Engineering of factors determining α-amylase and cyclodextrin glycosyltransferase specificity in the cyclodextrin glycosyltransferase from Thermoaerobacterium thermosulfurigenes EM1

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The starch-degrading enzymes α-amylase and cyclodextrin glycosyltransferase (CGTase) are functionally and structurally closely related, with CGTases containing two additional domains (called D and E) compared to the three domains of α-amylases (A, B and C). Amino acid residue 196 (Thermoaerobacterium thermosulfurigenes EM1 CGTase numbering) occupies a dominant position in the active-site cleft. All α-amylases studied have a small residue at this position (Gly, Leu, Ser, Thr or Val), in contrast to CGTases which have a more bulky aromatic residue (Tyr or Phe) at this position, which is highly conserved. Characterization of the F196G mutant CGTase of T. thermosulfurigenes EM1 revealed that, for unknown reasons, apart from the F196G mutation, domain E as well as a part of domain D had become deleted [mutant F196G(ΔDE)]. This, nevertheless, did not prevent the purification of a stable and active mutant CGTase protein (62 kDa). The mutant protein was more similar to an α-amylase protein in terms of the identity of residue 196, and in the domain structure containing, however, some additional C-terminal structure. The mutant showed a strongly reduced temperature optimum. Due to a frame-shift mutation in mutant F196G, a separate protein of 19 kDa with the DE domains was also produced. Mutant F196G(ΔDE) displayed a strongly reduced raw-starch-binding capacity, similar to the situation in most α-amylases that lack a raw-starch-binding E domain. Compared to wild-type CGTase, cyclization, coupling and disproportionation activities had become drastically reduced in the mutant F196G(ΔDE), but its saccharifying activity had doubled, reaching the highest level ever reported for a CGTase. Under industrial production process conditions, wild-type CGTase converted starch into 35% cyclodextrins and 8% into linear oligosaccharides. These biochemical characteristics indicate a clear shift from CGTase to α-amylase specificity.

Keywords: α-amylase; cyclodextrin glycosyltransferase; domain structure; site-directed mutagenesis; product specificity.

Cyclodextrin glycosyltransferase (CGTase) and α-amylase both belong to glycosyl hydrolase family 13 (the α-amylase family), which represents a group of (βα)8-barrel proteins (Svenson, 1994). These enzymes are functionally closely related, both catalyzing the degradation of starch by cleavage of α-1,4-glycosidic bonds. CGTase converts starch mainly into cyclodextrins, cyclic oligomers of 6–8 glucose molecules linked via α-1,4-glycosidic bonds (α-, β- and γ-cyclodextrin, respectively). Cyclodextrins have the ability to form inclusion complexes with a wide range of small hydrophobic molecules and may find applications in the food, cosmetic and pharmaceutical industries (Pedersen et al., 1995; Szejtli, 1982). CGTase catalyzes four different reactions, namely cyclization, coupling, disproportionation and hydrolysis (Penninga et al., 1995). α-amylase converts starch into linear oligosaccharides, resulting in a rapid decrease in viscosity (Antranikian, 1991; Vihinen and Mäntsälä, 1989). The enzyme has found numerous applications in commercial processes, including thinning and liquefaction of starch in the alcohol, brewing and sugar industries.

The crystal structures of several CGTase (Harata et al., 1996; Klein and Schulz, 1991; Kneget al., 1996; Kubota et al., 1991; Lawson et al., 1994) and α-amylase (Mačiūnas et al., 1995; Matsuura et al., 1984) proteins have been determined. The primary structures of α-amylases and CGTases show limited similarity (≤30%). In contrast, the three-dimensional structures of the A, B and C domains of CGTases and α-amylases are quite similar. Compared to α-amylases, CGTases are much larger and contain two additional domains (D and E). Domain E is involved in raw starch binding (Penninga et al., 1996; Svenson et al., 1989); the precise functions of the D domain remain to be clarified.

Analysis of sequence data has revealed several examples of incorrect classification of CGTases as α-amylases (Janeček et al., 1995). The α-amylases from Bacillus circulans strain F2 (Fig. 1, Nishizawa et al., 1987) and Bacillus sp. strain B1018 were later shown to be CGTases. The α-amylase from Thermoaerobacterium thermosulfurigenes EM1 has recently been reclassified as a CGTase with an unusually high hydrolytic activity (Wind et al., 1995). The active site amino acids Asp230, Glu258 and Asp329 (T. thermosulfurigenes EM1 CGTase numbering), directly involved in catalysis, are fully conserved among the different α-amylase and CGTase enzymes (Nakamura et al.,...
Fig. 1. Active-site sequences and catalytic residues. (A) Alignment of part of the active-site amino acid sequences of several CGTases and α-amylases (from Penninga et al., 1995 with modifications). * Indicates an exact match; TT, *T*, Thermosulfurigenes EM1; BM, Bacillus macerans (Takano et al., 1986); KP, Klebsiella pneumoniae (Binder et al., 1986); BST, B. stearothermophilus (Kubota et al., 1991); BLI, B. licheniformis (Hill et al., 1990); BC251, B. circulans strain 251 (Lawson et al., 1994); BC8, B. circulans strain 8 (Bender, 1990b); BSP1011, Bacillus sp. strain 1011 (Kimura et al., 1987); BACCI, B. circulans strain F-2 (Nishizawa et al., 1992); TAA, Aspergillus oryzae Taka-α-amylase A (Nagashima et al., 1992); ANI, Aspergillus niger acid α-amylase (PDB entry 3AAA); AMYBLI, Bacillus licheniformis α-amylase (PDB entry 1VJS); AMYP1G, pig α-amylase (Nakajima et al., 1986); AMYHUMANS, human saliva α-amylase (Nakajima et al., 1986); AMYHUMANP, human pancreatic α-amylase (PDB entry 1HNY). Alignments with ANI, AMYBLI and AMYHUMANS were obtained by 3D structure alignment (Holm and Sander, 1996) with TT (PDB entry 1CIU). (B) Alignment of the catalytic residues of the CGTase from Thermococcaeaet with Thermosulfurigenes EM1 (Asp230, Glu230, Asp239; Knecht et al., 1995) and α-amylase from Aspergillus niger (Asp239, Glu230, Asp239, Brady et al., 1991). C backbone traces are shown. Active-site residues are presented in bold. Blue and yellow: Thermococcaeaet CGTase; purple and white: Aspergillus α-amylase. Residues Phe196 of the CGTase and Gly167 of the α-amylase are overlapping.

1992; Strokopytov et al., 1995; Svensson, 1994). It has remained unclear what determines the different product specificities of α-amylases and CGTases.

Several reports describe the effects of deletions in the C-terminus of CGTase. Deletion of 36, 84, 125 and 225 amino acids from the C-terminus of a B. circulans var. alkalophilus CGTase yielded inactive proteins (Hellman et al., 1990). Fusions with Escherichia coli alkaline phosphatase, however, increased the specific activity of these truncated proteins again, indicating that the deleted sequences may have a role in maintaining structural integrity. Also, the characteristics of site-directed mutants of the alkalophilic Bacillus sp. no. 1011 CGTase, with 10–13 amino acids deleted from the C-terminus, have been reported (Kimura et al., 1989). All mutants produced larger amounts of...
glucose, oligosaccharides and α-cyclodextrin from starch than the parental CGTase, suggesting that the C-terminal domain is important for an efficient cyclization reaction. In contrast, deletion of the C-terminal 90 amino acids from a Klebsiella pneumoniae CGTase yielded an active protein not very different from the wild-type enzyme (Bender, 1990a).

Alignment of amino acid sequences from CGTases and α-amylases suggested that residue 196 (T. thermosulfurigenes EM1 CGTase numbering) might play a role in cyclization of oligosaccharides (Penninga et al., 1995). Residue 196 is present at a dominant position in the active-site cleft (Schmidt et al., 1997). All α-amylases studied have a small residue at this position (Gly, Leu, Ser, Thr or Val; Nakajima et al., 1986), in contrast to CGTases, which have a more bulky aromatic residue (Tyr or Phe) at an equivalent position, which is highly conserved (Penninga et al., 1995). An alignment of part of the active-site amino acid sequences of several CGTases and α-amylases is given in Fig. 1A. A structural alignment of the catalytic residues of the CGTase from T. thermosulfurigenes EM1 and the α-amylase from Aspergillus niger showed that Phe196 of the CGTase is at an equivalent position with Gly167 of the α-amylase (Wind, 1997; Fig. 1B). Previous studies showed that the presence of an aromatic residue at position 196 is important for an efficient cyclization reaction (Fujiiwara et al., 1992; Nakamura et al., 1994; Penninga et al., 1995; Sin et al., 1994). Penninga and co-workers (1995) reported enhanced production of linear oligosaccharides (glucose through maltotetraose) by the site-directed mutants Y196G, Y196W and Y196L of the B. circulans strain 251 CGTase.

This study describes construction of a T. thermosulfurigenes EM1 mutant CGTase (Phe196Gly) using site-directed mutagenesis. Its characterization revealed that, for unknown reasons, domain E and a part of domain D had become deleted.

MATERIALS AND METHODS

Bacterial strains, plasminis and growth conditions. E. coli JM109 (Yanisch-Perron et al., 1985) was used for recombinant DNA manipulations. E. coli PC1990 (Lazzaroni and Portaliot, 1979), known to leak periplasmic proteins because of a mutation in its tolB locus, was used for (extracellular) production of CGTase (mutant) proteins. Plasmid pCT2, a derivative of pUC18 containing the amyA (cgt) gene of T. thermosulfurigenes EM1 (Haeckel and Bahl, 1989; Wind et al., 1995), was used for site-directed mutagenesis, sequencing and expression of wild-type and mutant CGTase proteins. Plasmid-carrying bacterial strains were grown on Luria Bertani medium with 100 µg/ml ampicillin. When appropriate, isopropyl-β-D-thiogalactopyranoside was added at a concentration of 0.1 mM for induction of protein expression.

DNA manipulations. DNA manipulations and transformations of E. coli were essentially as described by Sambrook et al. (1989). Electroporation of E. coli was performed using the Bio-Rad gene pulser apparatus (Bio-Rad). The selected conditions were 2.5 kV, 25 µF and 200 Ω.

Site-directed mutagenesis. The mutant CGTase gene (F196G) was constructed via a double PCR method using Pfu DNA polymerase (Stratagene). A first PCR reaction was carried out with the mutagenesis primer for the coding strand plus a primer 195–715-bp downstream on the template strand. The reaction product was subsequently used as primer in a second PCR reaction together with a primer 295–815-bp upstream on the coding strand. The product of the last reaction was cut with NcoI and MunI, and exchanged with the corresponding fragment (900 bp) from the vector pCT2. The resulting (mutant) plasmid was transformed to E. coli JM109 for sequencing and to E. coli PC1990 for production of the (mutant) proteins. The following oligonucleotide was used to produce the mutation:

F196G 5′-GCATTATACGTAACTAGTTAGTACGAGC-3′

Successful mutagenesis resulted in appearance of the underlined AvrII restriction site, which allowed rapid screening of potential mutants. The mutation was verified by DNA sequencing (Sanger et al., 1977). All 900 bp on the MunI–NcoI fragment obtained by PCR were checked by DNA sequencing.

Production and purification of CGTase proteins. For production of CGTase proteins, E. coli PC1990 (pCT2) was grown in a 2-liter fermentor at pH 7.0 and 30°C. The medium contained 2% (by mass) trypton (Oxoid), 1% (by mass) yeast extract (Oxoid), 1% (by mass) sodium chloride, 1% (by mass) casein hydrolysate (Merck), 100 µg/l ampicillin and 0.1 mM isopropyl-β-D-thiogalactopyranoside. Growth was monitored by measuring the absorbance at 450 nm. At an A560 nm of 2–3, 50 g trypton was added to the fermentor. Cells were harvested after 20–24 h growth (8000 g, 30 min, 4°C), at A560 values of 8–12. The supernatant was directly applied to an α-cyclodextrin–Sepharose 6F affinity column (Nonna et al., 1988) for further purification of CGTase proteins. After washing the column with 10 mM sodium acetate pH 5.5, the CGTase was eluted with the same buffer supplemented with 1% (by mass) α-cyclodextrin. The purity and molecular mass of the CGTase (mutant) proteins were checked on SDS/PAGE (Wind et al., 1995). 10 µl purified protein was applied to the SDS/polyacrylamide gel containing 3–5 µg protein. Protein concentrations were determined by the method of Bradford, using the Coomassie protein assay reagent of Pierce (Pierce Europe bv).

N-terminal amino acid sequences. For determination of the N-terminal amino acid sequences, proteins were cut out from SDS/PAGE gels. Elution was performed overnight in 0.1% SDS at 37°C. The N-terminal amino acid sequence was determined at the Gas Phase Sequenator Facility (Department of Medical Biochemistry, University of Leiden, The Netherlands). The instrument used was an Applied Biosystems model 470A protein sequencer, equipped on-line with a model-120A photodiode-dantol analyzer.

Enzyme assays. Specific assays were used to determine the activities of the four different reactions catalyzed by CGTases. In the cyclization reaction, the reducing end of a sugar is transferred to another sugar residue in the same oligosaccharide chain, resulting in the formation of cyclic compounds. Coupling is the reverse reaction in which a cyclodextrin molecule is linked to a linear oligosaccharide chain, producing a longer oligosaccharide chain. In the disproportionation reaction, part of a linear donor oligosaccharide is transferred to a linear acceptor chain. The saccharifying activity is the hydrolysis of starch into linear oligosaccharides.

All assays were performed at pH 5.9 and 60°C. In all cases, the initial enzyme activities were measured in the first 5 min of the reaction by taking samples every 1 min, to assure that the rate of the reaction was linear. Cyclization and saccharifying assays were performed as described by Penninga et al. (1995). Coupling activity was measured essentially as described by Nakamura et al. (1993). β-cyclodextrin (2.5 mM) was used as donor substrate and methyl α-D-glucopyranoside (100 mM) as acceptor substrate. The linear oligosaccharide formed in the reaction was converted to single glucose units by the action of amyloglucosidase (Sigma). Glucose was detected with the glucose/GOD-Perid method of Boehringer Mannheim. Disproportionation activity was measured as described by Nakamura et al. (1994). EPS, 4-nitrophenyl-α-D-maltohexaose-4-6-O-ethyl-
idene (3 mM, Boehringer Mannheim), was used as donor substrate and maltose (10 mM) as acceptor substrate. The reaction product containing the nitrophenyl group was cleaved by the action of α-glucosidase (Boehringer Mannheim). For each reaction, units were defined as the amount of enzyme producing/converting 1 μmol product/substrate at pH 5.9 and 60°C.

Raw starch-binding properties were studied at standard assay conditions as described by Penninga et al. (1996). An appropriate amount of CGTase was incubated with increasing amounts of granular potato starch (AVEBE) at 4°C for 1 hour (equilibrium was reached within 10 min). CGTase bound to the starch granules was spun down at 4°C for 1 min at 10000 g and the remaining cyclization activity in the supernatant was measured as described.

The pH optimum for cyclization was determined by incubating 0.1 U/ml (β-cyclodextrin-forming activity) of the enzyme with 5% Paselli SA2 (partially hydrolyzed potato starch, AVEBE) in a 10-mM sodium citrate solution set at a specific pH (range 4.0–8.0). For each pH, a new calibration curve was prepared with 0–2 mM β-cyclodextrin. The pH optimum for the saccharifying reaction was determined in a similar way.

**HPLC product analysis.** Formation of cyclodextrins was measured under industrial production process conditions by incubation of 0.1 U/ml CGTase (β-cyclodextrin-forming activity) with 10% Paselli WA4 (pregelatinized drum-dried starch with a high degree of polymerization; AVEBE) in 10 mM sodium citrate, pH 6.0, at 60°C for 45 hours. Samples were taken at regular time intervals and boiled for 10 min. Products formed were analyzed by HPLC, using a 25-cm Econosil-NH2, 10-μm column (Alltech Nederland bv) eluted with acetonitrile/water (65:45, by vol.) at 1 ml/min. Products were detected by a refractive index detector (Waters 410, Waters Chromatography Division). The temperature of the flow cell and column was set at 50°C to avoid possible precipitation of starch. Formation of linear products was directly analyzed. Formation of cyclodextrins was analyzed after incubation of the samples with an appropriate amount of β-amylase (type-IB from Sweet potato, Sigma), degrading linear sugars (but not cyclodextrins) to glucose. The retention times for α-, β- and γ-cyclodextrins were the same as those for maltotetraose, maltpentaose and maltotetraose, respectively.

### Results and Discussion

#### Construction of mutant F196G.
To study the role of residue 196 in the *T. thermosulfurigenes* EM1 CGTase, Phe196 was replaced by Gly (Table 1). The purity and molecular mass of wild-type CGTase and F196G mutant CGTase were checked on SDS/PAGE (Fig. 2). Wild-type CGTase has a molecular mass of 75 kDa, but displays a molecular mass of 68 kDa on SDS/PAGE (Wind et al., 1995). The minor protein bands were earlier shown to be CGTase degradation products (Fig. 2, lane 1; Wind et al., 1995). To our surprise the mutant F196G preparation displayed a major protein band with a molecular mass of 54 kDa and a minor protein band of 19 kDa on SDS/PAGE. DNA sequencing of mutant F196G revealed a 460-bp longer gene than that found for wild-type CGTase. For unknown reasons base pairs 1209–1669 of the cgt gene had become inserted again behind base pair 1669 in the cgt gene, causing a shift in the reading frame and resulting in the stop codon TAA after 11 amino acids (Fig. 3).

The expressed F196G protein hence contained 556 amino acids from the N-terminus and a tail of 11 amino acids at the C-terminus (KLLMVLSSNVG; 567 amino acids in total), whereas wild-type CGTase contains 683 amino acids (Fig. 3). The molecular mass of the obtained construct was calculated as 62 kDa, which is in good agreement with the size of the major protein band (54 kDa) found on SDS/PAGE (Fig. 2). The identity of the smaller upper band is unknown (Fig. 2, lane 2). The truncated mutant F196G (F196G[196DE]) thus lacked all 104 amino acids of domain E and the last 23 amino acids of domain D (out of a total of 84 amino acids), very similar to the situation in α-amylases in general.

The minor protein (19 kDa) found on SDS/PAGE resulted from a translational restart at Met508 of the cgt gene, yielding a protein of 175 amino acids containing the complete E domain and 71 amino acids of domain D. The N-terminal sequence of the 19-kDa protein was determined and confirmed the restart at Met508. Binding of the protein to the α-cyclodextrin–Sepharose 6FF affinity column might be explained by the presence of maltose-binding sites (MBS) in the E domain of the CGTase from

<table>
<thead>
<tr>
<th>CGTase</th>
<th>Supernatant activity</th>
<th>Purified protein fractions</th>
<th>Purification factor</th>
<th>Yield</th>
<th>Pure protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>specific total</td>
<td>specific total</td>
<td>-fold %</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.80 300</td>
<td>165 80</td>
<td>205 25 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F196G[196DE]</td>
<td>0.15 40</td>
<td>40 20</td>
<td>270 50 0.5</td>
<td></td>
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</tr>
</tbody>
</table>

### Table 1. Purification of *T. thermosulfurigenes* EM1 wild-type and mutant F196G[196DE] CGTase proteins.
2-L supernatant was used for protein purification. β-cyclodextrin-forming specific (sp. act.) and total activities (tot. act.) are shown.
acids involved in both binding sites (Trp609 and Trp655 in 1MBS1 B. circulans strain 25 strongly to MBS T. thermosulfurigenes 1∆ (Penninga et al., A The T. thermosulfurigenes B. stearothermophilus of CGTases known from literature are much lower, i.e. the wild- cyclization and hydrolysis (Wind et al., 1

There are multiple errors and corrections in the text, such as missing characters and typos. The text is difficult to read and understand due to these issues.

At optimal pH, the mutant enzyme displayed a saccharifying activity of 65 U/mg, the highest ever reported for a CGTase. The mutant enzyme appeared to be relatively stable since activities did not significantly decrease within one month of storage at 4°C. Mutant Y196G of B. circulans strain 251 also displayed severely reduced cyclization, coupling and disproportionation activities compared to the wild-type CGTase (van Alebeek, G., J., unpublished results; Penninga et al., 1995). The saccharifying activities of the B. circulans strain 251 wild-type CGTase, however, are relatively minor (3 U/mg), and this activity was enhanced by a factor 1.4 in mutant Y196G and by a factor of 1.6 in mutant Y169L (4.3 U/mg and 4.8 U/mg, respectively; Penninga et al., 1995). Both the F196G mutation and loss of (part of the D, E domains thus may contribute to the doubling of the saccharifying activity of T. thermosulfurigenes EM1 mutant F196G(∆DE).

The presence of an aromatic residue at CGTase position 196 thus is crucial for an efficient cyclization reaction. Mutations at position 196 also cause changes in cyclodextrin product ratios. In fact, the size of residue 196 may influence the size of the preferred cyclodextrin formed. Replacement of residue 196 by Leu indeed resulted in production of increased amounts of β-cyclodextrin and γ-cyclodextrin and decreased amounts of α-cyclodextrin in other CGTases (Nakamura et al., 1994; Penninga et al., 1995; Sin et al., 1994). The cyclodextrin product ratio of the F196G(∆DE) mutant enzyme had not significantly changed compared to the wild-type enzyme (Table 3, Fig. 4).

Similar observations were made for mutant Y196G of B. circulans 251 CGTase (Penninga et al., 1995).

Most CGTases (e.g. the B. circulans strain 251 enzyme; Penninga et al., 1995) incubated with starch under industrial process conditions produce only cyclodextrins and no or minor amounts of linear oligosaccharides. The wild-type T. thermosulfurigenes EM1 CGTase is quite exceptional, converting starch for 11% into linear sugars (glucose, maltose and maltotriose). This value is even higher for mutant F196G(∆DE) (18%; Table 3). Mutant Y196G of the B. circulans 251 CGTase also showed a drastically increased conversion of starch into linear saccharides (glucose, maltose, maltotriose and maltotetraose), from 0% for the wild-type enzyme to 16–20% for the mutant enzyme (Penninga et al., 1995).

The pH optimum for hydrolysis has shifted from pH 4.0 to pH 5.0 for wild-type CGTase, and from pH 5.0 to pH 5.5 for mutant F196G(∆DE) (Table 2). Also, the pH optimum for cyclization has shifted to a higher pH (from pH 4.5–6.5 to pH 5.5–6.5; Table 2). What is the cause of these shifts in pH? As site-directed mutations at position 196 in the B. circulans strain 251 CGTase did not cause structural rearrangements (Penninga et al., 1995), we expect that in the T. thermosulfurigenes EM1 CGTase a single F196G mutation would not cause any conformational changes that might affect the pH optima of the different reactions. In contrast, we cannot exclude that deletion of 127 amino acids from the C-terminus of CGTase could change the pH optimum for the mutant enzyme.

The pH optimum of the wild-type CGTase from T. thermosulfurigenes EM1 and mutant F196G(∆DE) (without signal peptides). The start and end of domains A–E and residue 196 are marked.

Characterization of mutant F196G(∆DE). Mutant F196G(∆DE) displayed reduced cyclization, coupling and disproportionation activities, compared to the wild-type CGTase. The T. thermosulfurigenes EM1 wild-type CGTase possesses an unusually high saccharifying activity, initially resulting in its misidentification as an α-amylase (Haeckel and Bahl, 1988; Knetgel et al., 1996). Saccharifying activities of CGTases known from literature are much lower, i.e. the wild-type CGTase from B. circulans strain 251 displays a saccharifying activity of 3.0 U/mg (Penninga et al., 1995) and the wild-type CGTase from B. steaothermophilus displays a saccharifying activity of 1.88 U/mg (Fujiwara et al., 1992).

We now observed that, compared to wild-type, the saccharifying activity of mutant F196G(∆DE) had doubled (Table 2).
Table 2. Specific enzyme activities and pH optima for *T. thermosulfurigenes* EM1 wild-type CGTase and mutant F196G(Δ′DE). Cyclization activity is shown as β-cyclodextrin-forming activity.

<table>
<thead>
<tr>
<th>CGTase</th>
<th>Specific enzyme activities</th>
<th>pH optima</th>
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<tbody>
<tr>
<td></td>
<td>cyclization</td>
<td>coupling</td>
</tr>
<tr>
<td>Wild-type</td>
<td>165</td>
<td>45</td>
</tr>
<tr>
<td>F196G(Δ′DE)</td>
<td>40</td>
<td>3</td>
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</table>

Table 3. Starch conversion by *T. thermosulfurigenes* EM1 wild-type CGTase and mutant F196G(Δ′DE). Proteins (0.1 U/ml β-cyclodextrin forming activity) were incubated for 45 hours with 10% Paselli WA4. Starch conversion into cyclodextrins or linear sugars (glucose, maltose and maltotriose) are shown relative to the initial amount of starch.

<table>
<thead>
<tr>
<th>CGTase</th>
<th>Conversion of starch into cyclodextrins</th>
<th>Conversion of starch into linear sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>Wild-type</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>F196G(Δ′DE)</td>
<td>21</td>
<td>30</td>
</tr>
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</table>

Fig. 4. Cyclodextrins formed during incubation of the wild-type CGTase from *T. thermosulfurigenes* EM1 (A) and mutant F196G(Δ′DE) (B). Proteins (0.1 U/ml β-cyclodextrin forming activity) with 10% (mass/vol.) Paselli WA4 starch for 45 hours at pH 6.0 and 60°C. ▲ β-cyclodextrin, ▼ γ-cyclodextrin.
C-terminal 90 amino acids of the \( K. \text{pneumoniae} \) CGTase had little effect on activity or product specificity (Bender, 1990a), whereas deletion of a mere 10–13 residues of the \( B. \text{circulans} \) sp. C-terminal domain had also been deleted from this protein. With respect to the domain structure and identity of the residue at position 196, this mutant CGTase is more similar to \( \alpha \)-amylases; however, it contains an additional C-terminal structure compared to \( \alpha \)-amylases. The C-terminal deletion yielded a protein unable to bind to raw starch and displaying a strongly reduced thermostability. Concomitantly, the cyclization, coupling and disproportionation activities became severely reduced, whereas a doubling of the saccharifying activity was observed. This resulted in a decreased conversion of starch into cyclodextrins and increased conversion into linear oligosaccharides. These biochemical characteristics indicate a shift from CGTase to \( \alpha \)-amylase specificity. Nevertheless, the mutant still produces cyclodextrins. The data provide a firm basis for analysis of other factors determining CGTase and \( \alpha \)-amylase product specificity in future work.

Thanks are due to Bart van der Veen for determination of the raw-starch-binding properties.

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**CONCLUSIONS**

Mutant F196G of the highly thermostable \( T. \text{thermosulfurigenes} \) EM1 CGTase was constructed using site-directed mutagenesis. Due to a frameshift mutation, the E domain and part of the D domain had also been deleted from this protein. With respect to the domain structure and identity of the residue at position 196, this mutant CGTase is more similar to \( \alpha \)-amylases; however, it contains an additional C-terminal structure compared to \( \alpha \)-amylases. The C-terminal deletion yielded a protein unable to bind to raw starch and displaying a strongly reduced thermostability. Concomitantly, the cyclization, coupling and disproportionation activities became severely reduced, whereas a doubling of the saccharifying activity was observed. This resulted in a decreased conversion of starch into cyclodextrins and increased conversion into linear oligosaccharides. These biochemical characteristics indicate a shift from CGTase to \( \alpha \)-amylase specificity. Nevertheless, the mutant still produces cyclodextrins. The data provide a firm basis for analysis of other factors determining CGTase and \( \alpha \)-amylase product specificity in future work. Fig. 5. Effect of temperature on cyclization activity of the wild-type CGTase from *T. \text{thermosulfurigenes} \) EM1 and mutant F196G(\( \Delta \)DE).

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