-CD was determined at 552 nm on the basis of its ability to form a stable, colorless inclusion complex with phenolphtalein [10]. γ-CD was determined measuring the color increase at 630 nm due to the formation of an inclusion complex with bromocresol green [19]. One unit of activity was defined as the amount of enzyme able to produce 1 μmol of cyclodextrin per minute under the corresponding conditions.

2.2.2. Coupling. The reaction was based on the method described by Nakamura [12]. α- or β-cyclodextrins (2.5 mM) are used as donors and methyl α-D-glucopyranoside (10 mM) as an acceptor. The linear oligosaccharide formed in the reaction was converted into single glucose units by the action of amyloglucosidase. The amount of glucose was accurately detected with the glucose/GOD-Perid reagent [20]. One unit of activity was defined as the amount of enzyme able to convert 1 μmol of cyclodextrin per minute under the corresponding conditions.

2.2.3. Disproportionation. The reaction was based on the method described by Nakamura [21]. EPS (*p*-nitrophenyl α-D-maltoheptaoside-4-6-*O*-ethylidene, 3 mM) is used as a donor and maltose (10 mM) as an acceptor. In this reaction EPS is cleaved and the maltose is coupled to the free reducing end. The *p*-nitrophenol may be cleaved from the reaction product by the action of α-glucosidase. One unit of activity was defined as the amount of enzyme able to release 1 μmol of *p*-nitrophenol per minute under the corresponding conditions.

2.2.4. Saccharifying. Hydrolytic activity was assayed using 2% potato soluble starch as a substrate, measuring the increase in the reducing ends. The reducing power was accurately measured with dinitrosalicylic acid [22]. One unit of activity was defined as the amount of enzyme able to release 1 μmol of reducing end per minute under the corresponding conditions.

2.3. Production assay

The production of CDs and oligosaccharides was also assayed under similar conditions to those used in the industry. The CGTase (7 μg) was incubated at 85°C with 25% (w/v) Paselli SA2 in 10 ml of 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. At different times, aliquots of 300 μl were taken and mixed with 300 μl of 0.4 N NaOH in order to quench the reaction. Samples were centrifuged during 15 min at 10 000×*g* and further analyzed by HPLC using two Aminex HPX-42A columns (300×7.8 mm, Bio-Rad) put in series. Water was used as the mobile phase (0.7 ml/min). The column temperature was kept constant at 85°C. Detection was performed using a refraction index detector.

2.4. Chemical modification of amino groups

The purified CGTase (2.5 mg) was incubated in 9 ml of 10 mM phosphate buffer (pH 8) at 4°C and 250 rpm. Acetic anhydride (6 μl) was added (a molar ratio 1000:1 of reagent with respect to the protein was used). The reaction was maintained in a pH stat at pH 8.0 with 0.2 N NaOH. Once the reaction was finished (approximately 1 h), the mixture was dialyzed against 10 mM phosphate buffer (pH 7.0) at 4°C in order to remove salt and an excess of reagents. The sample was treated with 0.5 M hydroxylamine (pH 7.0) for 5 h at 25°C and dialyzed again. The degree of modification was estimated using the fluorimetric method described by Stocks [23]. The intensity of the fluorescence emission was measured at 475 nm after excitation at 390 nm.

2.5. Protein concentration

The protein concentration was determined using the Bio-Rad method, with immunoglobulin G (IgG) as a standard.

3. Results and discussion

3.1. Chemical modification of CGTase

CGTase from *Thermoanaerobacter* was purified by affinity chromatography using α-cyclodextrin covalently coupled to a Sepharose matrix. The recovery of β-cyclodextrin forming activity was 64%. Pure enzyme (checked by SDS/PAGE) with a specific β-cyclodextrin forming activity of 252 U/mg was obtained.

The ε-amino groups of Lys (and the N-terminal α-amino group) of CGTase were chemically modified with acetic anhydride. This reaction converts the positive charge of the amino groups to zero charge. However, short chain acid anhydrides display a low selectivity towards the acylation of amino groups [24]. Thus, the modification of other functions, such as hydroxylic groups of Tyr, thiol groups of Cys and amino groups of His, is also possible. His and Cys residues are spontaneously deacylated under the reaction conditions [25]. Nevertheless, for the regeneration of Tyr residues, it is necessary to treat the protein with hydroxylamine [25]. The remov-

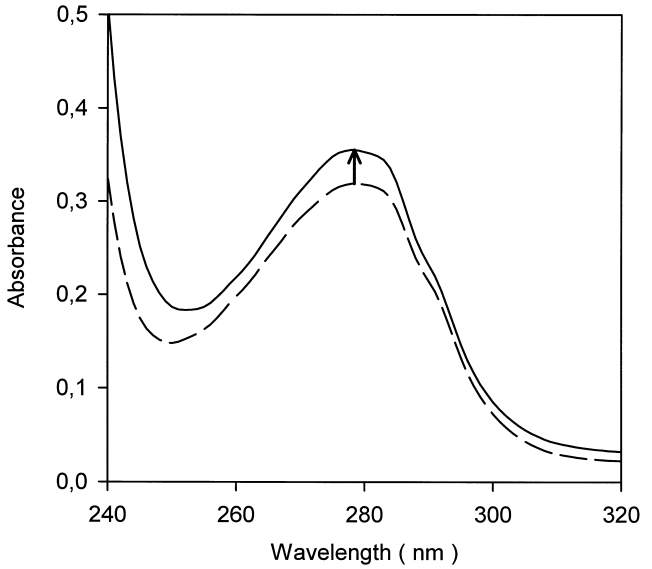

Fig. 1. Treatment of acetylated CGTase with hydroxylamine. UV-Vis spectra before (solid line) and after (dashed line) the treatment are shown. The arrow indicates the increment in the absorbance at 278 nm. Conditions: 0.5 M hydroxylamine, pH 7.0, 5 h.

al of acetic moieties is accompanied by an increase in the absorbance at 278 nm (ε_{278}:1160/M/cm). Fig. 1 shows the changes in the UV-Vis spectrum of chemically-modified CGTase upon treatment with hydroxylamine. The deacetylation was almost complete in 1 h: however, we maintained the reaction for 5 h to assure quantitative removal of acetyl groups from tyrosines. From the shift in the absorbance at 278 nm we calculated that 18% of the tyrosines (8 out of 44) were previously acylated and later deacetylated by the hydroxylamine treatment.

The CGTase from *Thermoanaerobacter* contains 23 Lys [7]. Fig. 2 illustrates the fluorimetric determination of the degree of substitution. Under the conditions described above, the acylation with acetic anhydride gives rise to a modification of the major part of the amino residues (85%, 20 NH_2 out of 24, including the N-terminal amino group). This large, nearly quantitative (and exclusive) modification of the amino groups in the protein assures that the product should be quite homogeneous. On the contrary, a low degree of modification would give a heterogeneous mixture of products, the resulting preparations more likely having a range of properties. As a consequence of the transformation of the positively charged amino groups into neutral acetamide moieties, a shift of the isoelectric point of CGTase from neutral (pI 6.3–6.7) to moderately acid values (pI 5.2–5.7) was observed (Fig. 3).

3.2. Effect of acetylation on activity

The cyclization, coupling, disproportionation and saccharifying activities of native and chemically-modified CGTases were first evaluated (Table 1). All the assays are referred to a short time (5–10 min) to avoid the interferences between the different processes. A blank of the modification process was also studied in the absence of acetic anhydride: it was concluded that the conditions of the reaction do not affect the CGTase properties.

Table 1 confirms that the main reaction catalyzed by CGTase is disproportionation [1]. Interestingly, the acetylation of CGTase amino groups produces a significant decrease in the specific activities, with the exception of the hydrolysis of starch. The rates of CD formation using acetylated CGTase were between 3- and 4-fold lower than those observed with the native glycosyltransferase and a similar effect takes place in the disproportionation process. Furthermore, the coupling of α- and β-CD with methyl α-D-glucopyranoside is 5 and 12 times faster, respectively, for the native enzyme compared to the acetylated preparation.

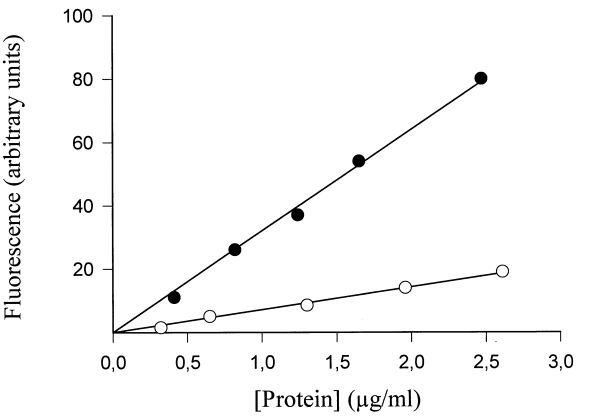

Fig. 2. Fluorimetric determination of the degree of modification using the fluorescamine method. Fluorescamine was bound to the free amino groups of the native (●) and acetylated (○) enzymes. The degree of substitution was estimated from the ratio of slopes of the plots representing the fluorescamine emission versus the protein concentration.

Surprisingly, the chemical modification of CGTase did not give rise to a significant decrease of the hydrolytic activity: the acetylated enzyme displayed a saccharifying activity even higher than the native CGTase. It is important to note that the saccharifying activity of native and chemically-modified CGTase (82 and 127 U/mg, respectively) are the highest values ever reported for a CGTase (a mutant of Tabium CGTase

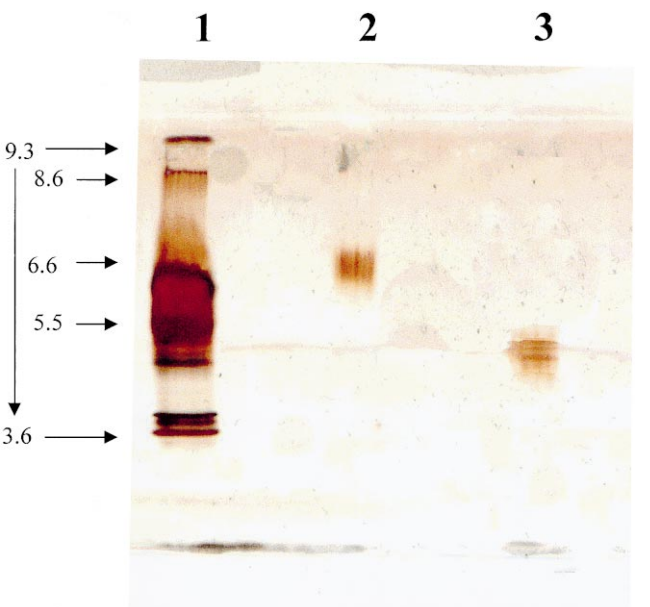

Fig. 3. Isoelectric focusing profile at pH 3–9 of native and acetylated CGTase. Lane 1, markers (pI 3.6–9.3); lane 2, native enzyme; lane 3, acetylated enzyme. Bands are visualized by silver staining.

Table 1
Initial activities of native and acetylated CGTases*

Activity (U/mg protein)							
	Cyclization			Coupling		Saccharifying	Disproportionation
	α	β	γ	α	β		
Native	289	252	149	270	48	82	1530
Acetylated	70	74	50	56	4.0	127	510

*Conditions described in Section 2.

displayed a hydrolytic activity of 65 U/mg [4] and the activity of most of the CGTases is very low, in the range of 1–3 U/mg).

The time course of CD formation, under similar conditions to those of industry, was analyzed for native and acetylated CGTase. The extraordinary saccharifying activity of CGTase from *Thermoanaerobacter* is definitely related to the presence of a high amount of short chain gluco-oligosaccharides in the production assay. Fig. 4 shows the HPLC chromatograms of the reaction mixture at 24 h and 8 days using native CGTase. After one day of reaction, the appearance of α-, β- and γ-CD is clearly far more significant than with respect to the formation of short oligosaccharides. However, when the reaction is allowed to progress, the production of oligosaccharides becomes more important. In conclusion, CGTase from *Thermoanaerobacter* is a glycosyltransferase exhibiting an exceptionally high α-amylase activity. The group-specific chemical modification of its amino groups with acetic anhydride produces a further significant shift towards α-amylase.

Regarding the effect of chemical modification on enzyme selectivity, Fig. 5 shows the production of α-, β- and γ-CD with time for native and acetylated CGTase. As expected from the short time activities in Table 1, the initial production of CDs is notably diminished upon acetylation. For the native CGTase the maximal CDs production is reached at 24 h, whereas for the acetylated enzyme the production of CDs is still increasing even after 8 days. This seems to be related to the low coupling activity of the acetylated CGTase (Table 1), which minimizes significantly the transformation of CDs into oligosaccharides. For the acetylated CGTase, the molar ratio of α:β:γ-CD after 8 days is 4.6:9.8:1 (at this point 22% of the initial Paselli is converted into CDs), whereas for the native enzyme, in the point of maximal cyclodextrin productivity (24 h) the ratio is 2.9:5.6:1 (20.5% Paselli converted into CDs). Regarding the formation of oligosaccharides, the modified enzyme exhibited a higher productivity, probably because of the higher saccharifying activity of the acetylated CGTase.

These results suggest the participation of one or more amino groups (normally as hydrogen bond donors) in the transglycosylation processes. In the cyclization process, amylose in the spiral state binds to the CGTase due to the ability of Phe-195 (the cyclization axis) to form an inclusion complex with amylose. The active center of CGTase has been proposed to contain at least eight subsites (designated as +5 to −3). The strength of the interactions of the glucose units with these subsites are believed to be responsible for the CD specificity in the cyclization process [5].

The amino acid sequences of CGTases from *Thermoanaerobacter* and Tabium display a great homology (91%, data not shown). In consequence, a similar overall architecture may be

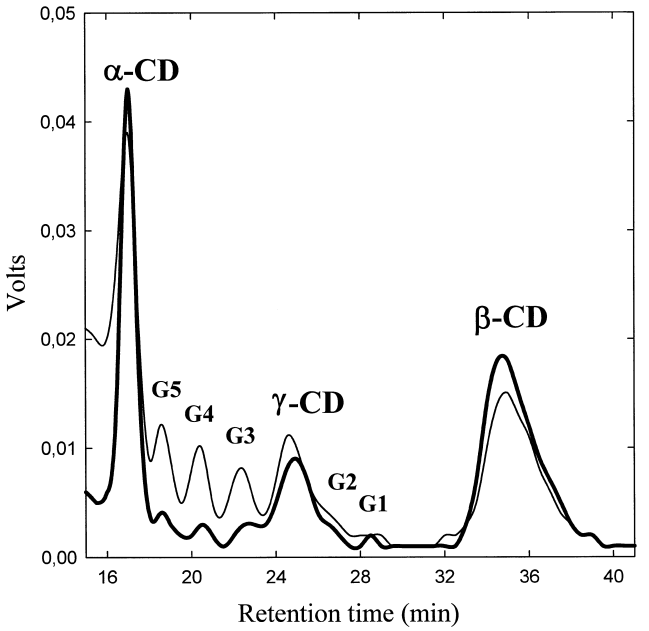

Fig. 4. HPLC chromatograms at 24 h (thick line) and 8 days (thin line) of the reaction mixture corresponding to the production assay using native CGTase. Conditions: 25% Paselli, 85°C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$.

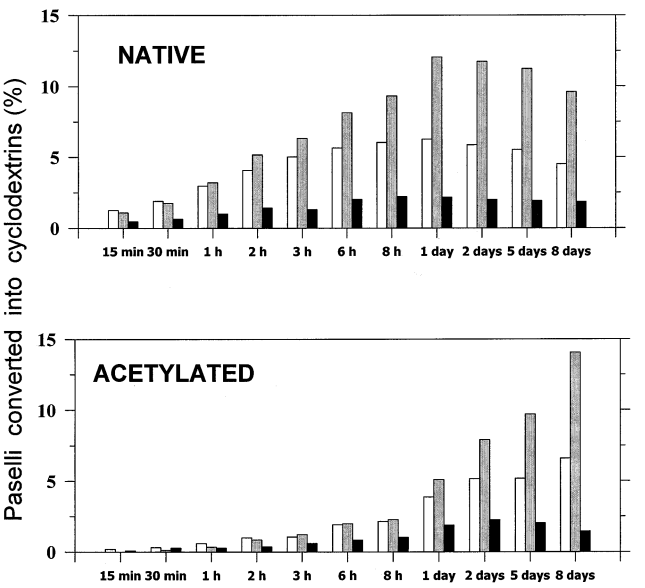

Fig. 5. Production of α- (□) β- (▨) and γ-CD (■) with time for native and acetylated CGTases. Conditions: 25% Paselli SA2, 7 μg CGTase, 85°C, 10 ml of 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$.

Table 2
Accessibility of the polar head of lysine residues of CGTase from *Thermoanaerobacter*. Calculations were made under the assumption that the three dimensional structure of this enzyme is similar to that of CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1

Residue	Relative accessibility (%)	Residue	Relative accessibility (%)
Lys-47	67	Lys-393	50
Lys-48	24	Lys-410	77
Lys-60	23	Lys-510	0
Lys-107	27	Lys-547	87
Lys-108	93	Lys-549	59
Lys-132	3.2	Lys-557	2.6
Lys-213	31	Lys-563	37
Lys-217	22	Lys-617	100
Lys-233	13	Lys-648	12
Lys-241	27	Lys-651	44
Lys-289	15	Lys-652	43
Lys-392	100	α-NH_2	75

assumed for both enzymes. Wind et al. [5] reported that Lys-47 and Lys-233 (also lysines in *Thermoanaerobacter*, with the same numbering) stabilize the conformation of subsites +3 and −2, respectively, in Tabium CGTase. In our work, the loss of cyclization activity might be related to the probable acetylation of these residues.

The only difference between the mechanisms of hydrolysis and intermolecular transglycosylation catalyzed by CGTase is the acceptor molecule, a water molecule (in the case of hydrolysis) or a carbohydrate C-4 hydroxyl group (in the case of transglycosylation) [4]. Considering that water has less space requirements and is much more easily activated, a feasible explanation for the low coupling and disproportionation activity of acetylated CGTase might be the involvement of any of the lysine residues in the acceptor binding site.

We calculated the accessibility for chemical modification of lysine residues from *Thermoanaerobacter* CGTase using the NACCESS software [26]. The calculations were made under the assumption that the three dimensional structure of this enzyme (not solved) is similar to that of Tabium CGTase [27]. In fact, 21 lysines (out of 23) occupy the same position in the amino acid sequences of both enzymes. As shown in Table 2, Lys-47 shows a high accessibility, supporting our hypothesis for the loss of cyclization activity. On the contrary, Lys-132, Lys-510 and Lys-557 are, theoretically, the less probable side chains to be modified (accessibility lower than 3.5%).

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