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Crystal Structure at 2.3 Å Resolution and Revised Nucleotide Sequence of the Thermostable Cyclodextrin Glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1

Ronald M. A. Knegtel¹, Richèle D. Wind², Henriëtte J. Rozeboom¹
Kor H. Kalk¹, Reinetta M. Buitelaar², Lubbert Dijkhuizen³ and Bauke W. Dijkstra*¹

¹BIOSON Research Institute and Laboratory of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4 9747 AG Groningen The Netherlands

²Agrotechnological Research Institute, P.O. Box 17, 6700 AA Wageningen The Netherlands

³Department of Microbiology Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30 9751 NN Haren The Netherlands

The crystal structure of the cyclodextrin glycosyltransferase (CGTase) from the thermophilic microorganism *Thermoanaerobacterium thermosulfurigenes* EM1 has been elucidated at 2.3 Å resolution. The final model consists of all 683 amino acid residues, two calcium ions and 343 water molecules, and has a crystallographic R-factor of 17.9% (Rfree 24.9%) with excellent stereochemistry.

The overall fold of the enzyme is highly similar to that reported for mesophilic CGTases and differences are observed only at surface loop regions. Closer inspection of these loop regions and comparison with other CGTase structures reveals that especially loops 88–95, 335–339 and 534–539 possibly contribute with novel hydrogen bonds and apolar contacts to the stabilization of the enzyme. Other structural features that might confer thermostability to the *T. thermosulfurigenes* EM1 CGTase are the introduction of five new salt-bridges and three Gly to Ala/Pro substitutions. The abundance of Ser, Thr and Tyr residues near the active site and oligosaccharide binding sites might explain the increased thermostability of CGTase in the presence of starch, by allowing amylase chains to bind non-specifically to the protein. Additional stabilization of the A/E domain interface through apolar contacts involves residues Phe273 and Tyr187. No additional or improved calcium binding is observed in the structure, suggesting that the observed stabilization in the presence of calcium ions is caused by the reduced exchange of calcium from the protein to the solvent, rendering it less susceptible to unfolding.

The 50% decrease in cyclization activity of the *T. thermosulfurigenes* EM1 CGTase compared with that of *B. circulans* strain 251 appears to be caused by the changes in the conformation and amino acid composition of the 88–95 loop. In the *T. thermosulfurigenes* EM1 CGTase there is no residue homologous to Tyr89, which was observed to take part in stacking interactions with bound substrate in the case of the *B. circulans* strain 251 CGTase. The lack of this interaction in the enzyme-substrate complex is expected to destabilize bound substrates prior to cyclization. Apparently, some catalytic functionality of CGTase has been sacrificed for the sake of structural stability by modifying loop regions near the active site.

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Keywords: X-ray structure; cyclodextrin glucanotransferase; cyclodextrin glycosyl transferase; thermostability; nucleotide sequence

Abbreviations used: BC8, *Bacillus circulans* strain 8; BC251, *Bacillus circulans* strain 251; CGTase, cyclodextrin glycosyltransferase; Hapes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; r.m.s., root-mean-square; α, β, γ-CD, α, β, γ-cyclodextrin.
Introduction

The commercial production of cyclodextrins (CDs) for use as complexing agents in food, pharmaceutical and cosmetic applications (Széchthl, 1982), makes use of bacterial enzymes called cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19). Three different types of CDs, consisting of six, seven or eight glucopyranose units, respectively, find applications in industry and these are commonly referred to as α-, β- or γ-cyclodextrins. Depending on the major product of the cyclization reaction, CGTases are classified as α-, β- or γ-CGTases (Schmid, 1989). Besides producing mixtures of differently sized cyclodextrins from starch, CGTases are capable of performing coupling, disproportionation and hydrolytic reactions (Penneinga et al., 1995). The limited product specificity, stability and the inhibition of the enzyme by its products have instigated us to perform protein engineering and crystallographic studies of CGTases with the aim of rationally designing enzymes with improved properties. Recently, we have reported the three-dimensional structure of the CGTase from Bacillus circulans strain 251 (Lawson et al., 1994) and its complexes with inhibitors (Strokopytov et al., 1995; B. Štrokopytov et al., unpublished results) and natural substrates and products (Knegtel et al., 1995). The enzyme was found to consist of five domains, designated A through E with the catalytic carboxylates Asp229, Glu257 and Asp328 being located at the C-terminal end of the α-strands of the (β/α)8-barrel A-domain. Three oligosaccharide binding sites located in the E and C domains were observed to bind three maltose molecules by means of hydrogen bonds and stacking interactions with aromatic residues (Lawson et al., 1994). Maltose binding sites 1 (involving residues near Trp662 and Trp616) and 3 (involving residues near Trp413) are thought to attach the enzyme to raw starch granules, while maltose binding site 2 (located near Tyr633) is thought to guide the starch chain towards the active site (unpublished results). These studies have provided a structural explanation for the catalytic mechanism and product inhibition, and have provided insight into the factors determining product specificity.

Besides improving the catalytic properties of CGTase we are interested in increasing the stability of this enzyme under conditions of the industrial production of CDs. In order to gain more insight into the factors determining structural stability in this class of enzymes we have studied the thermostable CGTase from the thermophilic anaerobic microorganism Clostridium thermosulfurigenes EM1, lately reclassified as Thermoaerobacterium thermosulfurigenes EM1 (Lee et al., 1993). This enzyme has been shown to be a CGTase rather than an α-amylase, as it was originally characterized (Wind et al., 1995). The enzyme has a temperature optimum for the cyclization reaction at 85°C and a broad pH optimum around pH 6. Its thermostability is reflected by the retention of cyclization activity greater than 90% when kept at 80°C for five hours in the presence of starch. Besides starch, calcium has been shown to improve the enzyme’s thermostability. Compared with the CGTase from B. circulans strain 251, however, the T. thermosulfurigenes EM1 CGTase has a twofold decreased cyclization activity and a sixfold increased hydrolytic activity at 60°C (Wind et al., 1995).

The structural basis for the thermostability of proteins isolated from thermophilic microorganisms has been the subject of an increasing number of studies due to the importance of stable enzymes for use in industrial biotechnological applications. A number of factors have been suggested to play a role in protein thermostability. On the basis of sequence and structure comparisons between mesophilic enzymes and their thermophilic counterparts, it was concluded that an increase of the Ala content of α-helices at the expense of Gly, Ser, Val and Lys residues as well as Gly to Ala or Pro mutations could improve thermostability of proteins (Argos et al., 1979). Replacement of Lys by Arg has also been implicated in this respect (Mrabet et al., 1992). Structural features such as an increased number of disulphide bridges and extension of the hydrophobic core have also been suggested to improve thermostability (Fontana, 1991). Introducing disulphide bridges, however, has not always proven to be successful. For instance, although the introduction of additional disulphide bridges in bacterio- phage T4 lysozyme increased its thermostability (Matsumura et al., 1989), the same strategy failed for a neutral protease (van den Burg et al., 1993). The recent analysis of the structures of aldehyde ferredoxin oxidoreductase and rubredoxin from Pyrococcus furiosus (Chan et al., 1995; Day et al., 1992) suggested that an increase of salt-bridges located at the protein surface, the shortening of loop regions and a reduction of the total protein surface contribute to the extreme stability of these proteins. Tight binding of calcium ions has been implicated in stabilizing protein structure (Fontana, 1991), as was, amongst others, observed in CGTase where the presence of calcium increases thermostability (Wind et al., 1995). In general, thermostable enzymes appear to be more rigid than mesophilic homologues as indicated by hydrogen exchange measurements, resistance against proteolytic degradation (Fontana, 1991) and a correlation between structural stability and reduced crystallographic B-factors (Vihinen, 1987). This rigidity has been suggested to explain the low catalytic efficiency of thermostable enzymes at room temperature, since they achieve the flexibility required for catalysis only at elevated temperatures. Although many different factors have been proposed to contribute to the thermostability of proteins, it generally cannot be explained by a single factor. Thermostability seems rather to be caused by a complex and subtle interplay of many different factors, and it is often dependent on the function and environment of the protein (Fontana, 1991; Jaenicke, 1991).
Besides allowing the rationalization of the observed thermostability in the presence of starch and calcium, the elucidation of the three-dimensional structure of the *T. thermosulfuri-genes* CGTase could provide a structural rationale for the differences in product ratio and hydrolytic activity compared with the CGTase from *B. circulans* strain 251 (Wind et al., 1995). Here, we present the corrected nucleotide sequence of the *T. thermosul-furigines* EM1 CGTase and its three-dimensional crystal structure at 2.3 Å resolution.

**Results and Discussion**

**DNA sequencing**

Figure 1 lists the revised amino acid sequence of the CGTase from *T. thermosulfurigines* EM1. When compared with the sequence reported by Bahl et al. (1991) differences are observed at residues 101 (Tyr instead of a deletion), 106 to 111 (FKRTNP instead of L-REQS) and 363 to 371 (EQYMTGNGD instead of V-Y-DRQWR). Figure 2 depicts the electron density in two regions in the *T. thermosulfurigines* EM1 CGTase structure where the corrected amino acid sequence deviates from that reported by Bahl et al. (1991). It is clear that the corrected amino acid sequence fits well in the electron density derived from the X-ray diffraction data. Only the side-chain of Arg108 has a reduced electron density. This residue sticks out into the bulk solvent and is likely to undergo motional averaging. Besides the corrected regions shown in Figure 2, no other discrepancy was observed between sequence and electron density during refinement.

**Quality and general features of the structure**

After refinement the final model had a crystallographic R-factor of 17.9 % (for all data in the 6.0 to 2.3 Å range with \( |F_o| > 0 \)), a free R-factor of 24.9% and excellent stereochemistry as is shown in Table 2. It contains all 683 amino acid residues, 2 calcium ions and 343 water molecules. The Ramachandran plot shows no outliers except for Phe196, which is located in a generously allowed region of the Ramachandran plot. Phe196 is placed centrally in the active site, analogously to residue Tyr195 in the CGTase from *Bacillus circulans* strain 251 (Lawson et al., 1994), which has similar phi-psi torsion angles. Gly654 deviates from the phi-psi torsion angle distribution for glycine residues as given by PROCHECK. This residue is located in the Lys652 to Ile657 surface loop region, which displays poor quality electron density, probably due to motional averaging.

**Crystal packing**

In contrast to the CGTase from *B. circulans* strain 251, the CGTase from *T. thermosulfurigines* EM1 crystallizes without the requirement for maltose or α-CD being present in the crystallization medium, similar to the CGTase from *B. circulans* strain 8 (Klein & Schulz, 1991). The intermolecular contacts observed in the *T. thermosulfurigines* EM1 crystals differ, however, from those observed for the *B. circulans* strain 8 CGTase where only two of the surrounding four protein molecules of the \( P_{2_1} 2_1 2_1 \) space group were contacted. As shown in Table 1 the *T. thermosulfurigines* EM1 CGTase is involved in direct hydrogen bonds with three surrounding protein molecules, one of which is its own copy in the neighbouring unit cell in the direction of the a axis. There is no significant hydrophobic interaction involved in crystal packing.

**Comparison with other CGTase structures**

The availability of the crystal structures at 2.0 Å resolution of the CGTases of the mesophilic *B. circulans* strains 251 (BC251) (Lawson et al., 1994) and 8 (Klein & Schulz, 1991) (BC8, PDB entry codes 1CGD and 1CGT, respectively), and at 2.5 Å resolution of the thermophilic *Bacillus stearother-mophilus* CGTase (Kubota et al., 1991: PDB entry code 1CYG) allows for a detailed structural comparison of the thermolabile and thermostable CGTase molecules. The overall fold of the CGTase of *T. thermosulfurigines* EM1 is highly similar to that of the other CGTase structures. When the *B. circulans* and *B. stearothermophilus* CGTase structures are superimposed on the Cα atoms of the structurally homologous regions of the *T. thermosul-furigines* EM1 CGTase, the r.m.s. deviations are 0.60 Å and 0.63 Å for the CGTases from BC251 and BC8, and 0.59 Å for the *B. stearothermophilus* CGTase, respectively. Significant differences occur only at loop regions near residues (using *T. thermosulfurigines* EM1 numbering) 88 to 95, 335 to 339, 494 to 499, 534 to 540 and 654 to 657. The *B. circulans* strain 8 CGTase structure has an additional deletion and insertion in loops 472 to 476 and 616 to 618, respectively. Figure 3 shows the superposition of the Cα backbones of the *B. circulans* and *B. stearothermophilus* CGTases onto that of *T. thermosulfurigines* EM1. As can be seen in Figure 3, the non-superimposable loops in the thermostable CGTase are all located at the surface of the protein. The role of these loop regions in the thermostability of CGTase will be discussed in more detail in the sections below.

The hydrophobic core is well conserved in all four proteins and the two calcium binding sites are practically identical in all four cases. No additional calcium binding site is observed. The stabilizing effect of the presence of calcium ions on the *T. thermosulfurigines* EM1 CGTase reported by Wind et al. (1995) could be due to a shift in the equilibrium between the apo and calcium-bound state of the enzyme towards the latter, rendering it less susceptible to unfolding. Also, long-range electro-
Crystal structure of T. thermosulfurigenes EM1 CGTase

Figure 1. (a) Alignment of amino acid sequences of bacterial CGTases. BC251, B. circulans strain 251 (Lawson et al., 1994); BC8, B. circulans strain 8 (Nitschke et al., 1990); BOHB, B. ohbensis (Sin et al., 1991); BSP382, Bacillus sp. strain 38-2 (Kaneko et al., 1988); BST2, B. stearothermophilus strain 2 (Fujiiwiara et al., 1992); TBACTERIUM, T. thermosulfurigenes EM1 (this study; Bahl et al., 1991). Amino acid numbering is started after the signal peptide and according to TBACTERIUM. Similarity scores are given compared with the CGTase from T. thermosulfurigenes EM1. (b) Differences between the amino acid sequence reported by Bahl et al. (1991) and the revised sequence of the T. thermosulfurigenes EM1 CGTase.
static effects and stabilization of calcium binding loops by, for instance, the stacking of Tyr112 onto Pro34 at the second calcium binding site could contribute to tight calcium binding.

The identity and conformation of active site residues near the catalytic carboxylates Asp230, Asp329 and Glu258 is highly similar to that observed in the other CGTase structures. At position 193 near the active site Lys is replaced by Arg with respect to the \textit{B. circulans} CGTases, which may render the enzyme less sensitive to chemical modification by glucose (Mrabet \textit{et al}., 1992). A second difference seen in the active site of the \textit{T. thermosulfurigenes} EM1 CGTase is that the hydrogen bond between the side-chain carboxyl groups of Glu257 and Asp328, which is present in the \textit{B. circulans} strain 251 and the other two CGTase structures, is lost. Instead the Glu258 side-chain has rotated about its \( \chi_2 \) angle to assume a conformation similar to that observed in the complex between the \textit{B. circulans} CGTase and the inhibitor acarbose (Strokopytov \textit{et al}., 1995). The aromatic residues that take part in oligosaccharide binding at the maltose binding sites in the BC251 CGTase are all conserved in the \textit{T. thermosulfurigenes} EM1 CGTase. Leu600, however, which was observed to be near the centre of the ring of an \( \alpha \)-CD molecule bound at maltose binding site 2 of the BC251 CGTase (Knegtel \textit{et al}., 1995), has been replaced by Tyr597.
**Figure 3.** Stereo view of the superimposed Cα backbone traces of the crystal structures of the CGTases from *B. circulans* strains 8 and 251, *B. stearothermophilus* and *T. thermosulfurigenes* EM1. The backbone of the *T. thermosulfurigenes* EM1 CGTase is drawn with thick lines. Maltose binding sites 1 to 3 (MBS1 to 3) and variable loop regions are indicated. The centre of the active site is indicated with an asterisk (*).

**Structure and thermostability**

The origins of thermostability in proteins have been subject of extensive debate in the literature (Fontana, 1991; Jaenicke, 1991; Rees & Adams, 1995). We have examined several proposed causes for the enhanced stability of thermostable proteins. An increase in the number of disulphide bridges has in some cases increased the stability of proteins. The two cysteine residues that formed a disulphide bridge in the *B. circulans* CGTases are replaced by His43 and Phe50, respectively, in the *T. thermosulfurigenes* EM1 CGTase. These two residues stack onto each other to form a small hydrophobic core together with Met377, while additional rigidity in this region is provided by the presence of Pro41. The CGTase from *B. stearothermophilus* still has the disulphide bridge present, although in this case the free cysteine residue at position 400 in the BC251 CGTase has been deleted from its sequence.

Recent structural studies of thermophilic enzymes have suggested that an increase in surface salt-bridges could be responsible for structural stability of proteins at elevated temperatures (Chan *et al.*, 1995; Day *et al.*, 1992). The heat-labile CGTases from *B. circulans* strains 251 and 8 have a total of 27 and 16 salt-bridges, respectively (using a 3.5 Å distance limit for the Arg, Lys, Asp and Glu charged side-chain atoms), while the *T. thermosulfurigenes* EM1 CGTase has 20. Although the BC251 CGTase has a larger total number of ionic interactions, the *T. thermosulfurigenes* EM1 CGTase has five salt-bridges that have no homologues in the *B. circulans* CGTases. These salt-bridges involve residues 47/89, 189/193, 228/230, 245/510 and 276/557, all of which are located near or at the protein surface and possibly stabilize the B/E and A/D domain interfaces. The thermophilic *B. stearothermophilus* CGTase has a total of 18 salt-bridges, of which six unique salt-bridges are located at its surface, involving residues 113/116, 336/461, 99/149, 205/241, 212/241 and 406/408. Only the 99/149 salt-bridge appears to be involved in stabilization of a domain interface. Thus, it is not only the number of surface salt-bridges that is important for conferring stability, but probably also the precise location of a salt-bridge might contribute to the overall stability of the enzyme (e.g. by providing interactions between domains or surface loops).

Other domain-interface stabilizing interactions are observed near Phe237, which assumes a conformation 120° rotated compared with that of the corresponding phenylalanine residue in the BC251 CGTase, as shown in Figure 4. This change in conformation results from a mutation of Thr to Tyr at position 187, and as a consequence a small hydrophobic core is formed involving Tyr272 and Phe273. Tyr272 is absent in both *B. circulans* CGTases and the observed contacts appear to improve the packing of two adjacent α-helices in the A domain. A similar arrangement of aromatic residues is observed in the *B. stearothermophilus* CGTase. The space that is occupied by Phe237 in the BC251 CGTase structure is taken by Tyr187 in the *T. thermosulfurigenes* EM1 CGTase, where its side-chain makes van der Waals contacts with Phe623 and a water-mediated hydrogen bond with the hydroxyl group of Tyr635. The latter two residues are located in the E domain and these interactions may stabilize the domain interface.

An increased overall rigidity and compactness have been suggested to improve the stability of proteins under extreme conditions (Fontana, 1991; Vihinen, 1987). Thermostable proteins are assumed to be more rigid at moderate temperatures. A decreased flexibility is faintly reflected in the lower average B-factor for the protein atoms of the *T. thermosulfurigenes* EM1 (20.6 Å²) and the *B. stearothermophilus* CGTases (12.7 Å²), compared with those of the *B. circulans* strains 251 (26.1 Å²) and 8 (22.2 Å²) CGTases. The differences are quite small, however, and could equally well be caused by differences in crystal packing and solvent content. Of the loops with the largest conformational differences, three have clearly reduced
temperature factors (residues 42, 89 to 93 and 335 to 336) but, in contrast, the temperature factors of loop 655-656 are twice as high (58.3 Å² compared with 29.8 Å²). Some rigidity may be conferred to the enzyme by the Gly to Ala or Pro substitutions at positions 41, 472 and 555. The compactness of thermostable protein structures or the reduction of cavity sizes and total volume of cavities has been shown to affect the stability of aldehyde ferredoxin oxidoreductase from *P. furiosus* (Chan et al., 1995) and citrate synthase from *T. acidophilum* (Russell et al., 1994). We calculated cavity sizes and total volumes with VOIDOO, using the program’s standard settings (Kleywegt & Jones, 1994). The results of these calculations do not agree with the expected trend where the thermostable protein has the smallest cavity and total volume. For instance, the thermostable BC251 CGTase had the smallest total cavity volume of 45 Å³ compared with 117 Å³ for the *T. thermosulfurigenes* EM1 CGTase, while the latter had a slightly larger total volume of 69,060 Å³ (68,290 Å³ for the BC251 CGTase).

As overall structural features conferring thermostability to the *T. thermosulfurigenes* EM1 CGTase, we considered loop regions that differ from those in the thermostable CGTases. On the basis of the amino acid sequence no obvious trend in reduction or increase of the length of loops in the thermostable CGTases is observed. When the structures of the loops carrying insertions or deletions are examined in more detail, some stabilizing new contacts are observed. The loop region consisting of residues 88 to 95 is one residue longer compared with the same loop in the *B. circulans* CGTases (Figure 1). Therefore one might expect that this loop plays no significant role in the stabilization of the enzyme. The conformation of this loop, however, allows Phe92 to form a small hydrophobic core with Pro372, Tyr373, Leu46 and the methylene groups of the Lys47 side-chain. In the *B. circulans* CGTases there is no apolar contact attaching this loop to the core of the protein. The 335-339 loop region is one residue shorter than the equivalent *B. circulans* loop regions but the thermostable *B. stearothermophilus* CGTase has no deletion in this region. In the *T. thermosulfurigenes* EM1 CGTase additional stabilization of this loop is achieved by hydrogen bonds between Asn335 and Asn369/Arg295, and hydrophobic contacts between Tyr334 and Gly336, Thr339 and Tyr365. The loop region consisting of residues

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**Figure 4.** Stereo view of the rearrangement of aromatic residues at the A/E domain interface. The *T. thermosulfurigenes* EM1 structure is drawn in bold, the *B. circulans* strain 251 CGTase structure with thin lines. Aromatic residues of the thermostable protein are labelled. The side-chain of Phe237 is rotated such that it contacts Tyr272 and Phe273, while Tyr187 contacts Phe623 and Tyr635 from the E domain.

**Figure 5.** Stereo view of the Cα trace of the loop region Phe531 to Trp541 in four different CGTase crystal structures. CGTases are labelled with their PDB entry code as follows: *B. circulans* strain 8 (1CGT), *B. circulans* strain 251 (1CGD), *B. stearothermophilus* (1CYG) and *T. thermosulfurigenes* EM1 (TtEM1). The thermostable CGTases 1CGD and 1CGT carry a three residue insertion in this region, which disrupts a β-sheet that is completed in the thermostable enzymes.
Thr534 to Val539 in the thermostable CGTases contains the largest deletion (three residues) with respect to the thermolabile CGTases (Figure 5), thereby completing an anti-parallel β-sheet in the D-domain that is disrupted in the B. circulans CGTases. The thermolabile CGTase from B. ohbensis (Figure 1) has a similar deletion at this position but the remaining loop contains larger residues (Asp, Phe) than in the thermostable proteins (Ser, Gly, Ala, Thr) which could influence the conformation and stability of this loop. In conclusion, it appears that especially loops 88-95, 335-339 and 534-539 may contribute to the thermostability in CGTases.

Argos et al. (1979) suggested that the exchange of Ser by Thr, Gly or Ala, and of Gly and Val by Ala stabilizes α-helices and thus the overall protein structure. Inspection of the amino acid composition of the thermostable CGTases compared with that of the mesophilic enzymes shows that in fact a decrease of almost 45% of the alanine content is observed for the thermophilic CGTases. In terms of absolute numbers the alanine residues are replaced by Thr (Val in the case of the B. stea rothermophilus CGTase) and to a lesser extent by Ser. At nine positions (residues 210, 214, 246, 249, 287, 306, 310, 313 and 400) Ser is introduced in α-helical regions in the T. thermosulfurigenes EM1 CGTase, suggesting that the replacement of Ala by Ser and/or Thr is important enough for overall thermostability to compensate possible destabilization of α-helices. A similar trend in amino acid composition has been observed for α-amylases (Ikai, 1980). Both α-amylases and CGTases are extracellular proteins that bind and convert long oligosaccharide chains and the preference for a higher Ser and Thr content might be related to their functionality. Most Ser and Thr residues in the T. thermosulfurigenes EM1 CGTase are positioned on one side of the protein surface, near the catalytic site and sugar binding domains. In general, reduction of the hydrophobicity of the protein exterior is thermodynamically favourable due to the increased solvent entropy. Alternatively, the increased number of hydrogen bonding surface residues such as Ser and Thr near the active site could provide tighter non-specific binding of the enzyme to raw starch granules at high temperatures. Indeed, a sharp increase in the thermostability of CGTase has been observed in the presence of soluble starch (Wind et al., 1995). It seems unlikely that this is caused by the binding of substrates to the maltose binding sites, since these are structurally conserved in the T. thermosulfurigenes EM1 CGTase, except for Tyr597 in maltose binding site 2, which is Leu in all other structures. Aromatic residues like Trp and Tyr have been observed previously to be involved in substrate binding at the maltose binding sites of CGTase (Lawson et al., 1994). In the T. thermosulfurigenes EM1 CGTase the number of tyrosine residues located at the protein surface is increased with respect to the B. circulans CGTases. A total of 11 tyrosine residues has been introduced at the protein surface compared with the BC251 CGTase. Only one of these are, however, present in both the B. stea rothermophilus and T. thermosulfurigenes EM1 CGTases; namely, Tyr272, which assists in stabilizing the A/E-domain interface and Tyr442, which is located near maltose binding site 3 (near Trp413). In conclusion, the observed increased stability in the presence of starch could be caused by increased non-specific binding of amylose chains at the surface of CGTase.

Increased hydrolytic activity of the T. thermosulfurigenes EM1 CGTase

Kinetic studies of the CGTase from T. thermosulfurigenes EM1 showed that it has a fivefold increased hydrolytic activity at 60°C compared with the CGTase from B. circulans strain 251 at 50°C, while its cyclization activity is reduced by almost 50% (Wind et al., 1995). The coupling and disproportionation activities appear to be very similar. Does the structure of the enzyme offer an explanation for the observed differences in activity? The absence of the hydrogen bond between the active site carboxylates Asp328 and Glu257 (BC251 numbering) could be due to the crystallization conditions. The pH range of 7.6 to 8.0 that was used for crystallization is beyond the pH optimum, and the other three CGTase structures were obtained at lower pH values (Klein & Schulz, 1991; Kubota et al., 1991; Lawson et al., 1994). On the other hand, this hydrogen bond is absent in BC251 CGTase complexed with acarbose (Strokopytov et al., 1995) or substrate (Knegtel et al., 1995) and in the complex with a G9 inhibitor (B. Strokopytov et al., unpublished results).

A possible explanation for the higher hydrolysis rate and lower cyclization activity of the CGTase from T. thermosulfurigenes EM1 compared with that of B. circulans strain 251 is the change of the size and conformation of the loop region consisting of residues 88 to 95. The structure of BC251 CGTase complexed with a maltononaose inhibitor was recently solved and revealed a stacking interaction between the aromatic side-chain of Tyr89 and glucose 3 and 4 of the inhibitor (B. Strokopytov et al., unpublished results). As is shown in Figure 6, where the structure of the maltononaose inhibitor is superimposed on the T. thermosulfurigenes EM1 CGTase structure, this interaction is no longer possible. In the thermostable enzyme the 88-95 loop is directed away from the inhibitor and no aromatic residue is at a position capable of interacting with the inhibitor. Bound oligosaccharides will thus lack an important stabilizing interaction and, certainly at elevated temperatures, cyclization will be reduced due to the increased flexibility of the accepting end of the amylose chain. A decrease in cyclization activity implies an increase in hydrolytic activity, which is in agreement with the experimental results (Wind et al., 1995). In the B. stea rothermophilus CGTase the role of Tyr89 in the B. circulans CGTases appears to be taken over by Met84 (Met87 in Figure 1), of which the sulphur atom is at the same position as the centroid of the aromatic ring of Tyr89.
Figure 6. Stereo views of a close-up of the active site of the CGTases from (a) *B. circulans* strain 251 and (b) *T. thermosulfurigenes* EM1 with a maltononaose inhibitor in the active site (Strokopytov et al., 1996). BC251 CGTase complexed with the maltononaose inhibitor was superimposed on the *T. thermosulfurigenes* EM1 CGTase and the maltononaose inhibitor was transferred to the *T. thermosulfurigenes* EM1 CGTase structure. Catalytic site residues and residues involved in substrate binding and stabilization of the 88-95 (*T. thermosulfurigenes* EM1 numbering) loop are labelled and the Cα trace of the loop region is drawn in bold. The *T. thermosulfurigenes* EM1 CGTase lacks the hydrophobic interaction of Tyr89 with the substrate resulting in a reduced cyclization activity.

Conclusions

The crystal structure of the cyclodextrin glycosyltransferase of the thermophilic *T. thermosulfurigenes* EM1 has been solved at 2.3 Å resolution. Its overall fold is highly similar to that reported for CGTases of mesophilic bacteria and structural differences are observed only at surface loops. The *T. thermosulfurigenes* EM1 CGTase structure provides another example of the subtle interplay of electrostatic and apolar interactions that causes thermostability in proteins. A number of structural features that could be responsible for the increased stability of this enzyme have been identified. Five or six salt-bridges, absent from the thermolabile CGTase structures, are introduced at the protein surface near domain interfaces. Loop regions consisting of amino acid residues 88 to 95, 335 to 339 and 534 to 539 also appear to add to the general stability of the enzyme, either by introducing new hydrophobic interactions or hydrogen bonds or by removing flexibility existing in the less stable CGTases. Rearrangement of aromatic residues near Phe237 appears to stabilize the A domain and its interface with the E-domain. Finally, the increased incidence of Ser, Thr and Tyr residues at the protein surface near the active site and oligosaccharide binding sites could improve non-specific binding of the enzyme to raw starch granules. This might explain the dramatic increase of thermostability upon addition of soluble starch. Other factors that have been implicated in conferring thermostability to proteins, like reduced cavity volumes, disulphide bridges and an increase in the number of helix-stabilizing
amino acid residues appear not to be of major importance for the stability of CGTases.

The increased hydrolytic and decreased cyclization activity of the T. thermosulfurigenes EM1 compared with that of the CGTase from B. circulans strain 251 appears to be mediated mainly by the conformational changes in the loop region consisting of residues 88 to 95. A number of apolar interactions, not present in the thermolabile CGTase structures, fix this loop to the hydrophobic core of the enzyme. Apparently, stabilization of this region in order to increase the overall thermostability of the enzyme weighs more heavily on the hydrolytic activity but is more likely to be an artefact of the high pH of the crystallization conditions.

The availability of the crystal structure of the T. thermosulfurigenes EM1 CGTase can serve as a starting point for protein engineering studies aimed at increasing the thermostability of industrially used CGTases and the manipulation of the ratio between cyclization and hydrolytic activity in this class of enzymes.

**Materials and Methods**

**Enzyme preparation and activity assays**

Production and purification of the CGTase from T. thermosulfurigenes EM1 was done according to Wind et al. (1995). Activity measurements were performed according to Pennings et al. (1995) at the temperature optimum of 50°C for the B. circulans 251 CGTase and at 60°C for the T. thermosulfurigenes EM1 enzyme, respectively. The temperature optimum of this latter enzyme is 85°C.

**Crystallization**

Initial attempts at crystallizing the enzyme were performed with a Sparse Matrix (48 experiments) setup using hanging drops with a protein concentration of 3.4 mg/ml in 10 mM sodium acetate (pH 5.5) at room temperature. Intergrown crystals were obtained from either 0.1 M sodium citrate and 1.0 M ammonium phosphate in Hepes buffer at pH 7.6 or 0.8 M sodium potassium tartrate in Hepes buffer at pH 7.7. Refinement of the tartrate conditions showed that rod-like single potassium tartrate in Hepes buffer at pH 7.7. Refinement phosphate in Hepes buffer at pH 7.6 or 0.8 M sodium either 0.1 M sodium citrate and 1.0 M ammonium temperature. Intergrown crystals were obtained from using hanging drops with a protein concentration of

**Data collection and processing**

A data set was recorded on an Enraf Nonius FAST area detector system and processed with MADNES (Messer-

**DNA sequencing**

DNA sequencing was carried out by the dideoxy-chain termination method (Sanger et al., 1977), using the fluoro-dATP+ labelling mix in combination with the Autoread Sequencing kit and the Automated Laser Fluorescent (A.L.F.) DNA sequencer from Pharmacia. Plasmid pCT2 was used as template DNA (Bahl et al., 1991). Primers were provided by Dr H. Bahl or synthesized by Pharmacia. Plasmid DNA for sequencing was isolated with the plasmid midi protocol of QIAGEN (Cathsworth, USA). The (corrected) T. thermosulfurigenes EM1 sequence has been deposited with the EMBL sequence database (accession number M57580).

Alignments of CGTase sequences were performed with the program DNASIS for Windows (Hitachi Software, San Bruno, USA). The same program was used for the calculation of similarity scores. Sequences were obtained from the EMBL sequence database. Accession numbers for CGTases: T. thermosulfurigenes EM1, M57580 (this
study; Bahl et al., 1991); *B. circulans* strain 251, X78145 (Lawson et al., 1994); *B. circulans* strain 8, X68326 (Nitschke et al., 1990); *Bacillus* sp. 38-2, M19880 (Kaneko et al., 1988); *B. ohbensis*, D90243 (Sin et al., 1991); *B. stearothermophilus* no. 2, X59042 or S51853 (Fujiwara et al., 1992).

**Molecular replacement**

For molecular replacement a model was constructed based on the 2.0 Å structure of wild-type CGTase from *Bacillus circulans* strain 251. Based on the amino acid sequence derived from the original nucleotide sequence by Bahl et al. (1991), mutations to Ala or deletions were introduced with O (Jones et al., 1991) and all three maltose molecules and both calcium ions were removed from the starting model. Molecular replacement with the BIOMOL programs (Protein Crystallography Group, University of Groningen) FROT0-3 (fast rotation function according to Crowther (1972)) and TRAFUN (translation function according to Crowther & Blow (1967)) using diffraction data in the 8.0 to 3.5 Å resolution range yielded a unique solution at 9σ that rotated the starting model over \( \alpha = 7.5^\circ, \beta = 135^\circ \) and \( \gamma = 265^\circ \) and translated it over \(-8.53 \, \AA, -12.73 \, \AA \) and \(-39.51 \, \AA \) along the \( a, b \) and \( c \) axes, respectively.

**Structure refinement and quality assessment**

The resulting structure was submitted to rigid body refinement with TNT (Tronrud et al., 1987) followed by alternating cycles of manual rebuilding with O (Jones et al., 1991) and all-parameter refinement with TNT. Free R-factors (Brünger, 1993) were monitored using a randomly chosen subset of 10% of the reflections during all stages of the refinement. Strong restraints were applied to bond lengths and angles as well as planes and non-bonded contacts using the reference values reported by Engh & Huber (1991). No restraint was applied to dihedrals or hydrogen bonds. During the refinement it became clear that the published amino acid sequence did not fit with the observed electron density at several places. Some of these residues were highly conserved in other CGTases but according to the sequence should be absent in the CGTase from *T. thermosulfurigenes* EM1. To resolve these issues the protein was resequenced and a corrected sequence derived from the original nucleotide sequence strain 251. Based on the amino acid sequence derived from the original nucleotide sequence *Bacillus circulans* sp. 38-2, M19880 (Kaneko et al., 1988). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. sect. A*, 47, 392–400.


**References**


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