Chapter 6

The role of the acceptor binding site in reaction mechanism and reaction specificity of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251

Bart A. van der Veen, Hans Leemhuis, Slavko Kralj, Joost C. M. Uitdehaag, Bauke W. Dijkstra, and Lubbert Dijkhuizen

Cyclodextrin glycosyltransferases (CGTase) (EC 2.4.1.19) belong to the α-amylase family (family 13 of glycosyl hydrolases). Whereas α-amylases usually catalyze hydrolysis reactions using water as acceptor, CGTases preferably catalyze transglycosylation reactions in which glucosyl residues are used as acceptor. The main differences between α-amylases and CGTases with respect to reaction specificity are thus likely to be found at the acceptor binding sites. CGTase residues His233 and Lys232 provide hydrogen bonding interactions with glucosyl groups at the +1 and +2 acceptor subsites. Identical residues are found in some α-amylases. Typical for CGTases is, however, the combination of two phenylalanines (Phe183 and Phe259) positioned at acceptor subsite +2. Also a subsite +3 (Glu264) has been identified in CGTase. Using site directed mutagenesis, the specific roles of these residues in the CGTase catalyzed reactions were identified.

Lys232 is specifically involved in binding the acceptor in the ternary complex in the coupling reaction and appears to be required for the role of His233 in catalysis. Glu264 is specifically involved in initial binding of the (maltose) acceptor in the disproportionation reaction. Phe183 and Phe259 play distinct roles in the transglycosylation reactions catalyzed by CGTase. Phe183 properly positions the acceptor glucose residues for interaction with Phe259, which subsequently induces them to adopt a conformation suitable for catalysis. Furthermore, the hydrophobicity of Phe183 and Phe259 limits the hydrolyzing activity of CGTase. Hydrolysis can be enhanced by mutagenesis of these residues, concomitantly decreasing the transglycosylation activities of the enzyme. An F183S/F259N double mutant was constructed, resulting in an enzyme preferring hydrolysis over cyclization (15 : 1), whereas wild type CGTase favours cyclization over hydrolysis (90 : 1).

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Introduction

Cyclodextrin glycosyltransferases (CGTase) (EC 2.4.1.19) belong to the α-amylase family (glycosyl hydrolase family 13) (Henrissat, 1991), an important group of starch converting enzymes. Catalysis in the α-amylase family proceeds via a covalently linked intermediate (Uitdehaag et al. 1999b), which basically divides the reaction in two steps. In the first step the donor substrate (starch or oligosaccharide) is processed, yielding the covalent intermediate (donor reaction). In the second step the acceptor reacts with this intermediate, resulting in product formation (acceptor reaction). Whereas α-amylases usually catalyze the hydrolysis reaction using water as acceptor, CGTases mainly catalyze transglycosylation reactions in which the acceptor is either the non-reducing end glucose of another oligosaccharide (disproportionation) or the non-reducing end glucose of the covalently linked oligosaccharide intermediate, resulting in formation of a cyclodextrin (cyclization). Also the reverse of cyclization, in which a cyclodextrin is cleaved and transferred to an accepting oligosaccharide (coupling) is catalyzed by CGTase (van der Veen et al. 2000c). The main determinants that cause the difference in reaction specificity between α-amylases and CGTases are likely to be found at the acceptor binding sites.

The crystal structures of the *B. circulans* strain 251 CGTase in complex with an acarbose-derived maltononaose inhibitor (Strokopytov et al. 1996) and a maltononaose substrate (Uitdehaag et al. 1999b) have revealed the nature of the acceptor site. In the maltononaose structures the glucose residue at subsite +1 has hydrogen bonding interactions with His233, similar to that in *Aspergillus oryzae* α-amylase complexed with an acarbose-derived maltohexaose inhibitor (Brzozowski and Davies, 1997). At subsite +2 a hydrogen bonding interaction occurs also in both enzymes (with Lys232, CGTase numbering)(Fig.1.a). In CGTase the +2 sugar residue is, however, sandwiched between phenylalanines 183 and 259 (see Fig. 1), which are well conserved in CGTases, whereas in the α-amylase structure only Leu232 (equivalent to Phe259 in CGTase) has hydrophobic interactions with this glucose residue (Brzozowski and Davies, 1997). Both aromatic residues have been shown to be important for the cyclization reaction (Nakamura et al. 1994a), but their specific functions have remained unclear. For the CGTase from *Thermoanaerobacter thermosulfurigenes* strain EM1 a third acceptor subsite has been identified, in which Glu265 (Glu264 in the *B. circulans* CGTase) is involved (Wind et al. 1998)(see Fig.1.a).

The crystal structures of the *B. circulans* strain 251 CGTase in complex with a γ-cyclodextrin (Uitdehaag et al. 1999a) revealed specific differences between the binding mode of linear and cyclic compounds. The glucose residue at subsite +1 is bound identically to the subsite +1 glucose of a linear maltononaose, however, hydrogen bonding interactions with the cyclodextrin at subsites +2 and +3 are not observed (Fig.1.b). Also the hydrophobic interactions with phenylalanines 183 and 259 show differences between the two binding modes (see Fig. 1). Whereas Phe183 has better stacking interactions with the linear compounds (Fig. 1.a), for Phe259 these interactions are better with the cyclodextrin (Fig. 1.b).
The present study provides a detailed analysis of the roles of amino acid residues in the acceptor subsites +2 and +3 of *B. circulans* strain 251 CGTase. It illustrates that the various residues have distinct roles, dependent on whether a disproportionation, cyclization, or coupling reaction takes place.

Figure 1. Schematic representation of the interactions between the *B. circulans* strain 251 CGTase and sugars bound at the active site. a) binding mode of a linear oligosaccharide (Strokopytov et al. 1996, Uitdehaag et al. 1999b). b) binding of a cyclodextrin (Uitdehaag et al. 1999a).

**Experimental procedures**

**Bacterial strains and plasmids**

*Escherichia coli* MC1061 [hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi] (Meissner et al. 1987) was used for recombinant DNA manipulations and site-directed mutagenesis. *E. coli* DH5α [F'endA1 hsdR17 supE44 thi1 recA1 gyrA (Nal') relA1 (lacZYA-argF) U196 (o80lacΔ(lacZ)M15)] (Hanahan, 1983) was used for the production of monomeric supercoiled plasmid DNA for sequencing. CGTase (mutant) proteins were produced with the α-amylase and protease negative *Bacillus subtilis* strain DB104A [amy nprR2 nprE18 aprA3] (Smith et al. 1988). Plasmid pDP66K (Penninga et al. 1996), with the cgt gene from *Bacillus circulans* strain 251 under control of the p32 promoter (van de Vossen et al. 1992), was used to introduce site-directed mutations and for production of the enzymes. Plasmid pBluescript KSII (Stratagene) was used for automated sequencing. DNA
manipulations and calcium chloride transformation of *E. coli* strains were performed as described (Sambrook et al. 1989). Transformation of *B. subtilis* was performed according to Bron (Bron, 1990).

**Site-directed mutagenesis**

Mutations were introduced with a PCR method using VENT-DNA polymerase (New-England Biolabs, Beverly, MA, USA) (Penninga et al. 1996). A first PCR reaction was carried out with a mutagenesis primer for the coding strand plus a primer downstream on the template strand. The reaction product was subsequently used as primer in a second PCR reaction together with a primer upstream on the coding strand. For the construction of F183/F259 double mutants the reaction product of the first PCR with the mutagenesis primer for F259 mutations was used as primer in a second PCR reaction together with the mutagenesis primer for F183 mutations. This reaction product was subsequently used as primer in a third PCR reaction together with a primer upstream on the coding strand. The products of the PCR reactions (1360 bp) were cut with *Pvu*II and *Sal*I and the resulting fragments (1210 bp) were exchanged with the corresponding fragment from the vector pDP66K. The resulting (mutant) plasmids were transformed to *E. coli* MC1061 cells. The following oligonucleotide was used to produce the Lys232 mutations: 5'-C ATG GAT GCA GTA C W G CAC ATG CCG-3'. The underlined cytosine is a replacement for the original thymine, resulting in removal of an *Nde*I site (CATATG). W can be an adenine or a thymine. An adenine results in the Lys232Gln mutation; a thymine results in the Lys232Leu mutation and introduction of an *Sca*I site (AGTACT). For the production of mutant Glu264Ala the primer 5'-C CTG GGC GTT AAC GCA GTG AGC CCG-3' was used, resulting in introduction of an *Hpa*I site (GTAAAC). Oligonucleotides used to produce the phenylalanine mutations were: for Phe183Ile,Thr,Asn, or Ser, 5'-GGC GGT ACC GAC A N T TCC ACG ACC-3', containing a *Kpn*I site (GGTACC); for Phe259Ile,Thr,Asn, or Ser, 5'-GGC GAA TGG A N C CTA GGC GTA AAT GAA-3', containing an *Avr*II site (CCTAGG). *N* can be any of the four bases, resulting in identical mutations in both primers. An adenine results in the Phe→Asn mutation; a cytosine in the Phe→Thr mutation; a guanine in the Phe→Ser mutation; a thymine in the Phe→Ile mutation. The built in restriction sites allowed rapid screening of potential mutants. A mutation frequency close to 70% was observed; all mutations were confirmed by restriction analysis and DNA sequencing.

**DNA sequencing**

Plasmids pDP66K carrying the correct restriction sites were cut with *Eco*RI and *Apa*I, and with *Apa*I and *Sal*I. The resulting fragments were cloned in the multiple cloning site of plasmid pBluescript, and the resulting plasmids transformed to *E. coli* DH5α cells. Dideoxy sequencing reactions were done using T7 DNA polymerase, with either 5'-end labelled primers or with unlabelled primers and fluorescein-labelled ATP (Voss et al. 1992; Zimmermann et al. 1990). Nucleotide sequencing was done with the Automated Laser Fluorescent DNA sequencer (Pharmacia). The nucleotide sequence data were compiled and analyzed using the programs supplied in the PC/GENE software package (Intelligenetics).
Growth conditions and purification of CGTase proteins

Plasmid carrying bacterial strains were grown on LB agar in the presence of the antibiotic kanamycin, at concentrations of 100 and 5 µg/ml for *E. coli* and *B. subtilis*, respectively (Sambrook et al. 1989). Agar plates also contained 1.5 % potato starch (Sigma) for the detection of CGTase secretion by the bacteria. CGTase activity is visible on these plates by the precipitation of (β-)cyclodextrins, resulting in halo formation. *B. subtilis* strain DB104A with plasmid pDP66K, carrying wild type or mutant cgt genes, was grown for 24 h in a 2 l fermentor, containing 1.5 l 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 kanamycin, to a final optical density at 600 nm of approximately 12. Under these conditions high extracellular CGTase levels were obtained reproducibly, allowing purification to homogeneity of up to 25 mg of CGTase protein per liter. The culture was centrifuged at 4°C for 30 min at 10,000 g. The (mutant) CGTases in the culture supernatants were further purified to homogeneity by affinity chromatography, using a 30 ml [α-cyclodextrin-Sepharose-6FF column (Pharmacia, Sweden) (Sundberg and Porath, 1974) with a maximal capacity of 3.5 mg protein per ml. After washing 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

For all assays and enzyme dilutions a 10 mM citrate buffer (pH 6) was used. All incubations were carried out at 50 °C.

**β-Cyclodextrin forming activity** was determined by incubating appropriately diluted enzyme (0.1-0.2 units of activity) for 2-4 min with a 5% solution of partially hydrolysed potato starch with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands) preincubated at 50 °C for 10 min. At regular time intervals samples were taken and the amount of β-cyclodextrin formed was determined based on its ability to form a stable colourless inclusion complex with phenolphthalein (Vikmon, 1982). One unit of activity is defined as the amount of enzyme able to produce 1 µmole of β-cyclodextrin per min.

**Coupling activities** were determined using the method described by Nakamura et al. (1993) with the modifications described by van der Veen et al. (2000c (chapter 3)). One unit of activity is defined as the amount of enzyme coupling 1 µmole of cyclodextrin to MαDG per min.

**Disproportionation activities** were determined using the method described by Nakamura et al. (1994b) was used with the modifications described by van der Veen et al. (2000c (chapter 3)). One unit of activity was defined as the amount of enzyme converting 1 µmole of EPS per min.

**The hydrolyzing activity** was determined as described before (Penninga et al. 1995): The hydrolysis of a 1% soluble starch (Lamers & Pleuger, Belgium) solution, preincubated at 50 °C for 10 min, upon addition of CGTase was followed by measuring the increase in reducing power (Bernfeld, 1955). One unit of saccharifying activity was defined as the amount of enzyme producing 1 µmole of reducing sugar per min.

**Kinetic analysis** of the two substrate reactions (coupling and disproportionation) was performed with SigmaPlot (Jandel Scientific). The following equations (Cornish-Bowden,
Chapter 6

1995) were used to fit the experimental data to determine which kinetic mechanism applies to the transglycosylation reactions catalyzed by CGTase:

1) substituted-enzyme mechanism:
   
   Ping-pong:
   \[ v = \frac{V \cdot a \cdot b}{K_{MB} \cdot a + K_{MA} \cdot b + a \cdot b} \] (Eq. 1.a)

   Ping-pong (with substrate inhibition):
   \[ v = \frac{V \cdot a \cdot b}{K_{MB} \cdot a + K_{MA} \cdot b \cdot (1 + b/K_i) + a \cdot b} \] (Eq. 1.b)

2) ternary complex mechanism:
   
   Random-order:
   \[ v = \frac{V \cdot a \cdot b}{K'_{MA} \cdot K_{MB} + K_{MB} \cdot a + K_{MA} \cdot b + a \cdot b} \] (Eq. 2)

In these equations \( v \) is the reaction rate, \( V \) is the maximal reaction rate, \( a \) and \( b \) are the donor and acceptor substrate concentrations, respectively, \( A \) and \( B \) are the donor and acceptor substrates, respectively, and \( K_m \) and \( K_m' \) are the affinity constants for the substrates in the absence and presence of the second substrate, respectively. \( K_{MB}' \) is lost in the derivation of equation 2, but it can easily be determined, since \( K_{MA}/K_{MB} = K_{MA}'/K_{MB}' \)

**Determination of protein concentration**

Protein concentrations were determined with the Bradford method using the Bio-Rad reagent and bovine serum albumin as a standard (Bio-Rad Laboratories, Richmond, CA, USA).

**RESULTS**

**Screening and identification of mutant enzymes**

Several variants of residues 183, 232, 259 and 264 were obtained. A first screening on starch containing agar plates revealed halo formation by all single mutants, indicating the production of \( \beta \)-cyclodextrin by the mutant CGTases. In contrast, several double mutants of residues 183 and 259 were incapable of halo formation. DNA sequencing of various selected clones resulted in identification of the following CGTase mutants: F183N, F183S, K232L, K232Q, E264A, F259N, F259S, and the double mutant F183S/F259N.

**Effects of the mutations on the cyclization reaction**

The \( \beta \)-cyclization activities of the wild type and mutant CGTase enzymes are shown in Table 1. Changing hydrogen bonding interactions at subsite +2 (K232 mutants) clearly resulted in decreased cyclization activity, especially with mutant K232Q. Likewise, substitution of F259 and F183 by hydrophilic residues reduced the activity. Of the single mutants F183N showed the highest reduction in cyclization activity (10 fold). The double mutant F183S/F259N showed even a 300 fold decrease in cyclization activity, which is much larger than what would
be expected from a combination of the single mutants, suggesting a concerted action of the two phenylalanine residues. In contrast, in the subsite +3 mutant E264A the cyclization activity was hardly affected.

**Effects of the mutations on the hydrolysis reaction**

The specific activities of hydrolysis of starch of the (mutant) enzymes are listed in Table 1. Especially the F259 mutants showed a significant increase in hydrolyzing activity on starch (10 to 20 fold), while mutation F183N resulted in a 3 fold increase. Starch hydrolysis activity of the double mutant F183S/F259N was intermediate compared to the single F183N and F259N mutants.

<table>
<thead>
<tr>
<th>(mutant) enzyme</th>
<th>β-cyclization(U/mg)</th>
<th>starch hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>270.0 ± 1.7</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>F183N</td>
<td>25.4 ± 1.7</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>K232L</td>
<td>122.9 ± 6.5</td>
<td>nd</td>
</tr>
<tr>
<td>K232Q</td>
<td>69.2 ± 3.5</td>
<td>nd</td>
</tr>
<tr>
<td>E264A</td>
<td>215.8 ± 11.9</td>
<td>nd</td>
</tr>
<tr>
<td>F259N</td>
<td>41.7 ± 0.2</td>
<td>60.3 ± 1.7</td>
</tr>
<tr>
<td>F259S</td>
<td>42.0 ± 1.8</td>
<td>32.6 ± 1.0</td>
</tr>
<tr>
<td>F183S/F259N</td>
<td>0.9 ± 0.1</td>
<td>14.3 ± 1.4</td>
</tr>
</tbody>
</table>

nd: not determined

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**Effects of the mutations on the disproportionation reaction**

For further characterization of the acceptor site the disproportionation reaction was analyzed (Fig. 2, Table 2). All mutations resulted in decreased disproportionation activities. For the K232 mutants and mutant F259N this reduction in activity was comparable to that in the cyclization activity, suggesting similar roles for these residues in the two reactions. Mutation F183N, however, showed only a twofold reduction in disproportionation activity compared to a tenfold reduction in cyclization activity, suggesting that this residue has a more significant role in cyclization. As for the cyclization reaction, the double mutant F183S/F259N resulted in a much larger reduction in disproportionation activity (75 fold) than would be expected from a combination of the single mutants. Also for the disproportionation reaction the results suggest a concerted action of the two phenylalanine residues. Finally, at subsite +3, mutant E264A showed a twofold reduction in disproportionation activity, indicating that this subsite, although somewhat remote from the catalytic site, contributes to this reaction. Surprisingly, all mutations, even the one at subsite +3, resulted in decreased $K_M$ values for maltose, and thus increased affinities. Furthermore, the data for mutant F183N in Fig. 2.a clearly indicates substrate inhibition exerted by the acceptor substrate (maltose), which may be a direct effect of the increased affinities. In the insert in Fig. 2.b the Lineweaver-Burk plot for the wild type is shown, the parallel lines indicating normal ping-pong types of kinetics, represented by equation 1.a.
Table 2. Kinetic parameters of the disproportionation reaction of wild type and mutant CGTases

<table>
<thead>
<tr>
<th>(mutant)enzyme</th>
<th>$V_{max}$ (U/mg)</th>
<th>$K_M$EPS (mM)</th>
<th>$K_M$Mal (mM)</th>
<th>$K_i$Mal (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>970.0 ± 17.6</td>
<td>0.22 ± 0.02</td>
<td>0.83 ± 0.05</td>
<td>nd</td>
</tr>
<tr>
<td>F183N</td>
<td>556.3 ± 16.0</td>
<td>0.26 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>K232L</td>
<td>627.3 ± 15.6</td>
<td>0.29 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>K232Q</td>
<td>303.3 ± 11.9</td>
<td>0.39 ± 0.04</td>
<td>0.29 ± 0.03</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>E264A</td>
<td>520.6 ± 10.6</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>5.1 ± 1.1</td>
</tr>
<tr>
<td>F259N</td>
<td>189.2 ± 4.2</td>
<td>0.10 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>4.9 ± 1.1</td>
</tr>
<tr>
<td>F183S/F259N</td>
<td>13.7 ± 0.9</td>
<td>0.70 ± 0.06</td>
<td>0.01 ± 0.08</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

nd: not detectable

Although acceptor concentrations were raised up to 1 M, no inhibitory effect of maltose could be detected for the wild type enzyme. For all mutants the experimental data could best be fitted with equation 1.b, resulting in the inhibition constants listed in Table 2. The substrate inhibition by maltose was most pronounced for mutant F183N (shown in Fig. 2), although this mutant did not result in the highest increase in affinity for maltose.

Fig. 2. The disproportionation reaction of mutant F183N. A: s versus v plot, clearly showing substrate inhibition by maltose. B: Lineweaver-Burk plot; the reciprocal of the specific activity ($1/v$) is plotted against the reciprocal of the EPS concentration at fixed maltose concentrations (0.1 (●), 0.25 (○), 0.5 (▼), 1 (▲), 2 (■), 5 (◇), and 10 (◆) mM). Linear regression results in the dotted lines, which clearly deviate from the parallel lines observed with the wild type enzyme (see insert). The calculated fit with the Sigma-Plot program is represented by solid lines.


**Effects of the mutations on the coupling reaction**

To delineate the roles of the acceptor site residues in the formation of the ternary complex in the coupling reaction (van der Veen et al. 2000c), the effects of the mutations on β-CD coupling were analyzed (Table 3). Drastically decreased coupling activities were observed with the F183 mutants, suggesting an important role for this residue in the coupling reaction. The F259 and K232 mutants more significantly affected the substrate affinities, suggesting specific roles for these residues in the formation of the ternary complex.

![Table 3: Kinetic parameters of the β-coupling reaction of wild type and mutant CGTases](image)

<table>
<thead>
<tr>
<th>(mutant) enzyme</th>
<th>Vmax (U/mg)</th>
<th>K_mCD (mM)</th>
<th>K_mACC (mM)</th>
<th>K'_mCD (mM)</th>
<th>K'_mACC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>294.0 ± 7.6</td>
<td>0.32 ± 0.02</td>
<td>18.1 ± 1.4</td>
<td>0.15 ± 0.04</td>
<td>8.5 ± 2.2</td>
</tr>
<tr>
<td>F183N</td>
<td>1.6 ± 0.06</td>
<td>0.15 ± 0.02</td>
<td>6.2 ± 0.9</td>
<td>0.13 ± 0.07</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>F183S</td>
<td>1.0 ± 0.03</td>
<td>0.41 ± 0.06</td>
<td>4.7 ± 0.6</td>
<td>0.43 ± 0.03</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>K232L</td>
<td>213.9 ± 15.6</td>
<td>0.47 ± 0.02</td>
<td>44.8 ± 4.3</td>
<td>0.35 ± 0.02</td>
<td>33.6 ± 4.3</td>
</tr>
<tr>
<td>K232Q</td>
<td>84.8 ± 15.6</td>
<td>0.49 ± 0.02</td>
<td>60.6 ± 4.3</td>
<td>0.32 ± 0.02</td>
<td>39.4 ± 4.3</td>
</tr>
<tr>
<td>E264A</td>
<td>282.7 ± 15.6</td>
<td>0.24 ± 0.02</td>
<td>17.8 ± 4.3</td>
<td>0.12 ± 0.02</td>
<td>8.7 ± 4.3</td>
</tr>
<tr>
<td>F259N</td>
<td>117.0 ± 6.2</td>
<td>0.38 ± 0.04</td>
<td>17.7 ± 2.1</td>
<td>0.49 ± 0.05</td>
<td>22.6 ± 2.0</td>
</tr>
<tr>
<td>F259S</td>
<td>239.0 ± 15.2</td>
<td>0.54 ± 0.05</td>
<td>79.5 ± 7.1</td>
<td>0.29 ± 0.03</td>
<td>42.1 ± 4.0</td>
</tr>
<tr>
<td>F183S/F259N</td>
<td>&lt;&lt; 1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd: not determined

**Discussion**

In the present study the acceptor binding site of CGTase was investigated in detail. Previously, structural and biochemical studies of CGTase had already provided evidence for the importance of His233 in the +1 acceptor binding subsite for catalysis. Here we concentrate on subsites +2 and +3, more distant from the catalytic site. Our results show that Phe183, Lys232, and Phe 259, which interact with substrates and/or products at subsite +2, are involved in all CGTase catalyzed transglycosylation reactions, whereas Glu264 at subsite +3 is only involved in the disproportionation reaction.

**The general importance of Lys232**

Mutations in Lys232 resulted in a similar decrease of activity for all CGTase catalyzed transglycosylation reactions (Tables 1-3). Mutant K232Q, designed to partially preserve the polar nature of the active site, resulted in more severely decreased activities than mutant K232L. The latter mutation better conserves the apolar aliphatic tail of lysine, which has hydrophobic interactions with Trp258, a residue neighbouring the acid/base catalyst Glu257 (J. Uitdehaag, unpublished). Another important neighbouring residue, which may become affected is His233. Interestingly, the dipeptide Lys232His233 is completely conserved throughout CGTases and non-plant α-amylases, indicating a structural and/or functional role...
of this pair of residues. In plant α-amylases the residue corresponding to His233 is typically a glycine, while the residue corresponding to Lys232 is either a lysine or an arginine (Matsui and Svensson, 1997). Mutational analysis of barley α-amylase revealed that replacement of the glycine (residue 184) severely reduced the (hydrolyzing) activity, whereas mutations of the arginine (residue 183) had little effect (Arg183Asn, Arg183Thr) or even improved the activity (Arg183Ser) (Matsui and Svensson, 1997). Therefore, the specific involvement of His233 of CGTases and non-plant α-amylases in catalysis may require the presence of Lys232. This is supported by mutational experiments on Lys210 of Saccharomyces fibuligera α-amylase, which corresponds to Lys232 in CGTases. Lys210Arg and Lys210Asn mutations both resulted in severely decreased (hydrolyzing) activities (Matsui et al. 1992a).

The importance of hydrophobic residues at subsite +2

Replacement of phenylalanines 183 and 259 by hydrophilic residues resulted in increased hydrolyzing activities and reduced transglycosylation activities. The physiological function of CGTase is to produce cyclodextrins, and not short, linear oligosaccharides, which can be utilized by competing organisms. Therefore, hydrolysis of the substrate should be prevented, which CGTase accomplishes by excluding water from the active site and making use of an intricate induced fit mechanism (Uitdehaag et al. 2000). Introduction of hydrophilic residues near the catalytic site renders the active site more favourable for water molecules that can react with the intermediate, which is then no longer available for transglycosylation and cyclization reactions, as shown by the increased hydrolyzing activities of the Phe183 and Phe259 mutants (Table 1). However, the mutations also resulted in increased affinities for the acceptor maltose, indicating that the lower transglycosylation activities may be caused by a negative effect on the induced fit mechanism (see below). The double mutant Phe183Ser/Phe259Asn showed an almost complete loss of transglycosylation activity combined with an increased hydrolysis, resulting in an enzyme preferring hydrolysis over cyclization (15:1), whereas the wild type favours cyclization over hydrolysis (90:1).

Phenylalanines 183 and 259 have distinct roles in the transglycosylation reactions

Mutations of Phe183 resulted in drastically decreased cyclization and coupling activities. These reactions require a conformational change of the covalently linked oligosaccharide, producing a circular product from a linear substrate (circularization) or vice versa (linearization), in contrast to the disproportionation reaction, which only involves linear substrates and products. Phe183 may thus be actively involved in the conformational change of the intermediate in the cyclization and coupling reactions.

In contrast, mutations in Phe259 affect cyclization and disproportionation to a similar extent, with less strong effects on the coupling reaction. This can be related to the acceptors used in the different reactions. In the coupling reaction the monosaccharide MαDG is used as the acceptor. This acceptor molecule only binds productively at subsite +1, and consequently does not interact with Phe259 at subsite +2 during the formation of the new glycosidic bond in the reaction. The acceptors for the cyclization reaction (non-reducing end of the covalently linked intermediate) and the disproportionation reaction (maltose), however, do bind at subsite
+2 at this stage and apparently require interactions with Phe259. The interaction with Phe259 may put the non-reducing end in a correct position for the formation of a new glucosidic bond. Crystal structures show that at subsite +2 Phe259 has ideal stacking interactions with cyclodextrins, whereas for Phe183 these interactions are better with linear substrates (see Fig. 1)(Uitdehaag et al. 1999a). This indicates that a cyclodextrin-like binding mode of the acceptor is required for efficient catalysis of both the cyclization and the disproportionation reactions.

Thus, whereas Phe183 appears to be actively involved in the circularization of the oligosaccharide chain, Phe259 serves as a docking site for the non-reducing end. This co-operative action of the subsite +2 phenylalanines explains why the double mutant, in which both steps in the cyclization reaction are affected, resulted in loss of virtually all cyclization activity.

Substrate inhibition by maltose in the disproportionation reaction

The disproportionation reaction operates via a ping-pong mechanism in which the processing of the first (donor) substrate is followed by binding of the second (acceptor) substrate (van der Veen et al. 2000c). The donor substrate EPS is bound in a fashion similar to the maltononaose (see Fig. 1a). After cleavage of the glucosidic bond between the residues bound at subsites -1 and +1, the product occupying the acceptor subsites dissociates, making the acceptor site available for maltose. Although the acceptor site is thus part of the donor site, in the wild type enzyme no inhibitory effect of maltose on binding of EPS is observed. This indicates that the CGTase active site is perfectly suited for the successive binding of donor and acceptor. Surprisingly, all mutations in the acceptor site described here, including Glu264Ala at subsite +3, resulted in substrate inhibition exerted by maltose. Thus, not only subsites +1 and +2, which are required for productive binding of the maltose acceptor, but also subsite +3 plays an important role in acceptor binding. We propose that in the wild type acceptor site a maltose can bind either at subsites +2 and +3 (Fig. 3.a), or at +1 and +2 (Fig. 3.b). The lack of substrate inhibition in the wild type enzyme, even at high maltose concentrations, indicates that the presence of the acceptor maltose does not affect binding of the donor EPS. The distribution of bound maltose over subsites +1/+2 and +2/+3 apparently facilitates replacement of this maltose by the donor substrate in such way that no inhibitory effect of maltose is observed. Mutations in the acceptor site affect this distribution, resulting in inhibition of binding of the donor substrate by the acceptor substrate.

Induced fit of the acceptor substrate

In an induced fit mechanism substrate binding energy is used for activation of catalysis, resulting in a low apparent affinity for this substrate. Thus, mutations of residues involved in such a mechanism may result in an increased affinity for the substrate involved (Cornish-Bowden, 1995). Recently evidence for an induced fit mechanism has been reported, which results from binding of an acceptor substrate in the active site of CGTase. In the structures of Asp229Ala/Glu257Ala CGTase complexed with maltohexaose and maltoheptaose the side chains of Tyr195 and His233 partially block the acceptor subsite +1. It was suggested that binding of an acceptor requires these residues to move, thereby activating the catalytic residues to perform the second step in catalysis (Uitdehaag et al. 2000). The
characteristics of the mutants described here, which all resulted in increased affinities for the acceptor maltose, suggest specific roles for the residues in the acceptor subsites in this induced fit mechanism. Mutants Glu264Ala and Phe183Asn showed a similar decrease of the disproportionation activity, and are thus of equal importance for the disproportionation reaction. Since both residues are involved in maltose binding at subsites +2/+3 (Fig. 3.a), binding of the acceptor at this position may be important for the induced fit of acceptor binding. The observation that mutation Phe183Asn results in a smaller increase in affinity for maltose than mutation Glu264Ala can be explained by the fact that in mutant Phe183Asn hydrophobic interactions with maltose are lost, also when it binds at subsites +1 and +2. Mutant Phe259Asn results in the largest reduction in disproportionation activity, indicating that interactions with Phe259 at subsite +2 are most important. As explained above, Phe259 specifically supports the “cyclodextrin” binding mode of the acceptor (Fig. 3.b) which appears to be required for efficient catalysis.

Thus, whereas Phe183 together with Glu264 provides an initial docking site for the acceptor maltose, Phe259 serves to put the maltose in a position suitable for catalysis. As for the cyclization reaction, this co-operative action of the subsite +2 phenylalanines explains why the double mutant Phe183Ser/Phe259Asn, in which both these steps of acceptor binding in the disproportionation reaction are affected, resulted in a very large decrease of disproportionation activity.

Figure 3. Schematic representation of acceptor binding at the active site of the B. circulans strain 251 CGTase. A) initial “unproductive” binding at subsites +2 and +3. B) “productive” binding at subsites +1 and +2.
The role of the acceptor site in the coupling reaction

The kinetic mechanism of the coupling reaction proceeds via a ternary complex (van der Veen et al. 2000c). A monosaccharide is used in the assay for the coupling reaction, requiring only binding at subsite +1 for formation of a new glycosidic bond. The binding modes of cyclodextrins, which bind similarly to linear substrates from subsites -2 to +1, imply the involvement of subsite +2 and/or +3 in binding the acceptor in the ternary complex (Uitdehaag et al. 1999a; Schmidt et al. 1998; van der Veen et al. 2000c). The present results show that all residues at subsite +2 contribute to the formation of the ternary complex, whereas Glu264 at subsite +3 is not involved. The effects of the Phe183 and Phe259 mutants do not allow a detailed analysis of their roles in the complex formation. For the Phe183 mutants the coupling activity had decreased much more than the cyclization activity, which indicates that, besides being involved in the conformational change of the oligosaccharide (see above), Phe183 may be of crucial importance in the formation of the ternary complex. For the Lys232 mutants the affinity for MαDG had decreased more than that for β-cyclodextrin, suggesting that this residue is especially involved in binding of the monosaccharide acceptor substrate at subsite +2.

Thus, in the coupling reaction all subsite +2 residues are important for the formation of the ternary complex. Furthermore the linearization of the cyclodextrin appears to be mediated by Phe183, similar to the circularization of the linear chain in the cyclization reaction.

Conclusions

The hydrolyzing activity of CGTase is limited by the hydrophobicity of Phe183 and Phe259, as shown by the increased hydrolysis resulting from the replacement of these phenylalanines by hydrophilic residues. By a combination of rational design and (semi) random mutagenesis we were able to select a double mutant (Phe183Ser/Phe259Asn) which significantly prefers hydrolysis over cyclization (15:1), whereas the wild type favours cyclization over hydrolysis (90:1). Furthermore, the roles of Phe183 and Phe259 in the transglycosylation reactions catalyzed by CGTase have been clarified. It appears that in both the disproportionation and the cyclization reactions Phe183 serves to position the acceptor glucose residues in such way that they can be picked up by Phe259, which subsequently induces them to adopt a conformation suitable for catalysis. Whereas the contribution of Phe259 is identical in both reactions, Phe183 is much more important for cyclization due to its intimate involvement in the 23Å relocation of the non-reducing end glucose of the covalently linked intermediate. In the disproportionation reaction Phe183 together with Glu264 provides a docking site for the acceptor (maltose) at subsites +2 and +3. This acceptor substrate is positioned in such way that it can easily overcome the blocking of subsite +1 by residues Tyr195 and His233. The involvement of this His233 in catalysis apparently requires the presence of Lys232 at subsite +2.
References

(References are listed in chapter 7)