Engineering reaction and product specificity of cyclodextrin glycosyltransferase from Bacillus circulans strain 251

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Chapter 5

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 (Leu, 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 size 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 provided valuable information about the processes taking place during cyclodextrin production assays. Mutant Arg47Leu 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 Arg47Gln 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.

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Introduction

Cyclodextrins are cyclic oligomers of 6 (α-), 7 (β-), 8 (γ-) (French, 1957)or even 9 (δ-), and 10 (ε-cyclodextrin) (Penninga et al. 1995) α(1-4) linked glucose residues. They can form inclusion complexes with small hydrophobic molecules (Saenger, 1980), and are used in industrial and research applications (Schmid, 1989; Allegre and Deratani, 1994). The enzyme cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) converts starch into cyclodextrins via an intramolecular transglycosylation reaction (cyclization). Other reactions catalyzed are: (i), disproportionation, transfer of part of a linear oligosaccharide to another oligosaccharide; (ii), coupling, opening of a cyclodextrin ring followed by transfer to a linear oligosaccharide; (iii), hydrolysis, transfer of part of a linear oligosaccharide to water (van der Veen et al. 2000c).

In all known CGTase structures (Hofmann et al. 1989; Klein and Schulz, 1991; Kubota et al. 1991; Knegtel et al. 1996; Harata et al. 1996) 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 (Lawson et al. 1994; Svensson et al. 1989; Jespersen et al. 1991) forming two maltose binding sites (MBS) that are responsible for starch binding (MBS1) and for guiding of the substrate into the active site (MBS2) (Penninga et al. 1996). These MBS's 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 non-competitive component. When MBS2 on the E-domain is impaired (mutant Tyr633Ala) non-competitive inhibition is drastically reduced, but competitive inhibition, affecting catalysis in the active site, clearly remains (Penninga et al. 1996).

CGTase X-ray studies (Strokopytov et al. 1996; van der Veen et al. 2000b; Uitdehaag et al. 1999b; Wind et al. 1998; Uitdehaag et al. 1999a; Schmidt et al. 1998) 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; 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/lentus* 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.

Here we report a detailed characterization of the CGTase mutants Arg47Leu and Arg47Gln, 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 thus is involved in product inhibition.

Table 1. Cyclodextrin product specificity of CGTases. Sequences were obtained from the SWISS-PROT/EMBL protein data base.

Abbreviation	Bacterial source	Main product[1]	Reference
KLEPN	*K. pneumoniae* strain M5a1	α	(Binder et al. 1986)
BMAC2	*B. macerans*	α	(Sakai et al. 1987)
BMACE	*B. macerans* strain NRRL B388	α	(Fujiwara et al. 1992b)
BSTEA	*B. stearothermophylus* strain NO2	α/β	(Fujiwara et al. 1992b)
BLICH	*B. licheniformis*	α/β	(Hill et al. 1990)
TBNOVO	*Thermoanaerobacter* sp. ATCC53.627	α/β	(Dijkhuizen et al. 1996)
TABIUM	*T. thermosulfurigenes* EM1	β/α	(Wind et al. 1994)
BCIR8	*B. circulans* strain 8	β	(Nitschke et al. 1990)
BC192	*B. circulans* strain E192	β	(Bovetto et al. 1992)[2]
B663	*Bacillus* sp. strain 6.6.3	β	*
BF2	*B. circulans* strain F2	β	(Kim et al. 1992)
BC251	*B. circulans* strain 251	β	(Lawson et al. 1994)
B1018	*Bacillus* sp. strain B1018	β	(Itkor et al. 1990)
B1011	alkalophilic *B.* sp. strain 1011	β	(Kimura et al. 1987)
B382	alkalophilic *B.* sp. strain 38.2	β	(Hamamoto et al. 1987)
B171	alkalophilic *B.* sp. strain 17.1	β	(Kaneko et al. 1989)
BKC201	*Bacillus* sp. strain KC201	β	(Kitamoto et al. 1992)
BSP11	alkalophilic *B.* sp. strain 1.1	β (no α)	(Schmid et al. 1988)
BOHB	*B. ohbensis* (strain C-1400)	β (no α)	(Sin et al. 1991)
BREV	*Brevibacillus brevis* strain CD162	β/γ	(Kim et al. 1998)
BF290	*B. firmus/lentus* strain 290-3	γ/β	(Englbrecht et al. 1988)[2]

1 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).

2 Sequence obtained from Roquette company

* Akhmetzjanov, A.A., ENTREZ-NCBI seq ID: 39839 (1992)

Experimental procedures

Bacterial strains and plasmids

Escherichia coli MC1061 [*hsd*R *mcr*B *ara*D139 Δ(*ara*ABC-*leu*)7679 Δ*lac*X74 *gal*U *gal*K *rps*L *thi*] (Meissner et al. 1987) was used for recombinant DNA manipulations and site-directed mutagenesis. *E. coli* DH5α [F'/*end*A1 *hsd*R17 *sup*E44 *thi*1 *rec*A1 *gyr*A (Nal^r) *rel*A1 (*lac*ZYA-*arg*F) U196 (o80d*lac*Δ(*lac*Z)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 npr*R2 *npr*E18 *apr*A3](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.

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              43       50
KLEPN          DPNNLKKYT
BMAC2          HS-NLKLYF
BMACE          RS-NLKLYF

TBNOVO         HT-SLKKYF
TABIUM         HT-SLKKYF
BSTEA          CT-NLRKYC

BLICH          CS-NLKLYC
BCIR8          CS-NLKLYC
BC192          CS-NLKLYC
B663           CS-NLKLYC
BF2            CSTNLKLYC
BC251          CT-NLRLYC
B1018          CT-NLRLYC
B1011          CT-NLRLYC
B382           CT-NLRLYC
B171           CT-NLRLYC

BKC201         CI-DLHKYC
BSP11          CI-DLHKYC
BOHB           CS-DLHKYC
BREV           CS-DLHKYC
BF290          CL-DLTKYC
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Figure 1. Sequence alignement 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 bold.

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). The product of the PCR reactions (1360 bp) was cut with *Pvu*II 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'-GC ACG AAC CTG C**W**G CTC TAT TGC GGC-3'. W can be an adenine or a thymine. An adenine results in the Arg47Gln mutation and introduction of a *Pst*I site (CTGCAG); a thymine results in the Arg47Leu mutation and introduction of a *Bcg*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 *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 labeled primers or with unlabeled primers and fluorescein-labeled 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). *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 4ºC 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 50ºC.

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 hydrolyzed potato starch with an average degree of polymerization of 50 (Paselli SA2; AVEBE, Foxhol, The Netherlands) preincubated at 50ºC 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.

Inhibition by cyclodextrins 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).

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 hydrolyzing activity was defined as the amount of enzyme producing 1 µmole of reducing sugar per min.

Production of cyclodextrins under industrial production process conditions was investigated by incubating a 10% solution of Paselli WA4 (pregelatinized starch), comparable to the jet-cooked starch used in industrial processes, with 2 units 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 NH_2 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 was determined by incubating properly diluted enzyme (1 U/ml 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 *Bacillus circulans* strain 251 and one structure of the wild type *Thermoanaerobacterium thermosulfurigenes* strain EM1 CGTase, different conformations of Arg47 (Lys47 in *T. thermosulfurigenes* strain EM1 CGTase) have been observed (Fig. 2). In the structure of the *B. circulans* strain 251 Tyr195Phe CGTase with a linear maltononaose inhibitor bound in the active site (Strokopytov et al. 1996), the Arg47 side chain is directed away from the oligosaccharide (Fig. 2A). Soaking of crystals of the Asp229Asn/Glu257Gln CGTase with a β-cyclodextrin resulted in appearance of a linear maltononaose in the active site (Uitdehaag et al. 1999b). In this structure the side chain of Arg47 was found to be pointing towards the glucose at subsite -3 (Fig. 2B). Although no hydrogen bonding interactions with the maltononaose could be observed, this conformation might be a remnant of interactions with the β-cyclodextrin. Structural analysis of the wild type *T. thermosulfurigenes* strain EM1 CGTase with a bound semicyclic maltohexaose inhibitor (Wind et al. 1998) showed that Lys47 is hydrogen bonded to the O2 atom of the sugar at

subsite -3 (Fig. 2C). Finally, the structure of the Asp229Asn/Glu257Gln mutant of *B. circulans* strain 251 CGTase complexed with a γ-cyclodextrin (Uitdehaag et al. 1999a) revealed hydrogen bonding interactions between the O2 and O3 atoms of the sugar at subsite -3 and Arg47 (Fig. 2D).

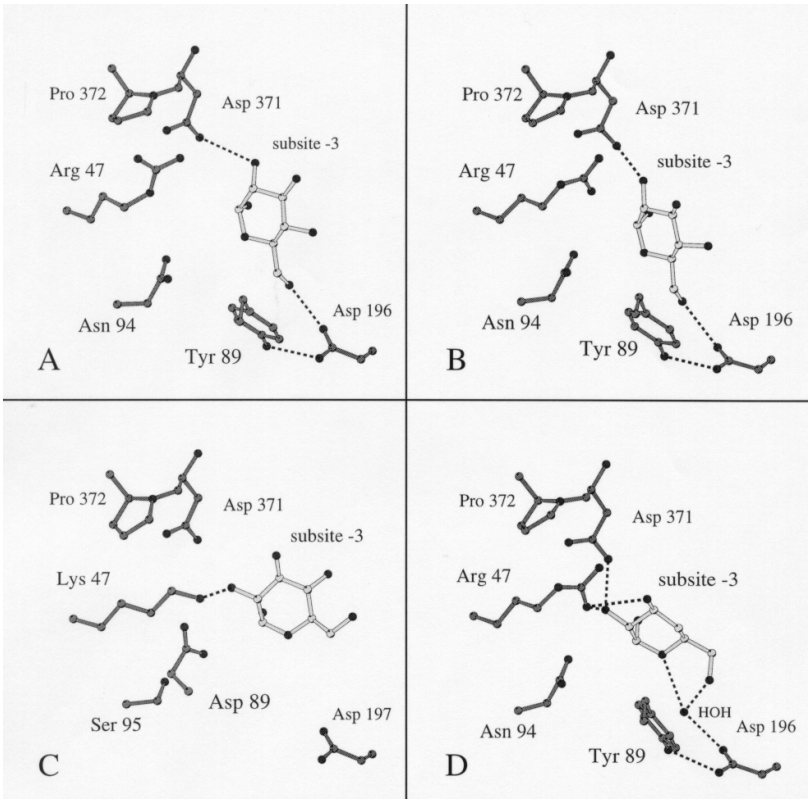

Fig. 2. Different conformations of the side chain of residue 47 in X-ray structures of various (mutant) CGTases complexed with different ligands. Ligands are shown in white, amino acid residues are shown in grey. A) *Bacillus circulans* strain 251 Tyr195Phe mutant CGTase with a maltononaose inhibitor in the active site (Strokopytov et al. 1996). B) *B. circulans* strain 251 Asp229Asn/Glu257Gln mutant CGTase with a natural maltononaose substrate in the active site (Uitdehaag et al. 1999b). C) *Thermoanaerobacterium thermosulfurigenes* strain EM1 wild type CGTase with a maltohexaose inhibitor in the active site (Wind et al. 1998). D) *B. circulans* strain 251 Asp229Asn/Glu257Gln mutant CGTase with a γ-cyclodextrin in the active site (Uitdehaag et al. 1999a). For details see text.

To further investigate the role of Arg47 in CGTase catalyzed reactions we introduced mutations Arg47Leu and Arg47Gln in *B. circulans* strain 251 CGTase. Leucine, with its short aliphatic side chain, was chosen in order to remove all potential hydrogen bonding interactions with cyclodextrins at position 47. Alternatively, a glutamine was selected, because it is shorter than arginine, but has intact hydrogen bonding capability.

Arg47 has no significant role in reactions involving only linear substrates and products

Mutations in Arg47 did not result in drastic changes in k_{cat} and K_M of the disproportionation reaction (Table 2). This is in agreement with the structural data (see above), which do not show interactions of Arg47 with linear oligosaccharides. The mutations have a more significant effect on the hydrolysis reaction, with a twofold increase in k_{cat} for the Arg47Leu mutant and a threefold decrease in k_{cat} for mutant Arg47Gln (Table 3). Hydrolysis, however, remains a minor activity compared to the other reactions.

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

(Mutant) Protein	k_{cat} (s^{-1})	K_M (mM EPS)	k_{cat}/K_M	Ki (mM α-CD)	Ki (mM β-CD)	Ki (mM γ-CD)
wild type	1130 ± 26	0.308 ± 0.022	3669	6.1 ± 0.8	0.54 ± 0.04	0.25 ± 0.04
R47L	1488 ± 38	0.482 ± 0.034	3087	10.6 ± 1.3	1.21 ± 0.08	1.00 ± 0.27
R47Q	1295 ± 33	0.517 ± 0.044	2505	7.7 ± 0.9	0.68 ± 0.05	0.38 ± 0.04

Arg 47 has an important role in the cyclization reaction

In Table 3 the β-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 Arg47Leu mutation, removing all possible hydrogen bonding interactions, resulted in a threefold decrease in k_{cat}. Mutant Arg47Gln, with intact hydrogen bonding capability, but a shorter side chain than the original Arg, shows an intermediate β-cyclization activity when compared to wild type and mutant Arg47Leu. This suggests that the interactions observed for Arg47 with cyclic compounds are at least partially conserved in this mutant.

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

(Mutant) Protein	Hydrolysis k_{cat} (s^{-1})	β-Cyclization k_{cat} (s^{-1})	k_{cat}/K_M*	Stability t½ (min)
wild type	3.2 ± 0.2	270 ± 1.7	1097	10.2
R47L	6.8 ± 0.4	87 ± 1.2	180	6.1
R47Q	1.0 ± 0.2	164 ± 1.8	317	5.7

* Putative kcat/K_M values, taking the K_M for EPS (Table 2) as a measure for the affinity of the active site for linear substrates.

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 analyzed (Table 4). The Arg47Leu 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 Arg47Leu 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 Arg47Gln (Table 3). Compared to the wild type enzyme the k_{cat} values of the coupling reaction of mutant Arg47Leu 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 Arg47Gln than with the Arg47Leu mutant. The catalytic efficiency, indicated by k_{cat}/K_M, however, had decreased more for the latter mutant.

Table 4. Kinetic parameters of the coupling reaction of wild type and mutant CGTase enzymes from *B. circulans* strain 251 (at 50°C).

(Mutant)	k_{cat} (s^{-1})			K_M (mM CD)			k_{cat}/K_M		
Protein	α	β	γ	α	β	γ	α	β	γ
wild type	$354^{(6)}$	$308^{(4)}$	$203^{(7)}$	$5.5^{(0.3)}$	$0.28^{(0.01)}$	$0.21^{(0.02)}$	64	1099	964
R47L	$189^{(9)}$	$111^{(1)}$	$56^{(2)}$	$22.3^{(2.0)}$	$0.96^{(0.02)}$	$0.60^{(0.04)}$	8.5	115	117
R47Q	$163^{(8)}$	$75^{(2)}$	$49^{(2)}$	$16.8^{(1.4)}$	$0.56^{(0.04)}$	$0.27^{(0.02)}$	9.7	133	227

(Values between brackets are the deviations)

Mutations in Arg47 decrease the inhibition by cyclodextrins

Inhibition by cyclodextrins was investigated using the disproportionation assay, since the high affinities of the mutant enzymes for the high molecular weight starch substrate used in the cyclization assay make it impossible to perform reliable kinetic studies of the cyclization reaction (van der Veen et al. 2000c). 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 only effects on K_M values (Fig. 3). The resulting Ki 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 Arg47Leu and Arg47Gln mutations resulted in a decreased competitive inhibition compared to the wild type (Fig. 3, Table 2). Especially for the Arg47Leu mutant inhibition by cyclodextrins has significantly decreased, which is very promising for an increased production of cyclodextrins from starch.

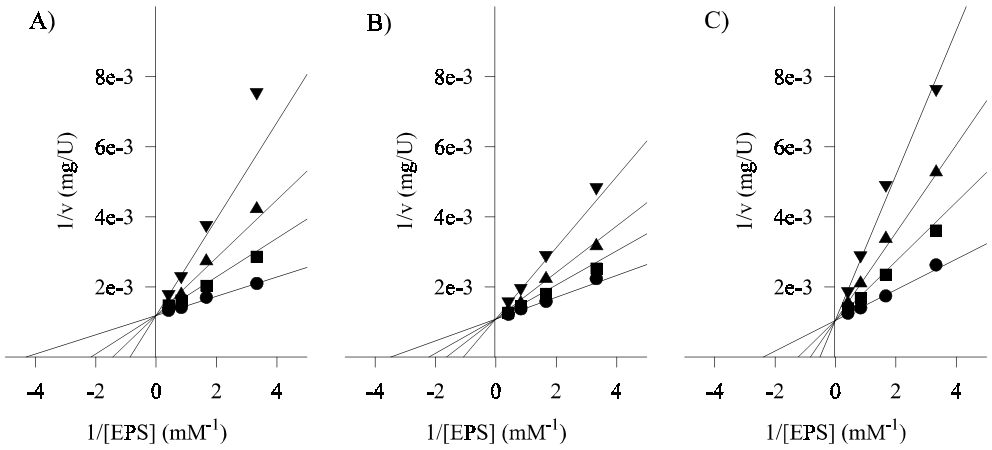

Figure 3. Lineweaver-Burk plots of the disproportionation reaction of CGTase from *B. circulans* strain 251 with β-cyclodextrin as inhibitor. EPS (donor) concentrations were varied (0.3, 0.6, 1.2, and 2.4 mM) at a constant maltose (acceptor) concentration (10 mM) and different β-cyclodextrin (inhibitor) concentrations (●, 0; ■, 0.5; ▲, 1; ▼, 2 mM). A) wild type, B) Arg47Leu and C) Arg47Gln CGTases.

The performance of the (mutant) enzymes under conditions resembling industrial production processes 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 (van der Veen et al. 2000b). In Fig. 4 (a) and Table 5 a typical cyclodextrin production profile for the wild type enzyme is shown. Initially especially β- and γ-cyclodextrin are 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 also linear products accumulated in the mixture (Table 5).

Compared to the wild type enzyme, the conversion of starch into cyclodextrins initially proceeded more rapidly with mutant Arg47Leu (Fig. 4 (b), Table 5; percentage of starch converted), although equal amounts (2 units) 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 Arg47Leu mutant was comparable to the wild type enzyme, but less cyclodextrins were produced, while more linear products accumulated.

Table 5. Production of cyclodextrins by wild type and mutant CGTase enzymes from starch (Paselli WA4) at 50°C.

(Mutant) Protein	Incubation time (min)	Fraction of total products formed (%) α	β	γ	δ	linear	Starch converted (%) CDs	total
	5	7.1±0.9	66.6±0.3	20.9±0.7	5.4±0.7		23±2	23±2
wild type	30	8.5±0.6	71.2±0.7	18.0±0.4	3.0±0.2		35±2	35±2
	180	16.6±0.1	58.7±0.2	14.7±0.7	2.0±0.4	8.9±0.4	50±2	55±2
	5	2.9±0.2	61.3±0.4	22.3±1.1	10.8±0.4	0.3±0.1	32±2	32±2
R47L	30	4.9±0.2	68.5±2.1	17.7±0.5	5.6±0.1	3.4±0.2	41±3	43±3
	180	11.5±0.1	54.1±0.4	12.0±0.2	2.5±0.2	20.0±0.5	43±2	54±2
	5	3.4±0.6	66.4±0.6	21.5±0.5	8.7±1.0		21±3	21±3
R47Q	30	4.3±0.2	65.3±3.0	17.5±1.5	4.9±0.5	3.0±0.6	36±6	37±6
	180	7.6±0.2	67.4±0.6	17.0±1.0	2.0±0.4	5.9±0.6	45±3	48±3

Mutant Arg47Gln initially also produced more of the larger size cyclodextrins than the wild type CGTase. The ratios of the cyclodextrins produced changed fairly little in time, with only an increased contribution of α-cyclodextrin at the expense of δ-cyclodextrin (Fig. 4 (c)). Only small amounts of linear products were produced by this mutant. Also lower amounts of cyclodextrin were produced, due to a decreased conversion of the starch used.

Stability tests of the (mutant) enzymes revealed that the Arg47 mutant CGTase proteins suffered from a twofold decreased 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 (van der Veen et al. 2000c; van der Veen et al. 2000b). 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 since mutant Arg47Gln (with conserved hydrogen bonding capacity) shows the largest decrease in affinity for EPS, while mutant Arg47Leu (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).

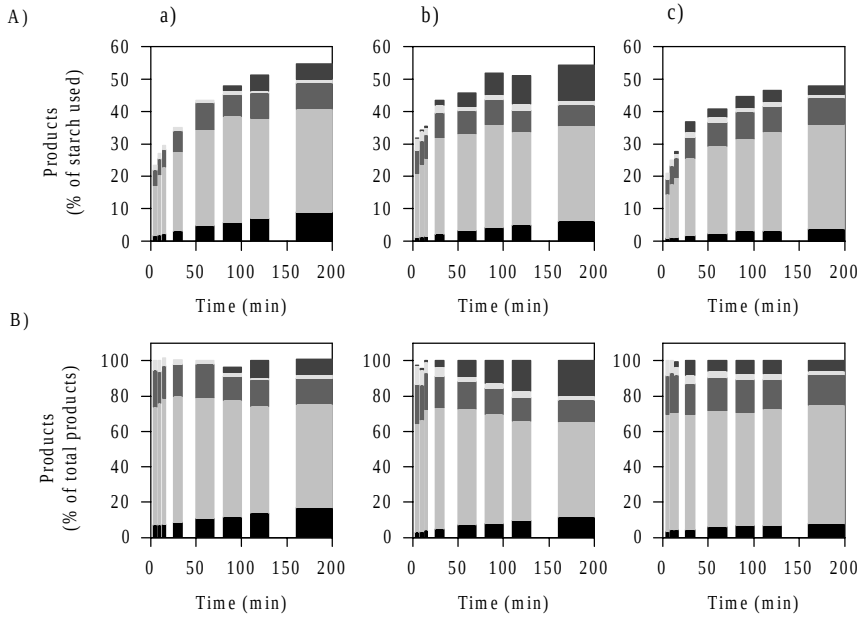

Figure 4. Product formation from 10% Paselli WA4 using 2 units of β-cyclization activity. A) products formed in percentages of the initial amount of starch used. B) products formed in percentages of the total amount of products formed. a) wild type, b) Arg47Leu, c) Arg47Gln CGTase. ■, α-CD; ■, β-CD; ■, γ-CD; ■, δ-CD; ■, linear products.

Arg47 stabilizes the transition state for coupling and cyclization reactions

The kinetic parameters of enzyme catalyzed reactions are measures for 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 weight 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 way similar to other short oligosaccharides, which includes binding at subsite -3, where Arg47 is positioned (Wind et al. 1998; van der Veen et al. 2000b). 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). Since 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 Arg47Leu and Arg47Gln 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.

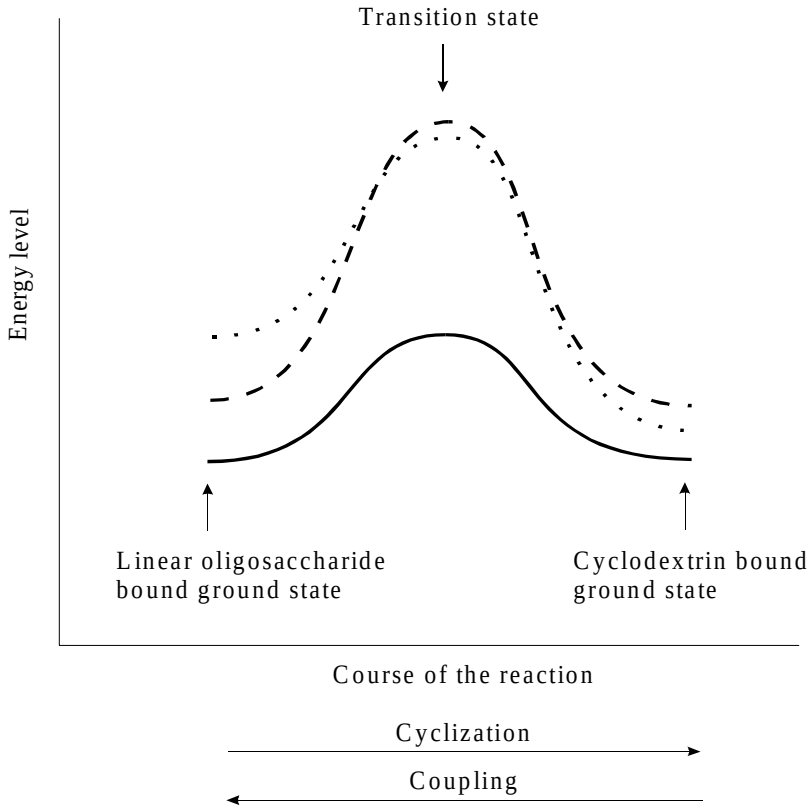

Fig. 5. Schematic representation of putative energy levels involved in CGTase catalyzed cyclization and coupling reactions. The lines indicate the course of the reactions through these energy levels. Solid line: wild type CGTase, dashed line: mutant Arg47Leu, dotted line: mutant Arg47Gln.

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

Although Gln47 has interactions with cyclodextrins, mutations Arg47Leu and Arg47Gln 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 Arg47Gln is higher than that for Arg47Leu, resulting in a higher energy level of the substrate bound ground state for cyclization, explaining the higher cyclization activity of mutant Arg47Gln compared to Arg47Leu. Similarly, the K_M for cyclodextrins of Arg47Gln is lower than that for Arg47Leu, resulting in a lower energy level of the substrate bound ground state for coupling, explaining the lower coupling activity of mutant Arg47Gln compared to Arg47Leu (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 Y633A mutant, affected in MBS2 on the E-domain (Penninga et al. 1996). The current data illustrate that this inhibitory effect is not merely caused by the reverse reaction (coupling), since 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 (Ki) 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 find applications 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 a ratio of the different cyclodextrins generated depending on the specific activities for formation of these cyclodextrins (van der Veen et al. 2000b). Clearly, the mutations in Arg47 affect this ratio, causing a shift in specificity towards the larger cyclodextrins, which is most apparent for mutant Arg47Leu. The rate of cyclodextrin formation gradually decreases in time due to product inhibition. This is nicely shown by mutant Arg47Leu, for which the significantly decreased competitive product inhibition allows

cyclodextrin production from starch to proceed faster. Although a minor activity, hydrolysis gives rise to the production of short linear oligosaccharides, ranging in size from maltose to maltopentaose mainly. These short oligosaccharides are excellent acceptors for the coupling reaction (van der Veen et al. 2000c), 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 (Arg47Leu (Table 5, Fig. 4). With mutant Arg47Gln the ratios of the products formed change less in 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.

Conclusions

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 (Arg47Gln) 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 (van der Veen et al. 2000b), to be used in industrial cyclodextrin production processes.

References

(References are listed in chapter 7)