The role of arginine 47 in the cyclization and coupling reactions of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251

Implications for product inhibition and product specificity

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Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) is used for the industrial production of cyclodextrins. Its application, however, is hampered by the limited cyclodextrin product specificity and the strong inhibitory effect of cyclodextrins on CGTase activity. Recent structural studies have identified Arg47 in the *Bacillus circulans* strain 251 CGTase as an active-site residue interacting with cyclodextrins, but not with linear oligosaccharides. Arg47 thus may specifically affect CGTase reactions with cyclic substrates or products.

Here we show that mutations in Arg47 (to Leu or Gln) indeed have a negative effect on the cyclization and coupling activities; Arg47 specifically stabilizes the oligosaccharide chain in the transition state for these reactions. As a result, the mutant proteins display a shift in product specificity towards formation of larger cyclodextrins. As expected, both mutants also showed lower affinities for cyclodextrins in the coupling reaction, and a reduced competitive (product) inhibition of the disproportionation reaction by cyclodextrins.

Both mutants also provide valuable information about the processes taking place during cyclodextrin production assays. Mutant Arg47→Leu displayed an increased hydrolyzing activity, causing accumulation of increasing amounts of short oligosaccharides in the reaction mixture, which resulted in lower final amounts of cyclodextrins produced from starch. Interestingly, mutant Arg47→Gln displayed an increased ratio of cyclization/coupling and a decreased hydrolyzing activity. Due to the decreased coupling activity, which especially affects the production of larger cyclodextrins, this CGTase variant produced the various cyclodextrins in a stable ratio in time. This feature is very promising for the industrial application of CGTase enzymes with improved product specificity.

*Keywords:* cyclodextrin glycosyltransferase; product inhibition; product specificity; site-directed mutagenesis; cyclodextrins.

Cyclodextrins are cyclic oligomers of 6 (α-), 7 (β-), 8 (γ-) [1], 9 (δ-), or 10 (ε-cyclodextrin) [2] α(1→4) linked glucose residues. They can form inclusion complexes with small hydrophobic molecules [3], and are used in industrial and research applications [4,5]. The enzyme cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) converts starch into cyclodextrins via an intramolecular transglycosylation reaction (cyclization). Other reactions catalyzed are: (a) disproportionation, transfer of part of a linear oligosaccharide to another oligosaccharide; (b) coupling, opening of a cyclodextrin ring followed by transfer to a linear oligosaccharide; and (c) hydrolysis, transfer of part of a linear oligosaccharide to water [6].

In all known CGTase structures [7–11], five domains (A–E) can be recognized. The three N-terminal domains (A–C) have structural similarity to the three α-amylase domains. Domain E contains a raw-starch binding motif [12–14] forming two maltose-binding sites (MBS) that are responsible for starch binding (MBS1) and for guiding of the substrate into the active site (MBS2) [15]. These MBSs also bind cyclodextrins and thus play an important role in the product inhibition of CGTase. Kinetically, this inhibition is of a mixed type, with both a competitive and a noncompetitive component. When MBS2 on the E-domain is impaired (mutant Tyr633→Ala) noncompetitive inhibition is drastically reduced, but competitive inhibition, affecting catalysis in the active site, clearly remains [15].

CGTase X-ray studies [16–21] show that Arg47 occupies a position allowing it to interact with cyclodextrins, but not with linear substrates (see below). This suggests that Arg47 is involved in (a) CGTase reactions with cyclic products (cyclization) or substrates (coupling) and (b) the competitive inhibition of CGTase by cyclodextrins. Sequence comparisons of CGTases from different sources also suggest that the identity of residue 47 affects cyclodextrin product specificities (Table 1;...
indicated in those cases where both cyclodextrins are formed in comparable amounts [however, with (slight) preference for the first one mentioned]. Vertical lines.

The CGTases are ordered according to their cyclodextrin product specificity as shown in Table 1. The position of residue 47 is shown in between two vertical lines.

Here we report a detailed characterization of the CGTase mutants Arg47→Leu and Arg47→Gln, including analysis of the four CGTase catalyzed reactions and sensitivity to competitive inhibition by cyclodextrins. The data provide clear evidence that Arg47 plays an important role in the CGTase catalyzed cyclization and coupling reactions and that it affects cyclodextrin product specificity. Moreover, Arg47 indeed interacts with cyclodextrins and is thus involved in product inhibition.

### EXPERIMENTAL PROCEDURES

#### Bacterial strains and plasmids

*Escherichia coli* MC1061 [hsdR mcrB araD139 Δ(araAB-leu)7679 ΔlacX74 galU galK rpsL thi] [22] was used for recombinant DNA manipulations and site-directed mutagenesis. *E. coli* DH5α [F′/endA1 hsdR17 supE44 thi recA1 gyrA (NalR) relA1 (lacZYA-argF) U196 (o80 dLacΔ(lacZ)M15] 23], 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 npr2 nprE18 aprA3] [24]. Plasmid pDP66K [15], with the cgt gene from *Bacillus circulans* strain 251 under control of the p32 promoter [25], was used to introduce site-directed mutations and for production of the enzymes. Plasmid p Bluescript KSI (Stratagene) was used for automated sequencing. DNA manipulations and calcium chloride transformation of *E. coli* strains were performed as described [26]. Transformation of *B. subtilis* was performed according to Bron [27].

#### Site-directed mutagenesis

Mutations were introduced with a PCR method using Vent DNA polymerase (New England Biolabs) [15]. The product of Table 1. Cyclodextrin product specificity of CGTases. Sequences were obtained from the SWISS-PROT/EMBL protein data base.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Bacterial source</th>
<th>Main product†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLEPN</td>
<td><em>K. pneumoniae</em> strain M5a1</td>
<td>α</td>
<td>[33]</td>
</tr>
<tr>
<td>BMAC2</td>
<td><em>B. macerans</em></td>
<td>α</td>
<td>[34]</td>
</tr>
<tr>
<td>BMACE</td>
<td><em>B. macerans</em> strain NRRL B388</td>
<td>α</td>
<td>[35]</td>
</tr>
<tr>
<td>BSTEA</td>
<td><em>B. steaerothermophilus</em> strain NO2</td>
<td>α/β</td>
<td>[35]</td>
</tr>
<tr>
<td>BLICH</td>
<td><em>B.licheniformis</em></td>
<td>α/β</td>
<td>[36]</td>
</tr>
<tr>
<td>TBNOVO</td>
<td><em>Thermoanaerobacter</em> sp. ATCC53.627</td>
<td>α/β</td>
<td>[37]</td>
</tr>
<tr>
<td>TABIUM</td>
<td><em>T. thermosulfurogenes</em> EM1</td>
<td>β/α</td>
<td>[38]</td>
</tr>
<tr>
<td>BCR8</td>
<td><em>B. circulans</em> strain 8</td>
<td>β</td>
<td>[39]</td>
</tr>
<tr>
<td>BC192</td>
<td><em>B. circulans</em> strain E192</td>
<td>β</td>
<td>[40]b</td>
</tr>
<tr>
<td>B663</td>
<td><em>Bacillus</em> sp. strain 6.6.3</td>
<td>β</td>
<td></td>
</tr>
<tr>
<td>BF2</td>
<td><em>B. circulans</em> strain F2</td>
<td>β</td>
<td>[41]</td>
</tr>
<tr>
<td>BC251</td>
<td><em>B. circulans</em> strain 251</td>
<td>β</td>
<td>[12]</td>
</tr>
<tr>
<td>B1018</td>
<td><em>Bacillus</em> sp. strain B1018</td>
<td>β</td>
<td>[42]</td>
</tr>
<tr>
<td>B1011</td>
<td>alkalophilic <em>B. sp.</em> strain 1011</td>
<td>β</td>
<td>[43]</td>
</tr>
<tr>
<td>B382</td>
<td>alkalophilic <em>B. sp.</em> strain 38.2</td>
<td>β</td>
<td>[44,45]</td>
</tr>
<tr>
<td>B171</td>
<td>alkalophilic <em>B. sp.</em> strain 17.1</td>
<td>β</td>
<td>[46]</td>
</tr>
<tr>
<td>BKC201</td>
<td><em>Bacillus</em> sp. strain KC201</td>
<td>β</td>
<td>[47]</td>
</tr>
<tr>
<td>BSP11</td>
<td>alkalophilic <em>B. sp.</em> strain 1.1</td>
<td>β (no α)</td>
<td>[48]</td>
</tr>
<tr>
<td>BOHB</td>
<td><em>B. ohbensis</em> (strain C-1400)</td>
<td>β (no α)</td>
<td>[49]</td>
</tr>
<tr>
<td>BREV</td>
<td><em>Brevibacillus brevis</em> strain CD162</td>
<td>β/γ</td>
<td>[50]</td>
</tr>
<tr>
<td>BF290</td>
<td><em>B. firmus/lenus</em> strain 290-3</td>
<td>γ/β</td>
<td>[51]b</td>
</tr>
</tbody>
</table>

† Single cyclodextrins are mentioned as main products for those CGTases that produce only small amounts of the other cyclodextrins. Two cyclodextrins are indicated in those cases where both cyclodextrins are formed in comparable amounts [however, with (slight) preference for the first one mentioned].

b Sequence obtained from Roquette comp. a A. A. Akhmetzjanov, ENTREZ-NCBI seq ID: 39839 (1992).

Fig. 1: Arg or Lys in CGTases producing mainly α- and/or β-cyclodextrin; His in CGTases producing virtually no α-cyclodextrin; Thr in the primarily γ-cyclodextrin producing CGTase from *Bacillus firmus/lenus* strain 290-3. Thus, a relatively short side-chain of residue 47 is accompanied by a clear preference for the production of the larger size cyclodextrins, most notably by a decrease in the formation of α-cyclodextrin.

Fig. 1. Sequence alignment of the region around residue 47 in CGTases. The CGTases are ordered according to their cyclodextrin product specificity as shown in Table 1. The position of residue 47 is shown in between two vertical lines.
the PCR reactions (1360 bp) was cut with *Pvu*I and *Sal*I and the resulting fragment (1210 bp) was exchanged with the corresponding fragment from the vector pDP66K. The resulting (mutant) plasmid was transformed to *E. coli* MC1061 cells. The following oligonucleotide was used to produce the mutations: 5′-GCACGAACTGCGCTTATTTGCGCC-3′, where W is A or T. An adenine results in the Arg47—Gln mutation and introduction of a *Pst*I site (CTGCAG); a thymine results in the Arg47—Leu mutation and introduction of a *Bgl*I site (CGANNNNNNTGC). This 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 *Apr*I, and with *Apr*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 carried out using T7 DNA polymerase, with either 5′-ended labeled primers or with unlabeled primers and fluorescent-labeled ATP [28,29]. 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 PCGene software package (IntelliGenetics).

**Growth conditions and purification of CGTase proteins**

Plasmid carrying bacterial strains were grown on Luria–Bertani agar in the presence of the antibiotic kanamycin, at concentrations of 100 and 5 μg·mL⁻¹ for *E. coli* and *B. subtilis*, respectively [26]. *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% tryptone, 0.5% yeast extract, 1% sodium chloride and 1% casamino acids (pH 7.0) with 10 μg·mL⁻¹ erythromycin and 5 μg·mL⁻¹ kanamycin, to a final *D₅₆₀* of ≈12. Under these conditions, high extracellular CGTase levels were obtained reproducibly, allowing purification to homogeneity of up to 25 mg of CGTase protein per litre. 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) [30] 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⁻¹ 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**

This was determined by incubating appropriately diluted enzyme (0.1–0.2 U, where one unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin per min) for 2–4 min with a 5% solution of partially hydrolyzed 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 [31].

**Coupling activity**

This was determined as described by van der Veen *et al.* [6]. The reaction mixtures containing cyclodextrin and methyl α-D-glucopyranoside (MeDG) were incubated for 10 min at 50 °C before the reaction was started with appropriately diluted CGTase. At regular time intervals (0.25 min) 100 μL samples were taken and the CGTase was inactivated. The linear products were converted to glucose residues through the action of amyloglucosidase. The glucose concentration was determined using the glucose/glucose oxidase/peroxidase method (Boehringer Mannheim Biochemica 124036). One unit of activity is defined as the amount of enzyme coupling 1 μmol of cyclohexrin to MeDG per min.

**Disproportionation activity**

This was measured using the method according to van der Veen *et al.* [6]. The reaction mixture contained up to 6 mM 4-nitrophenyl-α-D-maltoheptaoside-4,6-0-ethylidene [EPS; a maltoheptasaccharide blocked at the nonreducing end with a para-nitrophenyl group at its reducing end (Boehringer Mannheim Biochemica 1492977)] as donor and up to 10 mM maltose (Fluka 63418) as acceptor. After 10 min of preincubation at 50 °C, the reaction was started with appropriately diluted CGTase. At regular time intervals (0.25 min) 100-μL samples were taken and the CGTase was inactivated. Subsequently, the samples were incubated with α-glucosidase to liberate para-nitrophenol from the product of the disproportionation reaction, nonblocked linear oligosaccharide. After addition of 1 mL of 1 M sodium carbonate the absorbance of the samples was measured at 401 nm (*ε₄₀₁ = 18.4 mM⁻¹*). One unit of activity was defined as the amount of enzyme converting 1 μmol of EPS per min.

**Table 2. Kinetic parameters of the disproportionation reaction of wild-type and mutant CGTase enzymes from *B. circulans* strain 251 (at 50 °C).**

<table>
<thead>
<tr>
<th>(Mutant) Protein</th>
<th><em>kₐₜ</em> (s⁻¹)</th>
<th><em>Kₘ</em> (nm EPS)</th>
<th><em>kₐₜ/Kₘ</em></th>
<th><em>Kₜ</em> (mm cyclodextrin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1130 ± 26</td>
<td>0.308 ± 0.022</td>
<td>3669</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>R47L</td>
<td>1488 ± 38</td>
<td>0.482 ± 0.034</td>
<td>3087</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>R47Q</td>
<td>1295 ± 33</td>
<td>0.517 ± 0.044</td>
<td>2505</td>
<td>7.7 ± 0.9</td>
</tr>
</tbody>
</table>

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Inhibition by cyclodextrin

This was measured using the disproportionation assay with 5, 10 or 20 mM α-cyclodextrin, 0.5, 1 or 2 mM β-cyclodextrin or 0.2, 0.4 or 0.8 mM γ-cyclodextrin present in the reaction mixture.

The results obtained with the above mentioned enzyme assays were subjected to kinetic analysis using SIGMAPLOT (Jandel Corporation).

Hydrolyzing activity

This was determined as described previously [2]. The hydrolysis of a 1% soluble starch (Lamers & Pleuger) solution, preincubated at 50 °C for 10 min, upon addition of CGTase was followed by measuring the increase in reducing power [32]. One unit of hydrolyzing activity was defined as the amount of enzyme producing 1 μmol of reducing sugar per min.

Production of cyclodextrins

The process under industrial production conditions was investigated by incubating a 10% solution of Paselli WA4 (pregelatinized starch), similar to the jet-cooked starch used in industrial processes, with 2 U of β-cyclization activity of (mutant) enzyme for 3 h. At regular time intervals, samples were taken and analyzed for the presence of cyclodextrins and linear products by HPLC, using an econosphere NH2 column (Alltech). The concentrations of β-cyclodextrin were measured using the phenolphthalein assay and used as an internal standard for the determination of α- and γ-cyclodextrin formation.

Stability of the (mutant) enzymes

This was determined by incubating properly diluted enzyme (1 U·mL⁻¹ of β-cyclization activity) at 50 °C. At regular time intervals samples were taken and the remaining β-cyclization activity was measured.

RESULTS

Structural analysis of CGTases implies involvement of Arg47 in cyclodextrin binding at the active site. In several X-ray structures of mutant CGTases from B. circulans strain 251 and one structure of the wild-type T. thermosulfurigenes strain EM1 wild-type CGTase with a maltohexaose inhibitor in the active site [19], (D) B. circulans strain 251 Asp229→Asn/Glu257→Gln mutant CGTase with a γ-cyclodextrin in the active site [20]. For details see text.

Table 3. Hydrolyzing and β-cyclization activities and stability of wild-type and mutant CGTase enzymes from B. circulans strain 251 (at 50 °C).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrolysis</th>
<th>β-Cyclization</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kcat (s⁻¹)</td>
<td>kcat (s⁻¹)</td>
<td>kcat/Km²</td>
</tr>
<tr>
<td>Wild-type</td>
<td>3.2 ± 0.2</td>
<td>270 ± 1.7</td>
<td>1097</td>
</tr>
<tr>
<td>R47L</td>
<td>6.8 ± 0.4</td>
<td>87 ± 1.2</td>
<td>180</td>
</tr>
<tr>
<td>R47Q</td>
<td>1.0 ± 0.2</td>
<td>164 ± 1.8</td>
<td>317</td>
</tr>
</tbody>
</table>

² Putative kcat/Km values, taking the Km for EPS (Table 2) as a measure for the affinity of the active site for linear substrates.
resulted in appearance of a linear maltonaose in the active site [18]. In this structure the side-chain of Arg47 was found to be pointing towards the glucose at subsite $2^3$ (Fig. 2B). Although no hydrogen-bonding interactions with the maltonaose could be observed, this conformation might be a remnant of interactions with the $\beta$-cyclodextrin. Structural analysis of the wild-type T. thermosulfurigenes strain EM1 CGTase with a bound semicyclic maltohexaose inhibitor [19] showed that Lys47 is hydrogen bonded to the O2 atom of the sugar at subsite $2^3$ (Fig. 2C). Finally, the structure of the Asp229$\rightarrow$Asn/Glu257$\rightarrow$Gln mutant of B. circulans strain 251 CGTase complexed with a $\gamma$-cyclodextrin [20] revealed hydrogen-bonding interactions between the O2 and O3 atoms of the sugar at subsite $2^3$ and Arg47 (Fig. 2D).

Arg47 has an important role in the cyclization reaction

In Table 3 the $\beta$-cyclization activities of wild-type and mutant enzymes are shown. Although mutations in Arg47 do not affect the interconversion of linear compounds, the conversion of an oligosaccharide from a linear to a circular conformation is severely affected. The Arg47$\rightarrow$Leu mutation, removing all possible hydrogen bonding interactions, resulted in a threefold decrease in $k_{\text{cat}}$ for the Arg47$\rightarrow$Leu mutant and a threefold decrease in $k_{\text{cat}}$ for mutant Arg47$\rightarrow$Gln (Table 3). Hydrolysis, however, remains a minor activity compared to the other reactions.

Mutations in Arg47 decrease the affinity for cyclodextrins

To assess the importance of Arg47 interactions with cyclodextrins, the coupling reaction of the (mutant) enzymes was

![Fig. 3. Lineweaver–Burk plots of the disproportionation reaction of CGTase from B. circulans strain 251 with $\beta$-cyclodextrin as inhibitor. EPS (donor) concentrations were varied (0.3, 0.6, 1.2, and 2.4 mM) at a constant maltose (acceptor) concentration (10 mM) and different $\beta$-cyclodextrin (inhibitor) concentrations (●, 0; ■, 0.5; ▲, 1; ▼, 2 mM). (A) wild-type, (B) Arg47$\rightarrow$Leu and (C) Arg47$\rightarrow$Gln CGTases.](image)

<table>
<thead>
<tr>
<th>(Mutant) Protein</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM cyclodextrin)</th>
<th>$k_{\text{cat}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
<td>$\beta$</td>
<td>$\gamma$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>354 ± 6</td>
<td>308 ± 4</td>
<td>203 ± 7</td>
</tr>
<tr>
<td>R47L</td>
<td>189 ± 9</td>
<td>111 ± 1</td>
<td>56.1 ± 2</td>
</tr>
<tr>
<td>R47Q</td>
<td>163 ± 8</td>
<td>75 ± 2</td>
<td>49.1 ± 2</td>
</tr>
</tbody>
</table>
analyzed (Table 4). The Arg47→Leu mutation results in a loss of affinity similar for all cyclodextrins, indicating that the interactions of Arg47 observed with a γ-cyclodextrin bound in the active site also occur with an α- or β-cyclodextrin. When Arg47 was replaced by Gln, the $K_{m}$ values increased also, but especially for β- and γ-cyclodextrin this loss of affinity was less than with the Arg47→Leu mutation (Table 4). These data indicate that at least the larger cyclodextrins interact with the Gln residue at position 47, as was expected from the β-cyclization activity of mutant Arg47→Gln (Table 3). Compared to the wild-type enzyme, the $k_{cat}$ values of the coupling reaction of mutant Arg47→Leu showed a threefold decrease with β- and γ-cyclodextrin, whereas with α-cyclodextrin a twofold decrease was observed. Although the Gln at position 47 still interacts with cyclodextrins, the coupling reaction $k_{cat}$ values have dropped more with the Arg47→Gln than with the Arg47→Leu mutant. The catalytic efficiency, indicated by $k_{cat}/K_{m}$, however, had decreased more for the latter mutant.

Inhibition by cyclodextrins

This was investigated using the disproportionation assay, as the high affinities of the mutant enzymes for the high molecular mass starch substrate used in the cyclization assay make it impossible to perform reliable kinetic studies of the cyclization reaction [6]. Furthermore, cyclodextrins are used in the reverse (coupling) reaction, which may interfere with the determination of inhibition constants. Inhibition by cyclodextrins of the disproportionation reaction is of a competitive type, with effects only on $K_{m}$ values (Fig. 3). The resulting $K_{i}$ values correlate reasonably well with the $K_{m}$ values for the coupling reaction (Tables 2 and 4), showing that product inhibition is indeed linked to the coupling reaction. As hypothesized, the Arg47→Leu and Arg47→Gln mutations resulted in a decreased competitive inhibition compared to the wild-type (Fig. 3, Table 2). Inhibition by cyclodextrins significantly decreased, especially for the Arg47→Leu mutant, which is very promising for an increased production of cyclodextrins from starch.
The performance of the (mutant) enzymes under conditions resembling industrial production processes

This was studied using a 10% Paselli WA4 solution. In such an assay the ratio of cyclodextrins produced in the early stages reflects the difference in initial rates for the formation of the respective cyclodextrins, whereas in the later stages the effect of the other reactions (coupling, disproportionation, and hydrolysis) forces this ratio towards an equilibrium [17]. In Fig. 4A and Table 5 a typical cyclodextrin production profile for the wild-type enzyme is shown. Initially, β- and γ-cyclodextrin are mainly produced, while smaller amounts of α- and δ-cyclodextrin are produced. In the later stages, the contributions of γ- and δ-, and eventually also β-cyclodextrin in the total product decrease, while that of α-cyclodextrin increases. After prolonged incubation linear products also accumulated in the mixture (Table 5).

Compared to the wild-type enzyme, the conversion of starch into cyclodextrins initially proceeded more rapidly with mutant Arg47→Leu (Fig. 4B, Table 5; percentage of starch converted), although equal amounts (2 U) of β-cyclization activity were used in both cases. Initially more γ- and δ-cyclodextrin are produced by this mutant, when compared to wild-type, but their amounts decrease again very early in the production process with a concomitant increase of α-cyclodextrin and linear products. In the final stages, the amount of starch converted by the Arg47→Leu mutant was similar to the wild-type enzyme, but a lower amount of cyclodextrins were produced, while more linear products accumulated.

Mutant Arg47→Gln initially also produced more of the larger size cyclodextrins than the wild-type CGTase. The ratios of the cyclodextrins produced changed little over time, with only an increased production of α-cyclodextrin at the expense of δ-cyclodextrin (Fig. 4C). Only small amounts of linear products were produced by this mutant and lower amounts of cyclodextrin were produced, due to a decreased conversion of the starch used.

Stability tests of the (mutant) enzymes

This revealed that the Arg47 mutant CGTase proteins suffered from a twofold decrease in thermostability (Table 3).

DISCUSSION

The involvement of Arg47 in (de)circularization of oligosaccharides

Recently it has been suggested that the transition from a circular to a linear, and from a linear to a circular conformation of the substrate is the rate-limiting step in the coupling and cyclization reactions, respectively [6,17]. The data presented here indicate that Arg47 affects this transition, probably by hydrogen bonding to cyclic compounds. Although the structural data show no interactions between Arg47 and linear oligosaccharides, the affinities for the linear substrate EPS decrease...
upon mutation of this residue. This may be caused by effects on neighboring amino acids, especially as mutant Arg47→Gln (with conserved hydrogen-bonding capacity) shows the largest decrease in affinity for EPS, while mutant Arg47→Leu (with complete loss of hydrogen bonding capacity) shows the highest reduction in affinity for cyclodextrins. Thus whereas Arg47 does not hydrogen bond to linear oligosaccharides, such interaction may be initiated with an intermediate in the cyclization process by a slight change in the conformation of the Arg47 side-chain (see Fig. 2).

**Arg47 stabilizes the transition state for coupling and cyclization reactions**

The kinetic parameters of enzyme-catalyzed reactions are measures of the energy levels involved (shown in a simplified model of the CGTase reaction sequence; Fig. 5). The $K_m$ value is linked to the substrate-bound ground-state energy; a higher $K_m$ indicates a higher energy level. The $k_{cat}$ value is linked to the activation energy, the difference between the substrate-bound ground state level and the transition-state level; a higher $k_{cat}$ indicates a lower activation energy. Finally, $k_{cat}/K_m$ value indicates the energy level of the transition state with respect to free enzyme and free substrate; a lower transition-state energy results in a higher $k_{cat}/K_m$ value. Although no affinities could be determined for the high molecular mass substrate (starch) used in the cyclization reaction, relevant changes in $K_m$ and $k_{cat}/K_m$ values for this reaction can be deduced from a combination of the results obtained with the disproportionation and cyclization reactions. The short maltoheptaose substrate used in the disproportionation reaction (EPS) supposedly binds in a similar way to other short oligosaccharides, which includes binding at subsite $-3$, where Arg47 is positioned [17,19]. Effects of mutations in Arg47 on affinity for this short oligosaccharide thus reflect effects on the affinity of the active site for binding linear substrates for the cyclization reaction. The validity of this assumption is shown by the close correlation of the resulting $k_{cat}/K_m$ values for β-cyclization with those for β-coupling (see Tables 3 and 4). As coupling is the reverse reaction of cyclization, the transition state is expected to be the same for both reactions (Fig. 5). When comparing Tables 2–4, it is obvious that mutants Arg47→Leu and Arg47→Gln both drastically affect transition-state binding in the cyclization and coupling reactions, whereas for the disproportionation reaction relatively small effects are observed. Arg47 is therefore clearly involved in binding the transition state of cyclization and coupling, reducing its energy level and increasing the catalytic efficiency.

**Cyclization and coupling activities can be altered differently by affecting substrate binding**

Although Gln47 has interactions with cyclodextrins, mutations Arg47→Leu and Arg47→Gln result in similar decreases in $k_{cat}/K_m$ values, indicating that in both mutants (almost) all interactions of residue 47 with the transition state were lost. The differences in $k_{cat}$ values of the two mutants must then be explained by differences in the linear oligosaccharide and cyclodextrin bound ground states of the mutant enzymes. The $K_m$ for linear oligosaccharides (EPS) of Arg47→Gln is higher than that for Arg47→Leu, resulting in a higher energy level of the substrate-bound ground state for cyclization, explaining the higher cyclization activity of mutant Arg47→Gln compared to Arg47→Leu. Similarly, the $K_m$ for cyclodextrins of Arg47→Gln is lower than that for Arg47→Leu, resulting in a lower energy level of the substrate-bound ground state for coupling, explaining the lower coupling activity of mutant Arg47→Gln compared to Arg47→Leu (Fig. 5).

**Competitive inhibition of CGTase catalyzed reactions by cyclodextrins**

Previously, competitive product inhibition of the cyclization reaction by β-cyclodextrin was reported for the *B. circulans* strain 251 CGTase Tyr63→Ala mutant, affected in MBS2 on the E-domain [15]. The current data illustrate that this inhibitory effect is not merely caused by the reverse reaction (coupling), because an identical inhibitory effect of β-cyclodextrin on the disproportionation reaction (where β-cyclodextrin is not involved in the reaction) of the wild-type enzyme was observed. Nevertheless, inhibition by cyclodextrins is closely linked to the coupling reaction, as indicated by the correlation between the affinity constants ($K_m$) for cyclodextrins in the coupling reaction and the inhibition constants ($K_i$) in the disproportionation reaction. Moreover, the reduced affinities of the Arg47 mutants for cyclodextrins in the coupling reaction are reflected in similar reductions in the competitive inhibition exerted by these cyclodextrins. This indicates that the binding mode of the cyclodextrins resulting in the inhibitory effect is very similar if not identical to that in the productive enzyme-cyclodextrin complex in the coupling reaction.

**The influence of the various CGTase-catalyzed reactions on the production of cyclodextrins**

CGTases are used in the industrial production of cyclodextrins from starch. Therefore a prolonged incubation of Paselli WA4 (resembling the jet-cooked starch used in industry) was followed in time to analyze the performance of wild-type and mutant enzymes under conditions more similar to industrial production processes. The composition of the reaction mixture at a certain time point is the combined result of all the CGTase-catalyzed reactions, which explains the shifts in product ratios in time. At the start of the reaction, only the effect of cyclization is apparent, with the ratio of the different cyclodextrins generated depending on the specific activities for formation of these cyclodextrins [17]. Clearly, the mutations in Arg47 affect this ratio, causing a shift in specificity towards the larger cyclodextrins, most apparent for mutant Arg47→Leu. The rate of cyclodextrin formation gradually decreases in time due to product inhibition. This is nicely shown by mutant Arg47→Leu, for which the significantly decreased competitive product inhibition allows cyclodextrin production from starch to proceed more quickly. Although a minor activity, hydrolysis gives rise to the production of short linear oligosaccharides, ranging in size from maltose to maltoheptaose. These short oligosaccharides are excellent acceptors for the coupling reaction [6], especially with the larger cyclodextrins which have the highest affinity for the enzyme (Table 4), and thus are the first to be used in this coupling reaction. In the later stages of incubation, the ratios therefore shift toward formation of α-cyclodextrin (wild-type) or linear products (Arg47→Leu; Table 5, Fig. 4). With mutant Arg47→Gln, the ratios of the products formed change less over time. It has a lower hydrolyzing activity and significantly favors cyclization over coupling; in this mutant the coupling reaction thus interferes less with production of cyclodextrins. The decreased total production from starch is probably caused by the decreased stability of the enzyme.
In conclusion, more insights in the mechanisms of the CGTase-catalyzed (de)circularization reactions have been obtained. The conformational change in a bound oligosaccharide in the active site appears to be mediated by conformational changes in specific amino-acid residues as indicated here for Arg47, which is involved in stabilization of the transition state that characterizes the cyclization and coupling reactions specifically. As expected, the Arg47 mutations resulted in a shift in specificity towards production of the larger cyclodextrins. The data show that both hydrolysis and coupling interfere with cyclodextrin production. A single mutation (Arg47→Gln) resulted, in time, in a more stable composition of the cyclodextrin products during the conversion of starch.

These results offer possibilities for the design of highly specific CGTases based on initial cyclodextrin formation rates, as suggested recently [17], for use in industrial cyclodextrin production processes.

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