Protein engineering of cyclodextrin glycosyltransferase from Bacillus circulans strain 251
Penninga, Dirk

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 08-12-2019
SUMMARY
An enormous diversity of molecular functions in living organisms is carried out by proteins. Our studies have focused on the functional analysis of a starch-converting enzyme, cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* strain 251. All CGTase enzymes studied convert starch into mixtures of α-, β- and γ-cyclodextrins, with 6, 7 and 8 glucose molecules, respectively. These enzymes catalyze cyclization, disproportionation, coupling, and hydrolysis reactions. Starch (known as a very complicated substrate) or smaller glucose-oligosaccharides act as substrates in all four reactions, necessitating binding of these carbohydrate substrates at a suitable position in the active center. To elucidate the CGTase cyclization mechanism, we have initiated a study of the relationship between the CGTase structure, function and activity. Besides the CGTase catalytic mechanism, other factors determining its product specificity (i.e. size of cyclodextrins formed), its sensitivity to cyclodextrin product inhibition, and binding of CGTase protein to raw starch, required examination. For this study we have chosen a protein engineering approach.

Protein engineering is the design and construction of mutant proteins, usually by manipulation of their genes, and requires a multi-disciplinary approach. It involves recombinant DNA techniques (gene cloning, gene expression, and site-directed mutagenesis); biochemical techniques (protein purification and characterization); protein crystallography techniques (crystallization, soaking of crystals, X-ray analysis, and determination of three-dimensional structures); computer graphics techniques (docking of molecules in the protein structure), and identification of targets for protein mutagenesis.

In the course of this project a number of essential tools and techniques for studying CGTase were further optimized. A new gene-expression system was developed allowing extracellular production of large amounts of (mutant) CGTase proteins in the protease- and α-amylase-negative *Bacillus subtilis* strain DB104A. Also the CGTase protein purification protocol was optimized, a highly specific, single step, affinity chromatography method yielding large quantities of (mutant) protein. Furthermore, a reliable method for the construction of dozens of site-specific mutants of CGTase was adopted. For the characterization of the (mutant) CGTase proteins a number of enzyme assays were developed and optimized. For the cyclization, coupling, and disproportionation reactions, accurate and specific assays are now available. This set of techniques has allowed the characterization of biochemical properties and three-dimensional structures of various (mutant) CGTase proteins. The results of these studies are described in this thesis.

In chapter 2 the cloning and sequencing of the gene encoding CGTase from *B. circulans*
strain 251, and the crystallization of the CGTase protein, is reported. A transformant of the chromosomal genebank of B. circulans strain 251 CGTase DNA was found to contain a 2139 bp open reading frame in the reverse orientation with respect to the lacZ promoter of the cloning vector. Further analysis revealed an ATG initiation codon, a TAA termination signal, and a 27 amino acid signal peptide that is removed during export. The extracellular CGTase thus contains 686 amino acid residues encoded by 2058 bp of the cgt gene. The mature protein shows 75% identity to the CGTase from B. circulans strain 8. The X-ray structure of the CGTase protein was elucidated and refined against X-ray diffraction data to 2.0 Å resolution, showing an essentially similar three-dimensional structure as that of the B. circulans strain 8 enzyme, despite the 25% difference in amino acid sequence. Five distinct domains, labeled A through E, were observed in the CGTase protein. Domains A, B and C are structurally homologous to the equivalent domains of α-amylases. The E domain shows homology with the starch binding motif conserved in several starch converting enzymes.

High concentrations of maltose were absolutely required for crystallization of the B. circulans strain 251 CGTase protein. Three maltose binding sites (MBS) were observed at the protein surface, two in domain E and one in domain C. Two of the maltose binding sites are located at intermolecular crystal contacts. The maltoses bound in the E domain interact with the implicated raw starch binding motif.

Chapter 3 describes the effects of site-directed mutations in the amino acid Tyr195 located in the center of the CGTase active site. Alignment of amino acid sequences of several CGTases and α-amylases, and information about the binding mode of the substrate analogue acarbose in the active site cleft, suggested that Tyr195 plays an important role in cyclization of oligosaccharides. It has been postulated that oligosaccharides entering the active site fold around this residue, explaining the formation of cyclic products. Tyr195 was replaced with Phe (Y195F), Trp (Y195W), Leu (Y195L) and Gly (Y195G). This did not significantly affect the α-, β-, ε-cyclodextrin product ratios, except with Y195L which resulted in loss of ε-cyclodextrin formation and an unexpected shift towards the synthesis (in low yields) of larger cyclodextrins with up to 12 glucose molecules. These mutant CGTase proteins displayed a significant reduction in cyclodextrin forming and coupling activities, but not in disproportionation and saccharifying activities. Especially with the Y195L and Y195G mutant CGTase proteins a higher production of linear oligosaccharides was observed. Aromatic amino acids (Tyr or Phe) at the Tyr195 position in the CGTase protein thus appear to be of crucial importance for an efficient cyclization reaction, also preventing the formation of linear products. Aromatic residues favorably interact with the hydrophobic face of carbohydrate residues, e.g. with cyclic and linear oligosaccharides in the cyclization and
coupling reactions. Conceivably, the aromatic amino acid residues Tyr and Phe at position 195 in CGTases are most efficient in bending the non-reducing end of the acceptor oligosaccharide towards the reducing end of the donor substrate that is bound in the catalytic center, resulting in cyclodextrin formation.

The purified mutant CGTase proteins were crystallized, and their X-ray structures were determined at 2.5 to 2.6 Å resolution. Apart from their specific mutations, the structures of these mutant CGTase proteins were very similar to that of the wild-type CGTase protein. Comparison of the conformations of the amino acids surrounding residue 195 indicated clearly that no large structural rearrangements had taken place due to the mutations. Because the conformation of the mutant enzymes is not affected, no conformational feature can account for observed changes in the biochemical characteristics.

Asp229, Glu257 and Asp328 are presumed to constitute the catalytic residues of CGTase. In chapter 4 the effects of site-directed mutations D229N, E257Q and D328N, and the double mutant D229N/E257Q are reported. These mutant CGTase proteins displayed only a very low cyclization activity. The E257Q and D328N mutations caused a downward shift (0.5 unit) the pH optima. This suggests that Glu 257 and Asp328, but not Asp229, are both involved in proton transfer: Glu257 most likely acts as the proton donor in the reaction, while Asp328 is possibly involved in binding of the substrate and elevating the pKa of Glu257 through a direct hydrogen bond to this residue that exists only when no substrate or inhibitor is present. The role of Asp229 is most likely that of a general base or nucleophile.

Crystal structures were determined of wild type CGTase protein soaked at elevated pH with €-cyclodextrin and maltoheptaose. In addition, structures at cryogenic temperature were solved of the unliganded enzyme and the D229N/E257Q double mutant CGTase soaked with €-cyclodextrin. In the crystals soaked in €-cyclodextrin and maltoheptaose, a maltotetraose molecule had become bound in the active site, allowing identification of sugar-binding subsites -2 to 2. Maltotetraose is not cleaved by CGTase; probably the enzyme can not perform catalysis because certain contacts with sugars beyond subsites -2 and 2 are lacking.

Two €-cyclodextrin molecules were observed in the structure of the D229N/E257Q double mutant, bound at MBS1 and MBS2 in the E-domain. This demonstrated that cyclodextrins are able to bind strongly to this domain. Especially the cyclodextrin bound to MBS2 near Tyr633 might interfere with the catalytic activity of the enzyme since this MBS is part of the long groove on the protein surface leading into the active site.

Chapter 5: Crystals of a CGTase protein were subjected to a double soaking procedure with the inhibitor acarbose and with maltohexaose. The refined structure of the resulting
protein-carbohydrate complex was determined (at 2.6 Å resolution), revealing that a new maltotriose inhibitor had become bound in the active site of the CGTase protein. The four residues occupying subsites -2 to 2 correspond to acarbose. An oligosaccharide consisting of five glucose residues had become coupled to the non-reducing end of this acarbose. A total of nine sugar binding subsites were thus identified in the CGTase active site cleft. The glucose residues at subsite 1 through 4 form one half of a $\alpha$-cyclodextrin molecule but the glucose residues at subsites 5 through 7 are directed away from the active site cleft. Formation of a cyclic product from a normal amylose chain will involve hydrolysis of the glycosidic bond between the glucose molecules at subsites 1 and -1 (possibly an oxocarbonium intermediate is formed); next the non-reducing end of the amylose chain, which is pointing away, has to move 23 Å towards the intermediate, followed by ring closure. The number of amylose chain glucose units bound at the subsites in the CGTase active site thus may determine the size of the product formed. In order to manipulate the size of the cyclodextrins produced, the number of subsites that can be occupied by amylose should be changed, e.g. by altering the sugar binding capacities of the separate subsites via site-directed mutagenesis.

On the basis of the detailed knowledge of the nine subsites in CGTase gained in chapter 5, mutants Y89G, Y89D, S146P and the double mutant Y89D/S146P, involved in subsites 3, 6, and 7 were constructed to specifically increase $\epsilon$-cyclodextrin production (chapter 6). The Y89D/S146P mutant was crystallized and its X-ray structure, in a complex with a maltotriose inhibitor, present in a bent conformation, was determined at 2.4 Å resolution. Except for mutant Y89G, these mutants displayed significant differences in their initial cyclization activity from starch: An increase in the formation of $\epsilon$-cyclodextrin, and a decrease in the formation of $\epsilon$- and $\epsilon$-cyclodextrin. Especially mutant Y89D and double mutant Y89D/S146P also displayed increased coupling, disproportionation, and saccharifying activities. An increased production of $\epsilon$-cyclodextrin was also observed in incubations rather similar to industrial production process conditions. The $\epsilon:\epsilon:\epsilon$-cyclodextrin product ratio changed from 14:66:20 for wild-type CGTase into 30:54:19 for the double mutant Y89D/S146P, a 214 % increase in $\epsilon$-cyclodextrin synthesis. The data thus show that hydrogen bonds and hydrophobic (Tyr89) contacts of amino acids with the amylose chain more distant from the active site residues contribute strongly to binding of the amylose chain and thus to the size of cyclodextrins formed. The CGTase cyclodextrin production specificity can be changed in a rational manner by introducing specific mutations in the residues in the active site cleft which constitute these subsites.

The kinetic data and the structure of mutant Y89D/S146P complexed with the
maltohexaose inhibitor present in a bent conformation, in stead of a straight conformation as was observed in the structure of CGTase with a maltononaose inhibitor, provide evidence for the involvement of specific intermediates in the cyclization reaction leading to ζ-, η-, and ι-cyclodextrin.

The identification of two MBS’s in the E-domain of *B. circulans* strain 251 CGTase, and the homology with the raw starch binding motif observed in several starch converting enzymes, has led to the assumption that these sites are involved in raw starch binding and amylose binding at the E-domain. MBS1 includes tryptophan residues 616 and 662, which have hydrophobic contacts with a maltose unit. In MBS2, located near the groove leading to the active site, a maltose is stacked on Tyr633. A third MBS is located at the C-domain. CGTase is subject to non-competitive inhibition by cyclodextrins, displaying the highest sensitivity to ι-cyclodextrin, which binds at MBS1 and MBS2, but not (or less) at MBS3.

**Chapter 7** describes an analysis of the roles of the two MBS’s in the E-domain in raw starch binding and cyclization reaction kinetics, following construction of the W616A, W662A (both in MBS1) and Y633A (in MBS2) mutants. Characterization of the mutant proteins showed that MBS1 is most important for raw starch binding and that MBS2 is involved in guiding linear starch chains into the active site. The sensitivity of CGTase to cyclodextrin product inhibition appears to be of a mixed type of product inhibition. Non-competitive inhibition is caused by interference of cyclodextrin with starch binding at MBS2 in the E-domain. Competitive inhibition is probably based on the reverse reaction of cyclization, the coupling reaction. The W616A mutant CGTase has been crystallized as a carbohydrate independent dimer and the structure has been refined to 2.2 Å resolution. The 3D-structure shows that neither the absence of carbohydrates nor the W616A mutation cause significant conformational changes with respect to the wild-type enzyme.