Site-Directed Mutations in Tyrosine 195 of Cyclodextrin Glycosyltransferase from
Bacillus circulans Strain 251 Affect Activity and Product Specificity†

Dirk Penninga,‡ Boris Strokopytov,§ Henriëtte J. Rozeboom, Catherine L. Lawson,S,† Bauke W. Dijkstra,§
Jack Bergsma,‡ and Lubbert Dijkhuizen*‡

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen,
Kerklaan 30, 9751 NN Haren, The Netherlands, BIOSON Research Institute and Laboratory of Biophysical Chemistry,
Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4,
9747 AG Groningen, The Netherlands, and Department of Biotechnology, AVEBE Research & Engineering,
AVEBE-weg 1, 9607 PT Foxhol, The Netherlands

Received July 27, 1994; Revised Manuscript Received December 1, 1994‡§

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
α-amylases, and the analysis of the binding mode of the substrate analogue acarbose in the active site
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 Å 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
maltooligosaccharides. 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 long 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
β- and γ- (but no α-) cyclodextrins and minor amounts of δ-, ε-, ζ- and η-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
α(1,4) glycosic 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 (α-, β-, or γ-cyclodextrins). Selective crystallization steps are used to separate α-, β-, and γ-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 Å 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 α-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 α-amylases both degrade starch by cleavage of the α(1,4) glycosic bonds but produce virtually exclusively cyclic and linear products, respectively. The various CGTases studied can be further distinguished as α-, β-, and γ-CGTases on the basis of their main cyclodextrin product (Figure 1). The Bacillus macerans enzyme is the best studied example of an α-CGTase (Takano et al., 1986), whereas, for instance, the B. circulans strain 251 enzyme is a β-CGTase (Lawson et al., 1990). At present it is unclear what determines the differences in product specificity.
Mutations in Tyr195 of CGTase

between the various CGTases (α-, β-, and γ-cyclodextrin ratios) and α-amylases (cyclic versus linear maltooligosaccharides). Members of the CGTase family possess a high overall amino acid sequence identity (>60%) but only a fairly low sequence identity with α-amylases (~30%). Nevertheless, the active sites of CGTases and α-amylases, located in the A domain, are rather similar (Nakamura et al., 1992; Klebsiella pneumoniae; Nishizawa et al., 1987); (d) alkalophilic (Hill et al., 1990); (f) alkalophilic (Kimura et al., 1987); (g) alkalophilic (Nakamura et al., 1992; Sin et al., 1991); (i) alkalophilic (Binder et al., 1986); (j) alkalophilic (Kaneko et al., 1989); (e) alkalophilic (Kohno et al., 1992); (i) alkalophilic (Horikoshi, 1988); (l) alkalophilic (Takano et al., 1986); (k) alkalophilic (Schmid et al., 1988); (l) alkalophilic (Kubota et al., 1991); (m) alkalophilic (Kubota et al., 1991); (n) alkalophilic (B. maceruns sp. strain 17.1 (Kaneko et al., 1989); (o) alkalophilic (B. circulans sp. strain 8 (Bender, 1990); (p) alkalophilic (B. circulans sp. strain F-2 (Nishizawa et al., 1987); (q) alkalophilic Bacillus sp. strain 17.1 (Kaneko et al., 1989); (r) alkalophilic Bacillus sp. strain 3011 (Kimura et al., 1987); (s) alkalophilic Bacillus sp. strain 38.2 (Horikoshi, 1988); (t) alkalophilic Bacillus sp. strain 1-1 (Schmid et al., 1988); (u) alkalophilic Bacillus sp. strain 251 (Kimura et al., 1987); (v) alkalophilic Bacillus sp. strain F-2 (Hill et al., 1990); (w) alkalophilic Bacillus sp. strain 313 (Horikoshi, 1988); (x) Aspergillus oryzae Taka-amylase A (Nagashima et al., 1992); (y) pig α-amylase and (z) human saliva α-amylase (Nakajima et al., 1986).

Here we report the biochemical properties and crystal structures of mutant CGTase proteins in which the Tyr195 residue has been replaced by Phe, Trp, Leu, and Gly. Analysis of the products formed by mutant Y195L revealed a shift in product specificity toward larger cyclodextrins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Bacteriophage, and Plasmids.** Escherichia coli MC1061 [hsdR mcIR b araD139 Δ(araABC-leu)-7679 ΔlacX74 galU galK rpsL thi] (Meissner et al., 1987) was used for recombinant DNA manipulations. E. coli CJ236 [dar1 ung1 thi-1 relAI/pCJ105 (Cm' F')] (Kunkel et al., 1987) was used for site-directed mutagenesis. CGTase (mutant) proteins were produced with the α-amylase and protease negative Bacillus subtilis strain DB104A [amy his nprR2 nprE18 aprA3] (Smith et al., 1988). The bacteriofage M13K07 was used for preparing single-stranded DNA (Vieira & Messing, 1987). Plasmid pGKV432 [ErCm' pWV01-ori p32] (van de Vossen et al., 1992) was digested with HindIII and SmaI. The largest fragment was ligated with the B. circulans cgt gene from pDV58 (Lawson et al., 1994), digested with Asp718 (made blunt with Klenow polymerase) and HindIII. The resulting plasmid pDV66, with the cgt gene under control of the erythromycin-inducible p32 promoter (van de Vossen et al., 1992), was digested with HindIII, followed by incubation with Klenow polymerase (to produce a blunt end), and partially digested with HpaI. The 6.4 kb fragment obtained was ligated with the streptomycin/spectinomycin resistance (Sm'/Sp') gene from pH45Ω [Ap' Sm'/Sp'] (Fellay et al., 1987) digested with Smal and transformed to E. coli MC1061 under selection for erythromycin and spectinomycin resistance, yielding the CGTase protein expression vector pDP66S (Figure 3).

**Growth Conditions.** Plasmid-carrying bacterial strains were grown on LB medium in the presence of the antibiotics ampicillin (plasmid pDV58), erythromycin (pDV66), or erythromycin and spectinomycin (pDP66S) at concentrations of 100 and 5 μg/mL for E. coli and B. subtilis, respectively (Sambrook et al., 1987). When appropriate, agar plates contained 1% starch to screen for halo formation. B. subtilis strain DB104A was grown in a 1.5—3 L batch fermenter with aeration and with temperature and pH control, using a medium with 2% trypton, 0.5% yeast extract, 1% sodium chloride, and 1% casamino acids (pH 7.0) with 10 μg/mL erythromycin and 5 μg/mL spectinomycin.

**DNA Manipulations.** Restriction endonucleases and Klenow enzyme were purchased from Pharmacia LKB Biotechnology, Sweden, and used according to the manufacturer's instructions. DNA manipulations and calcium chloride transformation of E. coli strains were as described (Sambrook et al., 1989). Transformation of B. subtilis was performed according to the method of Bron (1990). **Site-Directed Mutagenesis.** The Kunkel method (Kunkel et al., 1987) was used for site-directed mutagenesis. Single-stranded DNA was prepared using plasmid pDV58 (carrying the fl origin from pBS+ and E. coli strain CJ236 after infection with bacteriophage M13K07. The following oligonucleotides were used to produce the mutations:

**Y195X:** 5'-TAC AAA AAC CTG NNA GAT CTC-3'  
5'-GCC GAC-3'

**Y195W:** 5'-TAC AAA AAC CTG NNA GAT CTC-3'  
5'-GCC GAC-3'

**Y195F:** 5'-TAC AAA AAC CTG NNA GAT CTC-3'  
5'-GCC GAC-3'

Successful mutagenesis resulted in the appearance of the underlined restriction sites, allowing rapid screening of potential mutants. For Y195X this restriction site was BgII, and for Y195W it was AlwI. 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
Penninga et al.

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 Phe183.

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).

Chain termination method (Sanger & Coulson, 1975) and the T7-sequencing kit from Pharmacia.

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
Mutations in Tyr195 of CGTase

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 & Pleugger, 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 & Pflugrath, 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, 1978) using σA-weighted (Read, 1986) (2mFo - DFo) exp(fo) 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 1CGV (Y195F), 1CGW (Y195G), 1CGX (Y195L), and 1CGY (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 streptomycinspectinomycin 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 amylose polymer) is transferred

<table>
<thead>
<tr>
<th>mutants</th>
<th>cyclization</th>
<th>coupling</th>
<th>disproportionation</th>
<th>saccharifying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y195F</td>
<td>620 ± 70</td>
<td>700 ± 80</td>
<td>650 ± 70</td>
<td>650 ± 80</td>
</tr>
<tr>
<td>Y195W</td>
<td>700 ± 80</td>
<td>650 ± 70</td>
<td>650 ± 80</td>
<td>650 ± 80</td>
</tr>
<tr>
<td>Y195L</td>
<td>650 ± 80</td>
<td>650 ± 80</td>
<td>650 ± 80</td>
<td>650 ± 80</td>
</tr>
<tr>
<td>Y195G</td>
<td>500 ± 70</td>
<td>4.3 ± 0.5</td>
<td>4.8 ± 0.5</td>
<td>4.8 ± 0.5</td>
</tr>
</tbody>
</table>

Table I: Specific Enzyme Activities of Bacillus circulans Strain 251 Wild-Type and Mutant CGTase Proteins
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 Gl-G4 oligosaccharides (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y195</td>
<td>39.3</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>Y195F</td>
<td>38.8</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td>Y195W</td>
<td>33.3</td>
<td>18</td>
<td>63</td>
</tr>
<tr>
<td>Y195L</td>
<td>24.4</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>Y195G</td>
<td>24.8</td>
<td>19</td>
<td>64</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.

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

resulted in considerably lower overall conversion of starch into cyclodextrins and in pronounced production of linear maltoligosaccharides (Table 2). A clear change in cyclodextrin product ratio was observed with mutant Y195L, which had completely lost the ability to produce α-cyclodextrin. 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.

**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 of 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.

---

**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>a (Å)</td>
<td>121.0</td>
<td>120.2</td>
<td>120.3</td>
<td>121.1</td>
</tr>
<tr>
<td>b (Å)</td>
<td>111.1</td>
<td>110.7</td>
<td>110.8</td>
<td>111.2</td>
</tr>
<tr>
<td>c (Å)</td>
<td>66.3</td>
<td>66.2</td>
<td>66.5</td>
<td>66.8</td>
</tr>
<tr>
<td>resolution range (Å)</td>
<td>29–2.50</td>
<td>29–2.50</td>
<td>29–2.53</td>
<td>29–2.53</td>
</tr>
<tr>
<td>total no. of observations</td>
<td>84 161</td>
<td>50 751</td>
<td>84 866</td>
<td>95 261</td>
</tr>
<tr>
<td>no. of unique observations</td>
<td>28 296</td>
<td>26 191</td>
<td>26 761</td>
<td>27 835</td>
</tr>
<tr>
<td>no. of discarded observations</td>
<td>3529</td>
<td>891</td>
<td>2734</td>
<td>3935</td>
</tr>
<tr>
<td>completeness of the data (%)</td>
<td>87.3</td>
<td>83.6</td>
<td>95.1</td>
<td>90.3</td>
</tr>
<tr>
<td>completeness in the last shell (%)²</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>
</tbody>
</table>

² The numbers in parentheses refer to the completeness in the last shell in angstroms.

**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 OA 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.
binding sites in the C and E domains of CGTase (Lawson in this study revealed strong negative effects of the mutations."

```
Aromatic residues such as Tyr, Phe, and Trp are much smaller than a similar position in the CGTase (Figure 1), doubled the production of γ-cyclodextrin. Replacement of Y195 of the 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 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 Bacillus stea rothermophilus 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

We thank Andries P. Bruins (Department of Analytical Pharmacy, University of Groningen) for mass spectrometric analysis of the larger cyclodextrins.

REFERENCES


Penninga et al.