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Concluding remarks
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This thesis describes investigations of cyclodextrin glycosyltransferase (CGTase) from Bacillus circulans strain 251. The enzyme is a member of the α-amylase family (family 13) of glycosyl hydrolases, containing many starch hydrolyzing enzymes. CGTase mainly catalyzes three different transglycosylation reactions. The most common transglycosylation reaction, also catalyzed by other members of the α-amylase family, is disproportionation, which can be described as: \( G(n) + G(m) \rightarrow G(n-x) + G(m+x) \) in which \( G(n) \) is the donor and \( G(m) \) the acceptor oligosaccharide, consisting of \( n \) and \( m \) glucose residues, respectively. The specific and characteristic CGTase reaction is the cyclization reaction in which the part of the donor substrate that has been cleaved also acts as the acceptor, resulting in formation of a cyclodextrin, described as: \( G(n) \rightarrow \text{cyclic}G(x) + G(n-x) \). The reverse reaction is also catalyzed by the enzyme and is referred to as the coupling reaction. Although the enzyme is strongly related to α-amylases, CGTase displays only a very weak hydrolytic activity.

The variety in catalyzed reactions, and the fact that CGTase specifically produces cyclodextrins consisting mainly of 6, 7, or 8 glucose residues (α-, β-, or γ-cyclodextrins, respectively), makes the enzyme extremely interesting for the study of reaction and product specificity in the α-amylase family. Amino acid sequence analysis has allowed identification of amino acids conserved in the whole α-amylase family, and other amino acids uniquely conserved in CGTases. This thesis presents a biochemical, mutational, and structural analysis of the role of selected CGTase residues in the CGTase reaction specificity, cyclodextrin product specificity, and cyclodextrin product inhibition. The data generated offer a detailed understanding of specific features of each of the CGTase catalyzed reactions. This has allowed rational construction of mutants displaying altered reaction specificity, modified cyclodextrin product specificity, or decreased cyclodextrin product inhibition. This may be of use for the industrial applications of CGTase, found in the production of cyclodextrins and CGTase limit dextrins (the high molecular residual starch molecules remaining after degradation of starch by CGTase). CGTase enzymes with a more economical production of cyclodextrins (increased product specificity, decreased product inhibition) or CGTases capable of producing the specific limit dextrins without the formation of cyclodextrins thus are of industrial interest. Detailed knowledge of the mechanisms of the CGTase catalyzed reactions is required for the construction of such enzymes via rational protein engineering.

The mechanisms of CGTase catalyzed reactions

Previous studies have shown that reactions catalyzed by members of the α-amylase family proceed via a double displacement mechanism involving a covalent enzyme intermediate complex using the invariant catalytic residues Asp229 and Glu257 (CGTase numbering) (Uitdehaag et al. 1999b). The kinetic mechanism is therefore expected to be a substituted enzyme (ping-pong) mechanism. Characterization of the three CGTase transglycosylation reactions, however, revealed that they differ in their kinetic mechanisms.
The cyclization reaction is a single-substrate reaction which proceeds according to Michaelis-Menten kinetics (when using short-chained substrates). The disproportionation reaction is a two-substrate reaction yielding two products and proceeds according to a ping-pong (substituted-enzyme) mechanism. The coupling reaction is a two-substrate reaction yielding one product and proceeds according to a random ternary complex mechanism (van der Veen et al. 2000d). A fourth reaction catalyzed by CGTase is hydrolysis, which is only a minor activity and was therefore not fully investigated.

**The cyclization reaction**

CGTases are capable of forming cyclodextrins from high molecular weight substrates (starch) via the unique cyclization reaction. The first step in this reaction, the binding of the substrate, is promoted by the presence of a raw starch binding domain (domain E). Two maltose binding sites (MBS) on this domain have been found to be involved in its role in raw starch binding. MBS1, containing Trp616 and Trp662, has the highest affinity for (raw) starch and is responsible for the initial binding. MBS2, containing Tyr633, is located at the beginning of a groove between domains A and B, which contains the active site. Starch chains bound at MBS1 are effectively guided to this groove by MBS2. This cooperative action of the MBSs on the E-domain severely enhances the enzyme’s affinity for high molecular weight substrates (Penninga et al. 1996). In the active site the starch chain subsequently binds to specific subsites as has been visualized in the structures of the CGTase from *B. circulans* strain 251 complexed with a maltononaose inhibitor (Strokopytov et al. 1996) and a natural maltononaose (Uitdehaag et al. 1999b). These linear oligosaccharides are bound in a “straight” conformation from subsite +2 to subsite -7. The significance of the binding mode of these maltononasaccharides has been shown by the effects on cyclodextrin product specificity (see below) of site directed mutagenesis of amino acid residues interacting with the maltononaoses at subsites -3 (Y89) and -7 (S146) (van der Veen et al. 2000c). Cleavage of the starch chain between subsites -1 and +1 results in formation of the covalently linked intermediate in the substituted-enzyme complex (Uitdehaag et al. 1999b) and is followed by a 23 Å migration of the non-reducing end of this intermediate to the acceptor site (circularization) to form a cyclodextrin.

During circularization the sugar bound at subsite -3 shifts its position, allowing Arg47 to hydrogen bond to its O2 and O3 atoms, as shown by the structure of *B. circulans* strain 251 CGTase with a γ-cyclodextrin bound in the active site (Uitdehaag et al. 1999a). Mutations Arg47Gln and Arg47Leu, removing interactions with the sugar residue at subsite -3 in the cyclodextrin conformation, show decreased cyclization reaction rates, indicating destabilization of the transition state compared to the substrate bound ground state (van der Veen et al. 2000b). Structural analysis of Tyr89Gly and Tyr89Asp mutants of CGTase show that the Tyr89Asp mutation stabilizes oligosaccharide binding in a more cyclodextrin-like “bent” conformation, compared to the maltononaose “straight” conformation. This mutant shows increased cyclization reaction rates, indicating stabilization of the transition state compared to the substrate bound ground state (van der Veen et al. 2000c). Therefore in the transition state of the cyclization reaction the cyclodextrin-like “bent” conformation of the oligosaccharide is preferred, indicating that circularization of the oligosaccharide is initiated.
during or prior to bond cleavage. X-ray crystallographic studies have shown that substrate binding up to at least subsite -6 activates the bond cleavage machinery; furthermore, it results in an induced fit, resulting in strain in specific loops in the enzyme which may trigger circularization (Uitdehaag et al. 1999a). Combination of the biochemical and crystallographic data suggest that bond cleavage and circularization are tightly synchronized, enhancing the efficiency of the cyclization reaction.

Comparisons of the maltonaose and γ-cyclodextrin binding modes in the CGTase active site show further remarkable differences at the acceptor subsite +2 (Uitdehaag et al. 1999a; Uitdehaag et al. 1999b). In both cases a glucose residue bound at this subsite is sandwiched between Phe183 and Phe259. Although Phe183 has ideal stacking interactions with the maltononaose and Phe259 with the cyclodextrin, site directed mutagenesis of these residues revealed a more important role for Phe183 in the cyclization reaction (van der Veen et al. 2000a). These results confirm Molecular Dynamics studies of the circularization route which show that Phe183 and Tyr195, a residue located centrally in the active site (Penninga et al. 1995), are intimately involved in the 23 Å displacement of the non-reducing end glucose from subsite -7 to subsite +1, whereas Phe259 acts as a final docking site for ring closure (Uitdehaag, 2000). It has been proposed from X-ray crystallographic studies of the CGTase from B. circulans strain 251 complexed with maltohexaose and maltoheptaose ligands bound from subites -1 to -6 and -7, respectively, that also in acceptor binding an induced fit mechanism is involved (Uitdehaag et al. 2000). The glucose residue binding at subsite +1 has to relocate residues Tyr195 and His233, which may lead to activation of the bond cleavage machinery, similar to binding of a glucose residue at subsite -6. This induced fit mechanism is clarified by the effects of mutating Phe183 and Phe259 at subsite +2 and Glu264 at subsite +3 (van der Veen et al. 2000a) on the disproportionation reaction (see below).

In conclusion, the CGTase enzyme is extremely specialized in the production of cyclodextrins from starch. The E-domain strongly enhances the affinity for high molecular weight substrates and efficiently guides these substrates into the active site. In the active site, substrate binding up to at least subsite -6 (required for the formation of cyclodextrins) stimulates bond cleavage, simultaneously initiating circularization, with important roles for amino acid residues at subsite -3 (Arg47 and Tyr89). Residues Tyr195 and Phe183 are intimately involved in completing this circularization, transferring the non-reducing end of the bound oligosaccharide chain to Phe259. Binding to this Phe259 subsequently enables activation of the second step of the reaction, cyclodextrin ring closure.

The disproportionation reaction

The disproportionation reaction of CGTase proceeds according to the ping-pong bi-bi mechanism typical for reactions involving a substituted-enzyme intermediate (van der Veen et al. 2000d). This mechanism implies that the first (donor) substrate is processed before the second (acceptor) substrate can bind. Biochemical (Bender, 1988) and structural (Strokopytov et al. 1996; Strokopytov et al. 1995; Kneetel et al. 1995; Uitdehaag et al. 1999b) studies with CGTase have shown that the donor substrate usually binds from subsite +2 down to subsites further in the active site. This binding mode of the donor substrate indicates that both acceptor subsites +1 and +2 are occupied by the donor. Thus, after cleavage of the glucosidic bond, the
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part of the donor occupying these subsites has to dissociate prior to binding of the acceptor (maltose). Mutational analysis of the acceptor site has shown that the maltose acceptor initially binds at subsites +2 and +3, after which it can move to subsites +1 and +2 (van der Veen et al. 2000a). Binding at subsites +2 and +3 involves specific stacking on Phe183 at subsite +2, which resembles the “straight” maltononaose binding mode, and hydrogen bonding interactions with Glu264 at subsite +3. Binding at subsites +1 and +2 of the acceptor site involves specific stacking on Phe259 at subsite +2 and hydrogen bonding interactions with His233 at subsite +1, thus resembling the cyclodextrin-like “bent” binding mode. This indicates that the roles of both phenylalanines in the disproportionation reaction are similar to those in the cyclization reaction; Phe183 positions the accepting glucose residue, which subsequently interacts with Phe259. This mechanism apparently helps the maltose to overcome the blocking of subsite +1 by Tyr195 and His233, thereby facilitating the induced fit of the acceptor substrate. Furthermore, the distribution of maltose binding at subsites +2 and +3, and at +1 and +2, prevents substrate inhibition by maltose. Mutations in the acceptor site affect this distribution, resulting in inhibition of binding of the donor substrate by the acceptor substrate (van der Veen et al. 2000a).

The coupling reaction

Whereas the double displacement mechanism suggests that the ping-pong mechanism would apply to all CGTase catalyzed reactions, the coupling reaction is operated by a random ternary complex mechanism (van der Veen et al. 2000d). This indicates that both the donor (α-, β-, or γ-cyclodextrin) and the acceptor (monosaccharide) are bound simultaneously to the active site cleft before the cyclodextrin is processed. Since the disproportionation reaction (see above) proceeds via a ping-pong mechanism, also when a monosaccharide is used as acceptor (results not shown), the deviating kinetic mechanism of the coupling reaction must result from the use of a cyclic donor substrate. Structural studies revealed that cyclodextrins bound in the CGTase active site lack certain hydrogen bonding interactions at the acceptor site (Uitdehaag et al. 1999a), notably at subsite +2 (Lys232), compared to the hydrogen bonding interactions with the linear substrate (Strokopytov et al. 1996). Bound cyclodextrins leave no room for glucose binding at subsite +1, since this subsite has to be occupied by the cyclodextrin in order to be cleaved. Binding of the acceptor at subsite +1 is required for catalysis, indicating that the cyclodextrin has to be cleaved before the acceptor can move to subsite +1. The monosaccharide acceptor must, therefore, bind sufficiently close to the catalytic residues to result in a random ternary complex mechanism. Mutational analysis of the acceptor subsite +2 has shown that indeed residues interacting with the glucose bound at this subsite (Lys232, Phe183, and Phe259) are involved in formation of the ternary complex (van der Veen et al. 2000a). Thus, although the events taking place at the active site resemble those of a ping-pong mechanism, the kinetic mechanism is characterized as a ternary complex. Presumably, the monosaccharide, bound at subsite +2, moves to subsite +1 after cleavage of the cyclodextrin. This is a small displacement, which is supposedly faster than the linearization of the covalently linked intermediate, explaining why it is not observed in the kinetic analysis. This linearization of the cyclic compounds is also actively mediated by the enzyme, as specifically shown by the effects of mutations of Arg47 (van der Veen et al. 2000b) and Phe183 (van der Veen et al.
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2000a), which are involved in the conformational change of the bound oligosaccharide. Mutation of Ser146, a residue specifically involved in binding linear compounds, also had a negative effect on the coupling \( k_{cat} \) values (van der Veen et al. 2000c). This indicates that for an efficient catalysis of the coupling reaction complete linearization of the cyclodextrin donor molecule is required.

**Hydrolysis**

The CGTase hydrolyzing activity may result in formation of linear products from starch. Under industrial cyclodextrin production conditions these linear oligosaccharides provide substrates for the coupling reaction, reducing cyclodextrin levels. Although the active sites of \( \alpha \)-amylase and CGTase are highly similar, CGTase has a relatively low hydrolytic activity compared to \( \alpha \)-amylase. In CGTase this is accomplished by excluding water from the active site; introduction of hydrophilic residues near the catalytic site renders the active site more favourable for water molecules that can react with the covalently bound reaction intermediate. This is shown by the replacement of phenylalanines 183 and 259 by hydrophilic residues, which resulted in increased hydrolyzing activities and reduced transglycosylation activities. 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) (van der Veen et al. 2000a). Mutants affected in subsite -7 (S146P and the double mutant Y89D/S146P) showed a doubling in the starch hydrolyzing activity (van der Veen et al. 2000c). Thus, binding of substrates of sufficient length to perform the cyclization reaction also limits hydrolysis.

**Cyclodextrin product specificity**

All CGTases produce mixtures of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-cyclodextrins from starch. Conceivably, the number of glucose residues (6, 7 or 8) binding in the active site cleft past the cleavage site determines which cyclodextrin is formed (\( \alpha \)-, \( \beta \)- or \( \gamma \)-cyclodextrin, respectively). The *Bacillus circulans* strain 251 CGTase has a high specificity for \( \beta \)-cyclodextrin formation, which may be explained by the fact that the active site cleft of this CGTase is specifically suited for binding 7 residues past the cleavage site (Strokopytov et al. 1996; Uitdehaag et al. 1999b). Indeed, disruption of the hydrogen bonding network at subsite -7 (mutation S146P) resulted in a specific decrease in the rate of formation of \( \beta \)-cyclodextrin (van der Veen et al. 2000c). Thus, initial substrate binding is very important for the cyclodextrin product specificity. However, also the rate of the subsequent circularization was found to be important. The conformational change in a bound oligosaccharide in the active site appears to be mediated by conformational changes in specific amino acid residues such as Arg47, which is involved in stabilization of the transition state that characterizes the cyclization and coupling reactions specifically. Mutations in Arg47 (van der Veen et al. 2000b), destabilizing this transition state, resulted in a decreased cyclization activity and a shift in specificity towards production of the larger size cyclodextrins. Similarly, mutation Tyr89Asp (van der Veen et al.
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2000c), which was found to stabilize the transition state, resulted in an increased cyclization activity and a shift towards formation of smaller cyclodextrins.

Cyclodextrin product inhibition

CGTase is inhibited by the cyclodextrin products it forms from starch. For wild type CGTase a mixed type of inhibition by β-cyclodextrin is observed when acting on high molecular weight substrates. This mixed inhibition can be divided in competitive inhibition, taking place at the active site, and non-competitive inhibition, which results from interference of β-cyclodextrin with the role of the E-domain in substrate binding. A β-cyclodextrin bound at MBS1 competitively inhibits binding of CGTase to raw starch granules, while a β-cyclodextrin bound at MBS2 hinders guidance of the substrate to the active site and may block the groove leading to the active site. Mutant Tyr633Ala, disrupting MBS2, had lost the non-competitive product inhibition of the cyclization reaction; the competitive inhibition, taking place in the active site, remained (Penninga et al. 1996).

The competitive inhibition of CGTase catalyzed reactions by cyclodextrins is not merely caused by the coupling reaction, since the inhibitory effect of β-cyclodextrin on the disproportionation reaction (where β-cyclodextrin is not involved in the reaction) of the wild type enzyme (van der Veen et al. 2000b) is identical to the competitive inhibition observed on the cyclization reaction of mutant Tyr633Ala. 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) for cyclodextrins 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.

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