Chapter 9

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Many plants produce a high molecular weight storage polymer of glucose (starch). Starch is mostly found in seeds (e.g. wheats) or roots (e.g. potato) in the form of granules consisting of two types of polymers; highly branched amylopectin (with $\alpha$(1-4) and $\alpha$(1-6) glucosidic bonds) and linear amylase (with $\alpha$(1-4) glucosidic bonds) (Chapter 1).

Many micro-organisms are able to use starch as carbon and energy source for growth. They convert the starch polymer extracellularly into smaller size molecules suitable for uptake and further conversion. In these organisms a whole range of starch degrading enzymes (mostly belonging to the $\alpha$-amylase family) with different specificities has evolved. A number of these enzymes find application in industrial processes, to generate a wide variety of products from starch. Examples are $\alpha$-amylase, producing short linear and branched oligosaccharides from starch, and cyclodextrin glycosyltransferase (CGTase), producing cyclic oligosaccharides consisting mainly of 6, 7, or 8 glucose residues ($\alpha$-, $\beta$-, or $\gamma$-cyclodextrins, respectively). The latter enzyme is unable to bypass branching points in the starch molecule and thus leaves a large part of it untouched (CGTase limit dextrin) (Chapter 1).

Whereas $\alpha$-amylases generally hydrolyze $\alpha$(1-4) glucosidic bonds, the basic reaction catalyzed by CGTases is transglycosylation. This reaction can be described as: $G(n) + G(m) - G(n-x) + G(m+x)$ in which $G(n)$ is the donor and $G(m)$ the acceptor oligosaccharide, consisting of n and m glucose residues, respectively. Disproportionation can be regarded as the default reaction, and is also catalyzed by several other members of the $\alpha$-amylase family (e.g. amylomaltase, disproportionating enzyme). The specific and characteristic CGTase reaction is the cyclization reaction in which the part of the donor substrate that has been cleaved off also acts as the acceptor, resulting in formation of a cyclodextrin, described as: $G(n) - cyclicG(x) + G(n-x)$. The reverse reaction is also catalyzed by the enzyme and is referred to as the coupling reaction (Chapter 1).

CGTase enzymes are used for the production of cyclodextrins, which have various applications in the food, cosmetic, pharmaceutical, and agrochemical industries. All known CGTases, however, produce mixtures of $\alpha$-, $\beta$-, and $\gamma$-cyclodextrins, requiring expensive and environmentally harmful purification procedures involving organic solvents to isolate the separate cyclodextrins. Furthermore, CGTase is subject to cyclodextrin product inhibition, limiting the formation of cyclodextrins from starch. Clearly, the availability of CGTase enzymes capable of producing an increased ratio of one particular type of cyclodextrin, and with reduced product inhibition, would be advantageous to the cyclodextrin producing industry. Also CGTase limit dextrins find industrial application, e.g. in surface sizing or coating of paper, to improve the writing quality and to obtain a glossy surface. Possible applications for CGTase in the preparation of doughs for baked products are under investigation. In such applications, however, cyclodextrins are unwanted side-products, requiring the availability of CGTase enzymes unable to produce cyclodextrins (Chapter 1).

In view of the above, protein engineering studies of CGTase of Bacillus circulans strain 251 have been initiated, with emphasis on structure-function relationships determining cyclodextrin product specificity and product inhibition, and the mechanisms of the CGTase.
catalyzed reactions. Site-directed mutagenesis, combined with biochemical and X-ray crystallographic analysis of (mutant) CGTase enzymes, has been applied to elucidate the complex mechanistic properties of CGTase.

Compared to α-amylases, CGTases contain two additional domains (D and E). The E-domain of CGTase has been recognized as a putative raw (native) starch binding domain and was investigated in chapter 2. β-cyclodextrin tightly binds to this E-domain, as observed in crystal structures, which may interfere with starch binding, providing at least part of the explanation for the observed cyclodextrin product inhibition. Analysis of a maltose-dependent CGTase crystal structure revealed that each enzyme molecule contained three maltose molecules, situated at contact points between protein molecules in the crystal. Two of these maltoses were bound to specific sites in the E-domain, the third maltose was bound at the C-domain. The roles of the two maltose binding sites in the E-domain in raw starch binding, cyclization reaction kinetics, and product inhibition by β-cyclodextrin were studied by replacing Trp616 and Trp662 of maltose binding site 1 and Tyr633 of maltose binding site 2 by alanines using site-directed mutagenesis. Characterization of purified (mutant) CGTases shows that maltose binding site 1 is most important for raw starch binding whereas maltose binding site 2 is involved in guiding linear starch chains into the active site groove. Inhibition of the cyclization reaction by β-cyclodextrin is of a mixed type, which can be divided in competitive inhibition interfering with catalysis in the active site and non-competitive inhibition interfering with the function of maltose binding site 2 in the E-domain.

In order to further investigate and eventually tailor the CGTase catalyzed reactions, it is useful to understand the mechanism by which these reactions proceed. Therefore a kinetic analysis of the transglycosylation reactions catalyzed by CGTase is presented in chapter 3. Previous studies have shown that all reactions catalyzed by CGTase proceed via a double displacement mechanism involving a covalent enzyme intermediate complex (substituted-enzyme intermediate). Characterization of the three CGTase catalyzed transglycosylation reactions revealed that they differ in their kinetic mechanisms, which can be largely explained by the different binding modes of the substrates used in the reactions. 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. The cyclization reaction is a single-substrate reaction with an affinity for the high molecular weight starch substrate used which was too high to allow elucidation of the kinetic mechanism. Michaelis-Menten kinetics is observed when using shorter amylose chains as substrate. In view of the different kinetic mechanisms observed for the various reactions it appears possible to manipulate CGTase in such a manner that a single reaction is affected most strongly, providing possibilities for the rational design of enzymes displaying desired specific activities. These new insights allow rational design of CGTase mutant enzymes with suitable characteristics for the industrial production of specific cyclodextrins or linear oligosaccharides from starch.
In chapter 4 cyclodextrin product specificity was further explored on the basis of the refined X-ray structure of the Y195F mutant of CGTase from *Bacillus circulans* strain 251 complexed with a maltononaose inhibitor (binding at subsites +2 to –7). This structure has provided detailed insight into the mode of binding of long amylose chains in the CGTase active site cleft, at sites distant from the catalytic residues. To probe the importance of these distant substrate binding sites for the α-, β-, and γ-cyclodextrin product ratios of CGTases, three single and one double mutant, Tyr89Asp, Tyr89Gly (subsite –3), Ser146Pro (subsite –7), and Tyr89Asp/Ser146Pro, were constructed using site-directed mutagenesis. The mutations had major effects on the cyclization, coupling, disproportionation, and hydrolysis reactions of the enzyme. The double mutant Tyr89Asp/Ser146Pro showed a twofold increase in the production of α-cyclodextrin from starch. This mutant protein was crystallized and its X-ray structure, in a complex with a maltotrihexaose inhibitor, was determined at 2.4 Å resolution. The maltotrihexaose molecule displays a binding mode different from the maltononaose inhibitor, allowing rationalization of the observed changes in product specificity. The ratio of the products formed in the cyclization reaction largely depends on hydrogen bonding interactions at specific subsites in the CGTase active site (Ser146 at subsite -7), determining how far the linear substrate will enter the active site. However, also the subsequent conformational change from linear substrate to cyclic product affects this ratio (Tyr89 at subsite -3). Destabilization of the linearly bound substrates can enhance the rate of this conformational change as shown by the increased β-, and γ-cyclization activities of mutant Tyr89Gly. Mutation Tyr89Asp, stabilizing a bent conformation of the bound ligand, resulted in an increase of cyclization reaction rates which was most apparent for α-cyclization (twofold). This indicates that the bent oligosaccharide conformation resembles a rate limiting reaction intermediate which is most strongly rate limiting for α-cyclization.

In chapter 5 the mechanism of competitive product inhibition exerted by cyclodextrins, taking place at the active site, is investigated. It is most likely linked to the CGTase coupling reaction, in which cyclodextrins are cut open and transferred to an oligosaccharide acceptor molecule, thus producing longer oligosaccharides. Residue Arg47 has been recognized as a CGTase active site residue in a position allowing it to interact with cyclodextrins, but not with linear oligosaccharides, thus most probably affecting CGTase reactions with cyclic substrates or products. In order to hydrogen bond to a cyclodextrin, Arg47 has to adapt its conformation compared to the unliganded and the linear substrate bound situation. The Arg47 residue has been replaced by Leu and Gln in order to investigate its role in the CGTase catalyzed reactions and in product inhibition. These Arg47 mutations indeed had a negative effect on the cyclization and coupling activities. The results show that Arg47 is involved in stabilization of the transition state that characterizes the cyclization and coupling reactions specifically. 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. The Arg47Leu and Arg47Gln mutants both showed lower affinities for cyclodextrins in the coupling reaction, and a reduced competitive (cyclodextrin product) inhibition. Product inhibition of CGTase thus is indeed linked to the coupling reaction. Interestingly, mutant Arg47Leu has increased hydrolysis activity, yielding large amounts of short oligosaccharides.
This increased presence of acceptor molecules for the coupling reaction may explain why Arg47Leu, despite its reduced product inhibition, produced lower final amounts of cyclodextrins from starch. The Arg47Gln mutant showed less reduction in product inhibition; this mutation, however, was shown to affect circularization and decircularization differently, resulting in an enzyme which significantly favours cyclization over coupling. This mutant has a decreased hydrolysis activity. As a consequence a more stable composition of the cyclodextrin products during the conversion of starch was obtained. These results allow design of highly specific CGTases based on initial cyclodextrin formation rates.

In the CGTase catalyzed reactions the acceptor binding site may have an important role in determining reaction specificity (transglycosylation versus hydrolysis). In chapter 6 the role of the acceptor binding site in in the different CGTase catalyzed reactions is investigated. His233, Lys232, and Glu264 have been identified as amino acid residues hydrogen bonding to glucose residues bound at the acceptor subsites +1, +2, and +3, respectively. At subsite +2 additional hydrophobic stacking interactions of the sugar with Phe183 and Phe259 have been observed. The His233 residue, involved in Ca\(^{2+}\) binding and hydrogen bonding interactions with the substrate residue bound at subsite +1, has already been shown to be of general importance for catalysis. Mutations Phe183Asn, Phe183Ser, Phe259Asn, Phe259Ser, and the double mutant Phe183Ser/Phe259Asn were introduced in order to define the roles of these residues at subsites +2 and +3, slightly more distant from the catalytic site. The two phenylalanines at the acceptor site were found to have specific roles in the cyclization and disproportionation reactions. Phe259 has a similar role in both reactions: correct positioning of the acceptor oligosaccharide in order to facilitate bond formation. In the disproportionation reaction Phe183 is involved in the “linear” binding mode of the acceptor substrate together with Glu264, thereby mediating the induced fit mechanism of the acceptor reaction. In the cyclization reaction Phe183 is actively involved in the movement of the non-reducing end of the covalently linked oligosaccharide, probably also activating the induced fit mechanism of the acceptor reaction. The mutant enzymes, with the phenylalanines changed into hydrophilic residues, creating a better environment for water acting as acceptor, possess an increased hydrolysis activity. The double mutant enzyme actually favours hydrolysis over cyclization (15 : 1), whereas the wild type favours cyclization over hydrolysis (90 : 1).

In conclusion, detailed information has been generated about structure-function relationships in cyclodextrin glycosyltransferase (CGTase) determining reaction/product specificity and cyclodextrin product inhibition. On the basis of this information the rational construction of mutants with optimized properties for various applications is possible.