ABSTRACT: Tyrosine 195 is located in the center of the active site cleft of cyclodextrin glycosyltransferase (EC 2.4.1.19) from Bacillus circulans strain 251. Alignment of amino acid sequences of CGTases and \( \alpha \)-amylases, and the analysis of the binding mode of the substrate analogue carbose in the active site cleft [Strokopytov, B., et al. (1995) Biochemistry 34, (in press)], suggested that Tyr195 plays an important role in cyclization of oligosaccharides. Tyr195 therefore was replaced with Phe (Y195F), Trp (Y195W), Leu (Y195L), and Gly (Y195G). Mutant proteins were purified and crystallized, and their X-ray structures were determined at 2.5–2.6 \( \AA \) resolution, allowing a detailed comparison of their biochemical properties and three-dimensional structures with those of the wild-type CGTase protein. The mutant proteins possessed significantly reduced cyclodextrin forming and coupling activities but were not negatively affected in the disproportionation and saccharifying reactions. Also under production process conditions, after a 45 h incubation with a 10% starch solution, the Y195W, Y195L, and Y195G mutants showed a lower overall conversion of starch into cyclodextrins. These mutants produced a considerable amount of linear maltodextrin. The presence of aromatic amino acids (Tyr or Phe) at the Tyr195 position thus appears to be of crucial importance for an efficient cyclization reaction, virtually preventing the formation of linear products. Mass spectrometry of the Y195L reaction mixture, but not that of the other mutants and the wild type, revealed a shift toward the synthesis (in low yields) of larger products, especially of \( \beta \)- and \( \gamma \)- (but no \( \alpha \)-) cyclodextrins and minor amounts of \( \delta \)-, \( \epsilon \)-, \( \zeta \)- and \( \eta \)-cyclodextrins. This again points to an important role for the residue at position 195 in the formation of cyclic products.

Cyclodextrins are cyclic oligomers of glucose linked via \( \alpha(1,4) \) glycosidic bonds (French, 1957). They are produced from starch by the enzyme cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19). Cyclodextrins can form inclusion complexes with many small hydrophobic molecules (Saenger, 1980) and find increasing use in industrial and research applications (Schmid, 1989).

CGTases from different bacterial sources (Bender, 1986; Schmid, 1989) all convert starch into a mixture of cyclodextrins consisting of six, seven, or eight glucose residues (\( \alpha \)-, \( \beta \)-, or \( \gamma \)-cyclodextrins). Selective crystallization steps are used to separate \( \alpha \)-, \( \beta \)-, and \( \gamma \)-cyclodextrins (Bender, 1986). To avoid these expensive procedures, and to produce cyclodextrins for applications involving human consumption, the development of a CGTase that produces only one particular form of cyclodextrin is desirable. Our current attempts to achieve this goal involve protein engineering of the CGTase from Bacillus circulans strain 251.

The CGTase encoding gene of B. circulans strain 251 has been cloned and sequenced, and the crystal structure of the protein has been determined at 2.0 \( \AA \) resolution (Lawson et al., 1990, 1994). The enzyme was found to consist of a single polypeptide chain of 686 amino acid residues; as in other known CGTase structures (Hofmann et al., 1989; Klein & Schulz, 1991; Kubota et al., 1991), five domains (A–E) can be recognized. The three N-terminal domains (A–C) have structural similarity with the three \( \alpha \)-amylase domains. Domain E contains a raw starch binding motif (Svensson, 1989; Jespersen et al., 1991; Lawson et al., 1994), but the precise functions of the D and E domains remain to be resolved.

CGTases and \( \alpha \)-amylases both degrade starch by cleavage of the \( \alpha(1,4) \) glycosidic bonds but produce virtually exclusively cyclic and linear products, respectively. The various CGTases studied can be further distinguished as \( \alpha \)-, \( \beta \)-, and \( \gamma \)-CGTases on the basis of their main cyclodextrin product (Figure 1). The Bacillus macerans enzyme is the best studied example of an \( \alpha \)-CGTase (Tukano et al., 1986), whereas, for instance, the B. circulans strain 251 enzyme is a \( \beta \)-CGTase (Lawson et al., 1990). At present it is unclear what determines the differences in product specificity.
Mutations in Tyr195 of CGTase

Y195F: 5'-TAC AAA AAC CTG TTC GAT CTC-3'  
Y195W: 5'-TAC AAA AAC CTG TGG GAT CTC-3'  
Y195X: 5'-TAC AAA AAC CTG NNA GAT CTC-3'  

Successful mutagenesis resulted in the appearance of the underlined restriction sites, allowing rapid screening of potential mutants. For Y195X this restriction site was BgsIII, and for Y195W it was AlwII. After mutagenesis, the DNA was transformed to E. coli MC1061 cells.

DNA Sequencing. DNA sequence determination was performed on supercoiled plasmid DNA using the dideoxy
Figure 2: Stereo picture of the interactions of the substrate analogue acarbose bound in the active site of CGTase (Strokopytov et al., 1995). The reducing end of acarbose is near Phe 183.

Figure 3: Construction of plasmids pDV66 and pDP66S. Subcloning steps are indicated adjacent to the arrows. Plasmid pDV58 consists of pBS and the cgt gene from R. circulans cloned as a Sau 3A insert (Lawson et al., 1994).

Production and Purification of CGTase (Mutant) Proteins. Plasmid pDV58, carrying positively characterized mutant cgt genes, and plasmid pDP66S were digested with PvuII and NarI. The 1207 base pair fragment from the expression vector pDP66S was replaced with the corresponding fragment containing the mutation from the mutagenesis vector pDV58, ligated, and transformed to E. coli strain MC1061. After isolation of pDP66S DNA and restriction analysis, the plasmid DNA was transformed to B. subtilis strain DB104A. The organism was grown to an optical density at 600 nm of 13 in a 1.5-3 L batch fermenter (for ~50 h). Every 12 h, additional erythromycin (10 μg/mL) was added to the medium. Under these conditions, high extracellular CGTase levels were produced. The culture was centrifuged at 4 °C for 30 min at 16,000 g. The supernatant proteins were concentrated either by ammonium sulfate (50%) precipitation or by ultrafiltration with a 10 kDa Omega mini-ultrasette (Filtron, The Netherlands). The (mutant) CGTases were further purified to homogeneity by affinity chromatography, using a 30 mL α-cyclodextrin Sepharose-6FF column (Pharmacia) (Sundberg & Porath, 1974) with a maximal capacity of 3.5 mg of protein/mL. After the column was washed with 10 mM sodium acetate buffer (pH 5.5), bound CGTase was eluted with the same buffer containing 10 mg/mL α-cyclodextrin.

Enzyme Assays. The various CGTase activities were measured by incubating appropriately diluted enzyme (cyclization, coupling, and saccharifying activities, 0.1-0.2 unit/mL; disproportionation activity, 0.2-0.5 unit/mL) for 5-10 min at 50 °C with substrate solutions in 10 mM sodium citrate (pH 6.0).

β-Cyclodextrin-forming activity was determined using 5% Paselli SA2, partially hydrolyzed potato starch with an average degree of polymerization of 50 (AVEBE, Foxhol, The Netherlands), as a substrate. The β-cyclodextrin formed was determined on the basis of its ability to form a stable, colorless inclusion complex with phenolphthalein (Vikmon, 1982). One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin per minute. Cyclodextrin formation was also measured under industrial production process conditions (Hokse et al., 1981). For this purpose, 0.1 unit/mL CGTase was incubated with 10% jet-cooked starch in a 10 mM sodium citrate buffer (pH 6.0) at 50 °C for 45 h. Samples were taken at regular time intervals and boiled for 5 min, and the products formed were analyzed by high performance liquid chromatography (HPLC) using a 25 cm Econosil-NH2 10 μm column (Alltech Associates Inc., Deerfield, IL) eluted with acetonitrile/water (60/40 v/v) at a flow rate of 1 mL/min.

Coupling activity was assayed with β-cyclodextrin plus linear maltotetraose (G4) as substrates (2% solutions). β-Cyclodextrin disappearance was measured with phenolphthalein. One unit of activity is defined as the amount of...
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enzyme coupling 1 μmol of β-cyclodextrin and G4 per minute.

Disproportionation activity was assayed with linear maltotetraose (G6) as substrate (2% solution). Reaction products were analyzed by HPLC. One unit of activity is defined as the amount of enzyme converting 1 μmol of G6 into other oligosaccharides per minute.

Saccharifying activity was assayed by measuring the increase in reducing power upon incubation of the enzyme with 1% soluble starch (Lamers & Pleuger, Belgium) (Bernfeld, 1955). After addition of 3,5-dinitrosalicylic acid and Rochelle salt (potassium sodium tartrate), the reaction was stopped by incubating the tubes for 5 min in a boiling waterbath. The contents of each tube were diluted 10 times with water before the absorbance at 540 nm was measured against water. A calibration curve of maltose was used to estimate the amount of reducing sugar. One saccharifying unit was defined as the amount of enzyme producing 1 μmol of reducing sugar (as maltose) per minute.

Conversion of Oligosaccharides. Oligosaccharide mixtures (6%) were prepared by fractionation of commercial syrups (AVEBE) on a Sephadex-G25 column. The mixtures were incubated with wild-type CGTase (0.1 unit/mL β-cyclodextrin-forming activity). Products formed were analyzed by HPLC and with phenolphthalein (see above).

Y195L Product Analysis. Purified Y195L mutant protein (0.1 unit/mL β-cyclodextrin-forming activity) was incubated with a 10% soluble starch solution in 10 mM sodium citrate buffer (pH 6.0) at 50°C for 30 min. Reaction mixtures subsequently were incubated with 5 units/mL β-amylase (Boehringer Mannheim) at 37°C for 1 h and concentrated 10-fold by freeze drying. Samples of 20 μL were analyzed by HPLC (see above) on line connected to an ion evaporation atmospheric pressure ionization tandem mass spectrometer (Huang & Henion, 1990).

Structure Determination of Mutant CGTase Proteins. (a) Crystallization. Purified CGTase mutant proteins (Y195F, Y195W, Y195L, and Y195G) were crystallized by vapor diffusion techniques as described (Lawson et al., 1990). Crystals reached their maximum size (0.2 × 0.15 × 1.0 mm3) within 3–4 weeks at room temperature and were approximately 2–3 times smaller by volume compared to native crystals. All crystals were isomorphous with the native crystals (space group P212121), displaying only small deviations in the unit cell dimensions of the native crystals. For crystal mounting, a standard mother liquor was used of 60% (v/v) 2-methyl-2,4-pentanediol (MPD) and 0.1% maltose (w/v) in 0.1 M Hepes buffer, pH 7.55.

(b) Diffraction Data Collection. Data were collected at room temperature with an Enraf Nonius FAST area detector system (Enraf Nonius, Delft, The Netherlands) with Cu Kα radiation from an Elliott GX21 rotating anode generator. The MADNES package (Messerschmidt & Pfugrath, 1987) was used for data collection and processing, with profile fitting and scaling of the data sets done according to Kabsch (1988). The data were merged with software from the Groningen BIOMOL protein crystallography package.

(c) Crystallographic Refinement. The four mutant CGTase structures were refined with the TNT package (Tronrud et al., 1987) using the refined 2.0 Å resolution structure of the wild-type CGTase (Lawson et al., 1994) as a starting model. For the Y195W, Y195F, and Y195L mutants, the residue at position 195 was replaced by Ala at the beginning of the refinement to verify the nature of the substitution and to avoid model bias. The refinement was started with 6–11 cycles of rigid body refinement to allow for the variations in the cell dimensions. This decreased the R-factor to the vicinity of 0.185 in all mutants. Subsequently, cycles of conventional coordinate and temperature factors were performed with manual interventions for minor adjustment of the models on an Evans and Sutherland PS390 graphics system with the program FRODO (Jones, 1986) using σA-weighted (Read, 1986) (2mFo-DFo) exp(iωcalc) electron density maps. Final results of the refinement are summarized in Table 3. The coordinates of the refined models have been deposited with the Brookhaven Protein Data Bank (Bernstein et al., 1977) under the entry codes lCGV (Y195F), lCGW (Y195G), lCGX (Y195L), and lCGY (Y195W).

RESULTS

Construction of Mutant CGTases. The vector pDV58 containing the cloned B. circulans cgt gene (Lawson et al., 1994) was used for the construction of mutants via site-directed mutagenesis. The oligonucleotide Y195X, which has two nucleotides randomly filled in, was designed as a primer for the conversion of Tyr195 into Ala, Arg, Gin, Glu, Gly, Ile, Leu, Lys, Pro, Ser, Thr, and Val. Only the properties of the Y195L and Y195G mutations have been studied and are described in this paper. The mutations Y195F and Y195W were constructed using specific oligonucleotide primers. In each case, a mutation frequency close to 40% was observed; all mutations were confirmed by restriction analysis and DNA sequencing.

As only relatively low expression levels were obtained with pDV58, pDV66 was constructed (Figure 3) with the cgt gene under the control of the strong p32 promoter (van de Vossen et al., 1992). Plasmid pDP66S was subsequently derived to obtain an additional antibiotic resistance marker. For this purpose the streptomycidspectinomycin resistance cassette described by Fellay et al. (1987) was used, with the additional advantage that this cassette contains two transcription terminators at both ends. The pDP66S vector thus carries a transcription terminator downstream from the cgt gene, preventing readthrough of the RNA polymerase. Using this expression vector, a high extracellular production of wild-type and mutant CGTase proteins was obtained reproducibly in batch fermentations with the α-amylase and protease negative B. subtilis strain DB104A. A single fermenter run with B. subtilis strain DB104A allowed purification to homogeneity of up to 100 mg of the (mutant) CGTase proteins.

Characteristics of CGTase (Mutant) Enzymes. CGTase catalyzes four transferase reactions (cyclization, coupling, disproportionation, and hydrolysis) (see Discussion) in which the donor substrate (e.g., an amyllose polymer) is transferred
FIGURE 4: Production of cyclodextrins (in % of converted starch) by wild-type (A) and Y195F (B), Y195W (C), Y195L (D), and Y195G (E) mutant CGTase proteins. •, α-, □, β-, and ▲, γ-cyclodextrin formation.

FIGURE 5: HPLC pattern of products formed after incubation of 10% jet-cooked starch with wild-type (A; for 45 h), Y195G (B; for 45 h), and Y195L (C, D; for 30 min) CGTase proteins. The profile shown in D was obtained after the Y195L reaction mixture (C) was treated with β-amylase.

Table 2: Starch Conversion and Product Specificity of Bacillus circulans Strain 251 Wild-Type and Mutant CGTases

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Conversion of starch into cyclodextrins (%)</th>
<th>Product ratio (%)</th>
<th>Conversion of starch into G1–G4 oligosaccharides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y195</td>
<td>39.3</td>
<td>13 64 23</td>
<td>0</td>
</tr>
<tr>
<td>Y195F</td>
<td>38.8</td>
<td>15 64 20</td>
<td>0</td>
</tr>
<tr>
<td>Y195W</td>
<td>33.3</td>
<td>18 63 19</td>
<td>2–4</td>
</tr>
<tr>
<td>Y195L</td>
<td>24.4</td>
<td>0 86 14</td>
<td>6–10</td>
</tr>
<tr>
<td>Y195G</td>
<td>24.8</td>
<td>19 64 17</td>
<td>16–20</td>
</tr>
</tbody>
</table>

*Results of 45 h incubations of CGTase proteins (0.1 unit of β-cyclodextrin-forming activity per milliliter) with 10% jet-cooked starch.

...to an acceptor substrate (Nakamura et al., 1993). Specific and sufficiently sensitive assays are required to discriminate between these different reactions. Incubations of wild-type CGTase with an oligosaccharide mixture (6%) composed of G1–G4 did not result in their conversion into other oligosaccharides (data not shown); G4 was therefore selected as the second substrate in assays of the coupling activity with β-cyclodextrin. Incubations of CGTase with an oligosaccharide mixture (6%) composed of G1–G10 showed conversion of especially G5–G7 into a range of other oligosaccharides (data not shown). The specific disproportionation activities measured with G5, G6, and G7 were 350, 620, and 400 units/mg protein, respectively. Therefore, G6 was selected as substrate for assaying the disproportionation activity.

All Tyr195 mutations studied resulted in a reduction in β-cyclodextrin-forming and -coupling activities (Table 1). The disproportionation activity with G6 was not affected;
Mutations in Tyr195 of CGTase

Table 3: Data Statistics and Quality of the Final Three-Dimensional Models for Y195 CGTase Mutants

<table>
<thead>
<tr>
<th>mutant</th>
<th>Y195F</th>
<th>Y195W</th>
<th>Y195L</th>
<th>Y195G</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell dimensions (Å)</td>
<td>121.0</td>
<td>120.2</td>
<td>120.3</td>
<td>121.1</td>
</tr>
<tr>
<td>a</td>
<td>111.1</td>
<td>110.7</td>
<td>110.8</td>
<td>111.2</td>
</tr>
<tr>
<td>b</td>
<td>66.3</td>
<td>66.2</td>
<td>66.5</td>
<td>66.8</td>
</tr>
<tr>
<td>c</td>
<td>28-2.50</td>
<td>28-2.50</td>
<td>29-2.59</td>
<td>29-2.53</td>
</tr>
<tr>
<td>resolution range (Å)</td>
<td>84 161</td>
<td>50 751</td>
<td>84 866</td>
<td>95 261</td>
</tr>
<tr>
<td>total no. of observations</td>
<td>28 296</td>
<td>26 191</td>
<td>26 761</td>
<td>27 835</td>
</tr>
<tr>
<td>no. of unique observations</td>
<td>3529</td>
<td>891</td>
<td>2734</td>
<td>3935</td>
</tr>
<tr>
<td>no. of discarded observations</td>
<td>87.3</td>
<td>83.6</td>
<td>95.1</td>
<td>90.3</td>
</tr>
<tr>
<td>completeness of the data (%)</td>
<td>45.0 (2.58–2.50)</td>
<td>32.5 (2.58–2.50)</td>
<td>78.1 (2.67–2.59)</td>
<td>30.9 (2.58–2.53)</td>
</tr>
<tr>
<td>completeness in the last shell (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71</td>
<td>173</td>
<td>184</td>
<td>179</td>
</tr>
<tr>
<td>no. of solvent sites</td>
<td>25.3</td>
<td>24.5</td>
<td>25.7</td>
<td>26.1</td>
</tr>
<tr>
<td>overall R-factor (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.155</td>
<td>0.160</td>
<td>0.152</td>
<td>0.160</td>
</tr>
<tr>
<td>final R-factor</td>
<td>0.010</td>
<td>0.015</td>
<td>0.011</td>
<td>0.011</td>
</tr>
<tr>
<td>bond lengths (Å)</td>
<td>2.10</td>
<td>2.43</td>
<td>2.18</td>
<td>2.25</td>
</tr>
<tr>
<td>van der Waals contacts (Å)</td>
<td>0.029</td>
<td>0.034</td>
<td>0.027</td>
<td>0.025</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numbers in parentheses refer to the completeness in the last shell in angstroms.

similar observations were made with G5 and G7 (data not shown). Reproducibly the Y195L and Y195G mutants were found to possess slightly enhanced saccharifying activities.

Under industrial cyclodextrin production conditions, CGTases are incubated for prolonged periods of time with starch. The performance and product specificity of the mutant enzymes under these conditions were studied in incubations with 10% jet-cooked starch over a 45 h period (Figures 4 and 5, Table 2). The Y195F and Y195W mutants caused relatively minor changes in the overall conversion of starch into cyclodextrins and in product specificity. The Y195L and Y195G mutations, however, resulted in considerably lower overall conversion of starch into cyclodextrins and in pronounced production of linear maltooligosaccharides (Table 2). A clear change in cyclodextrin product ratio was observed with mutant Y195L, which had completely lost the ability to produce α-cyclodextrin (Table 2, Figures 4D and 5c,d). HPLC product analysis revealed that this mutant CGTase early on (30 min incubation) accumulated several additional products (Figure 5c, retention times 20–40 min) that disappeared upon prolonged incubation. A similar phenomenon was also observed with γ-cyclodextrins, i.e., a large production early on followed by a gradual decrease (Figure 4D). Following incubation of the 30 min product samples of mutant Y195L with β-amylose, which degrades linear oligosaccharides but does not degrade cyclodextrins, several peaks still remained (Figure 5D, retention times 20–40 min). These products (0.1–0.7% of starch) were further investigated using HPLC coupled to a mass spectrometer (LC-MS). The spectra obtained showed mass peaks identical to those of cyclodextrins with 7–12 glucose molecules, 1134 for β-, 1296 for γ-, 1458 for δ-, 1620 for ε-, 1782 for ζ-, and 1944 for η-cyclodextrin. The data thus indicate that incubation of mutant Y195L CGTase protein with starch results in accumulation of these larger cyclodextrins.

Structure Determination of Mutant CGTase Proteins. To determine the structural basis for the observed changes in the reaction and product specificity, the three-dimensional structures of the four mutant CGTases were established by X-ray crystallography at 2.5–2.6 Å resolution. The final results of the structure determination and refinement are summarized in Table 3. The mean positional error in the atomic coordinates of the refined models is estimated to be around 0.3–0.4 Å from 〈R<sub>σ</sub>〉 plots (Read, 1986). The electron densities for the different amino acids at position 195 are depicted in Figure 6. Inspection of other parts of the structures, including the N- and C-termini, showed that no other substitutions (or deletions/insertions) detectable by X-ray crystallography at medium resolution had taken place in the structures. Furthermore, no large structural rearrangements compared to the wild-type structure had taken place. The root-mean-square differences with the wild-type structure are around 0.3 Å and are well within the limits of the accuracy of these structure determinations.

DISCUSSION

CGTase catalyzes the transfer of a newly made reducing end saccharide to an acceptor molecule. Depending on the nature of the acceptor molecule, four transferase reactions (cyclization, coupling, disproportionation, and hydrolysis) can be distinguished (Nakamura et al., 1993). (i) Cyclization is the transfer of the reducing end sugar to another sugar residue in the same oligosaccharide chain, thereby creating a cyclic compound. (ii) Coupling is the reaction where a cyclodextrin molecule is combined with a linear oligosaccharide chain to produce a longer linear oligosaccharide. (iii) Disproportionation is the transfer of a part of a linear oligosaccharide chain to a linear acceptor chain. Starting from a pure oligosaccharide, this reaction yields a mixture of smaller and longer oligosaccharides. (iv) Saccharifying activity is the transfer of the newly made reducing end to water, resulting in hydrolysis of the oligosaccharide. Only the hydrolysis reaction thus results in an increased number of reducing ends. The saccharifying activity assay, which estimates the number of reducing ends, is a direct measure for the hydrolyzing activity of CGTase. In this paper we also describe specific assays for the CGTase cyclization, coupling, and disproportionation activities (see Experimental Procedures). Applying these methods for the characterization of CGTase Y195 mutants has provided further insights into the function of this amino acid.
Mutations in Tyr195 of CGTase

E

FIGURE 6: Stereo diagrams of α_{i} weighted (Read, 1986) (2mF_{o} - DF_{c}) exp(iφ_{odi}) electron density maps of amino acid residues in the active site of wild-type (A), Y195F (B), Y195W (C), Y195L (D), and Y195G (E) CGTase of \textit{B. circulans} strain 251.

The CGTase reaction mechanisms, and the factors determining the specificities of the various reactions, are not understood at present. The aromatic amino acid Tyr195 is present at a dominant position in the center of the active site cleft of \textit{B. circulans} strain 251 CGTase, whereas α-amylases have a much smaller residue at this position (Figure 1). In view of the availability of a three-dimensional structure of this CGTase (Lawson et al., 1990, 1994), site-directed mutagenesis appeared the most straightforward approach to study the functions of Y195. The characterization of the four purified mutant CGTase proteins described in this study revealed strong negative effects of the mutations on the cyclization and coupling activities only (Table 1). The latter activities both involve an interaction with cyclodextrins, and they decreased to a similar extent (except for the Y195L mutant, which behaves anomalously in several respects). This suggests that coupling and cyclization activities are determined to at least some extent by similar factors. The data thus confirm that Y195 plays an important role in the CGTase reactions involving cyclodextrins.

The three-dimensional structures of the Y195 mutants are very similar to that of the wild-type (Figure 6, Table 3). Comparison of the conformations of the amino acids around residue 195 showed clearly that no large structural re-arrangements had taken place as a result of substitutions of Tyr by the other amino acids. No significant differences were found in the main chain φ/ψ angles. The enzyme conformation thus is not affected by these substitutions, and no conformational feature can directly account for the observed changes in properties compared with wild-type CGTase.

One possible explanation for the observed changes in CGTase properties is that the residue at position 195 interacts especially with cyclic substrates (in the coupling reaction) and with linear substrates which are converted into cyclic products. Aromatic residues such as Tyr, Phe, and Trp favorably interact with the hydrophobic face of carbohydrate residues, as has been observed, for instance, for the maltose binding sites in the C and E domains of CGTase (Lawson et al., 1994). Conceivably, the aromatic amino acid residues Tyr and Phe at position 195 in CGTases (Figure 1) are most efficient in bending the nonreducing end of the acceptor oligosaccharide toward the reducing end of the donor substrate that is bound in the catalytic center, resulting in cyclodextrin formation. This would explain, to at least some extent, the severe reduction in cyclization activity observed with mutant CGTases (Table 2). Likewise, in the coupling reaction, Tyr195 might provide a favorable interaction with the cyclic substrate.

Secondly, Sin et al. (1993) speculated that the size of the residue at position 195 also may influence the preferred cyclodextrin size. These authors observed that substitution of Y188 by Trp in the \textit{Bacillus ohbensis} CGTase, which is at a position equivalent to that of Y195 in the \textit{B. circulans} CGTase (Figure 1), doubled the production of γ-cyclodextrin. Replacement of Y195 of the \textit{B. circulans} CGTase by other amino acids, however, did not significantly affect the cyclodextrin product ratios, except with Y195L, which resulted in a shift toward the synthesis (in low yields) of larger products (Table 2; Figure 5). Interestingly, the γ-CGTase of \textit{B. subtilis} strain 313 is the only example of a CGTase with a Leu residue at this position (Figure 1). Also, the mutation F191Y at the similar position in the CGTase of \textit{Bacillus stearothermophilus NO2} had a minor effect only on product specificity (Fujiwara et al., 1992). Our initial expectation that mutant Y195W might lose the ability to produce α-cyclodextrins because Trp is too big to fit into the cyclodextrin cavity thus was not confirmed.

A third explanation for our observations may be that Tyr at position 195 is able to exclude water molecules from the active site, thus preventing hydrolysis. Conceivably, the wild-type enzyme is able to completely exclude water molecules from the active site when substrate is bound, whereas CGTases with a smaller residue at position 195 may be less adequate in this respect. Replacing Y195 with nonaromatic amino acids indeed resulted in strongly reduced total conversion of starch into cyclodextrins and a switch-over to synthesis of linear oligosaccharides upon incubation of these mutant CGTases with starch for 45 h (Table 2). This indicates that hydrolysis does indeed occur more often with these mutant enzymes, although their saccharifying activities remain relatively low (Table 1).

In conclusion, our data show that the residue at position 195 is important for the cyclization and coupling reactions, and to a lesser extent for the disproportionation and saccharifying activities. Nevertheless, even the Y195G mutant has a significant cyclization activity; additional factors thus also contribute to the efficiency of the conversion of starch into cyclodextrins. Further studies are needed to establish why Y195 and F195 are most effective in supporting formation of the cyclodextrin ring structure and/or in preventing hydrolysis. Clarification is also needed for the observation...
that the Y195L mutant produces virtually no α-cyclodextrin, but, in contrast to wild-type enzyme, produces larger cyclodextrins.

ACKNOWLEDGMENT

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REFERENCES


B1941723W