Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase family

Joost C.M. Uitdehaag[a,†], Bart A. van der Veen[b], Lubbert Dijkhuizen[b], Bauke W. Dijkstra[a,*]

[a]*Laboratory of Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*
[b]*Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

Abstract

The catalytic mechanism of cyclodextrin glycosyltransferase, a member of the α-amylase family, is reviewed. The focus is put on the bond cleavage mechanism, the nature of the transition state and of the covalent intermediate, and on the stereo-electronic and lateral protonation contributions to catalysis. The functions in catalysis of the absolutely conserved residues in this family are discussed. Finally, the fascinating capability of cyclodextrin glycosyltransferase to produce cyclodextrins from linear starch oligosaccharide chains is reviewed, together with protein engineering studies to modify the enzyme's product specificity. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Life on earth depends on the uptake of energy. While photosynthetic organisms can directly use the sun's light, all other forms of life obtain their energy from nutrients they take up from the environment. One of the most energy-rich sources of food is starch, which is a polymer of glucose molecules linked together by $\alpha(1{\rightarrow}4)$ and $\alpha(1{\rightarrow}6)$ glycosidic bonds [1,2]. It is consumed daily by a large part of earth's human population in the form of rice, wheat, maize, tapioca, or potatoes.

To degrade starch humans, but also other animals, fungi and bacteria possess a range of starch-degrading enzymes. Most important in this respect is a large and diverse group of hydrolytic enzymes, which have been classified in glycosyl hydrolase family 13, the α-amylase family [3,4]. This family comprises not only specialized enzymes for the hydrolysis of $\alpha(1{\rightarrow}4)$ and $\alpha(1{\rightarrow}6)$ glycosidic bonds, but also enzymes that make oligosaccharide products of a specific composition.

An example of such an enzyme that produces specific oligosaccharides is cyclodextrin glycosyltransferase (CGTase). CGTase is a 75 kDa bacterial enzyme that converts starch into cyclodextrins, which are cyclic oligoglucosides of six, seven or eight glucose residues (named α-, β-, and γ-cyclodextrin, respectively). Presumably, the bacteria that excrete CGTases do this to convert starch into compounds that can not be used by competing organisms. In this way they monopolize starch as a substrate for themselves [5].

However, besides its obvious functional importance for the bacteria that excrete it, cyclodextrin glycosyltransferase has also found important industrial applications. The major interest in the use of CGTase is for the large-scale production of cyclodextrins, but the enzyme is also applied for the liquefaction of starch [6]. A more recent development is the use of CGTase for the synthesis of modified oligosaccharides, such as the sweetener stevioside [6]. Stevioside, as isolated from the plant *Stevia rebaudiana*, has bitter taste and low solubility. CGTase catalyzed glycosylation decreases the bitterness and increases the solubility.

Despite its wide use in industry, CGTase suffers from several shortcomings that limit its applicability. All wild type CGTases produce mixtures of α-, β-, and γ-cyclodextrins, and the separate cyclodextrins have to be purified by selective crystallization using (expensive) organic solvents, that are sometimes difficult to remove. A second disadvantage of the CGTases commonly used in industry is that their activity is inhibited by the produced cyclodextrins [7]. Thus, there is a clear need for novel CGTases with improved

* Corresponding author. Tel.: +31-50-363-4381; fax: +31-50-363-4800.

E-mail address: bauke@chem.rug.nl (B.W. Dijkstra).

† Present address: Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom.
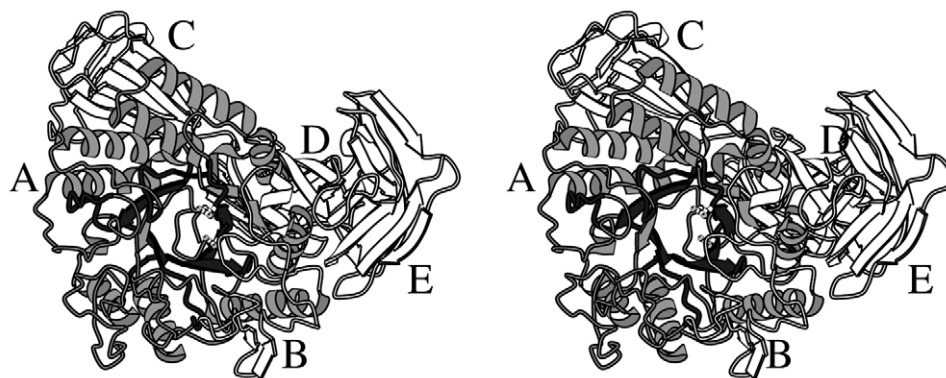
Fig. 1. Stereo-view of the structure of a representative member of the α-amylase family, *Bacillus circulans* strain 251 cyclodextrin glycosyltransferase (BC 251 CGTase) [11]. CGTase is a 75 kDa enzyme that consists of five domains. Domain A is the catalytic $(\beta/\alpha)_8$ barrel (gray), that CGTase shares with the other members of the α-amylase family. This domain contains the sequence regions that are conserved in the α-amylase family (black). In the center of the conserved regions indicated by the side chains of Asp229 and Glu257 in white ball-and-stick representation, the catalytic site is located. From domain A a loop protrudes that forms part of the active site (white; domain B), and which is also present in many other α-amylase family enzymes [18]. Behind the $(\beta/\alpha)_8$ barrel, three additional domains (C, D and E) are present (white). Domains C and E contain binding sites for raw starch [7,11]. This figure, and Fig. 4, were made with Molscript [74].

properties. In this article we review the existing literature on the catalytic mechanism of CGTase and related enzymes, and our protein engineering approach to improve the product specificity of the enzyme.

2. Three-dimensional structures of cyclodextrin glycosyltransferase

When in 1984 the first X-ray structure of an α-amylase family member, Taka-α-amylase, was elucidated, this revealed a catalytic domain with an $(\alpha/\beta)_8$, or TIM barrel, architecture [8]. At present 22 X-ray structures of α-amylase family members are known, among which are several of cyclodextrin glycosyltransferases [9–13]. CGTases have five domains, labeled A to E (Fig. 1). Domain A is the catalytic $(\alpha/\beta)_8$ domain, which CGTase has in common with the other α-amylase family members. Domain B is an extended loop region inserted after β-strand 3 of domain A. It contributes to substrate binding by providing several amino acid side chains alongside a long groove on the surface of the enzyme that interact with the substrate [14,

15]. Domains C and E have a β-sheet structure, and are specialized in binding to raw starch granules [7,11,16]. The function of domain D, which also has a β-sheet structure, remains to be elucidated.

The seven most conserved amino acid residues in the α-amylase family cluster together at the bottom of the substrate binding groove at the surface of the $(\alpha/\beta)_8$ barrel. They are in the center of the conserved regions (Fig. 1), where the catalytic site is located. Three of them are absolutely conserved, Asp229, Glu257 and Asp328, while Asp135, His140, Arg227 and His327 are almost completely conserved [3,17,18]. The evolutionary conservation of these residues can be explained because their common substrate, starch, contains only one basic chemical bond, the α-glycosidic bond. This bond occurs in several types, like $\alpha(1\rightarrow4)$, $\alpha(1\rightarrow6)$ or $\alpha(1\rightarrow1)$, depending on whether the C1 atom of a glucose is connected to the O4, O6 or O1 atom of a neighboring glucose, respectively (see Fig. 2, left part, for the glucose ring atom nomenclature).

α-Glycosidic bonds are very stable, having a rate of spontaneous hydrolysis of only about $2 \cdot 10^{-15}$ s^{-1} at room temperature [19]. Since for example CGTase has a rate in
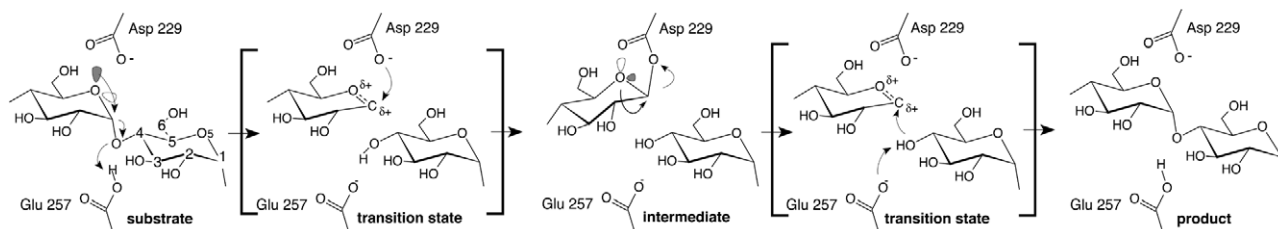

Fig. 2. Scheme of the α-retaining $\alpha(1,4)$ glycosidic bond cleavage mechanism as used by the α-amylase family, and as explained in the text. The glycoside ring atom nomenclature is incorporated in the left-most picture. The shaded orbitals represent the electrons that participate in the cleavage of the α-glycosidic bond according to the stereo-electronic theory (see text). Note that both the substrate and the product contain an identical $\alpha(1,4)$ glycosidic linkage. In the disproportionation, cyclization, and coupling reactions the part of the substrate that is not covalently bound to the enzyme during the reaction, is exchanged for a different saccharide, but the newly formed glycosidic bond remains an $\alpha(1,4)$ glycosidic bond.

the order of 3000 s^{-1} [20], this means that CGTase enhances the hydrolysis rate by 18 orders of magnitude, which ranks the enzyme among the most efficient ones known [19].

3. The reactions catalyzed by cyclodextrin glycosyltransferase

CGTase catalyzes four distinctly different reactions: three transglycosylation reactions (disproportionation, cyclization, and coupling), and a hydrolysis reaction [20]. Disproportionation is the major reaction. In this reaction a linear malto-oligosaccharide is cleaved and one of the products is transferred to another linear acceptor substrate. Cyclization is an intramolecular transglycosylation reaction, in which the non-reducing end of a linear malto-oligosaccharide is transferred to the reducing end of the same oligosaccharide chain. The products of this reaction are cyclodextrins. Coupling is the reverse of the cyclization reaction in which the opening of a cyclodextrin ring is followed by transfer to a linear oligosaccharide. Finally, hydrolysis is a reaction in which part of a linear oligosaccharide is transferred to water. These four reactions make use of the same basic mechanism, which is conserved throughout the α-amylase family; only the acceptor molecule differs. In the following we will use the standard sugar binding subsite nomenclature [21], in which the catalytic site is labeled -1, and the scissile bond is positioned between subsites -1 and $+1$. In this nomenclature the acceptor molecule will bind at the $+1$ subsite, after the bond cleavage reaction has taken place.

4. The bond cleavage mechanism of cyclodextrin glycosyltransferase

The catalytic mechanism of enzymes of the α-amylase family, which is now generally accepted, was first proposed by Koshland in 1953 [22], and is known as the α-retaining double displacement mechanism. This mechanism involves two catalytic residues, an acid/base catalyst (Glu257 in CGTase) and a nucleophile (Asp229) (Fig. 2). When substrate has bound, Glu257 (as acid) donates a proton to the scissile glycosidic bond oxygen. The scissile bond is then cleaved, during which reaction the -1 sugar goes through an oxocarbonium ion-like transition state (Fig. 2). This transition state is characterized by a partial double bond between the O5 and C1 atoms of the sugar over which a positive charge has delocalized. It has a planar configuration of the C2, C1, O5 and C5 atoms (Fig. 2).

In the subsequent reaction step, this oxocarbonium ion-like transition state collapses into a stable covalent glycosyl-enzyme reaction intermediate which is β-glycosidically linked to the nucleophile Asp229 (Fig. 2). The leaving group (at subsite $+1$) is exchanged for a new compound, the acceptor, which gets activated by Glu257 (now a base). This activated acceptor attacks the covalent intermediate, and, via another oxocarbonium ion-like transition state, the final α-glycosidic product bond is formed (Fig. 2). This α-retaining mechanism is closely related to the β-retaining mechanism of glycosyl hydrolases that act on β-glycosidic bonds. Both mechanisms have been extensively reviewed [23–29].

5. The nature of the transition state

For the α-amylase family enzymes, the transition state has been characterized by kinetic isotope experiments [30]. These studies have shown that it has an almost complete double bond between the O5 and C1 atoms, with the positive charge almost entirely localized on the O5 atom, the O4$'$ atom nearly fully protonated, and the C1–O4$'$ bond almost completely broken [24,30]. Thus, these measurements confirm an oxocarbonium ion-like structure. Such a transition state is further supported by the fact that the functional unit of the transition state mimicking inhibitor acarbose has a planar geometry, similar to that of an oxocarbonium ion [31].

6. The nature of the intermediate

The chemical nature of the intermediate has been the subject of an almost classical debate [26,32]. In his original proposal, Koshland drew the intermediate in α- and β-retaining mechanisms as covalent [22]. However, when the first structure of a glycosyl hydrolase was solved, the β-retaining hen egg white lysozyme, it was suggested that an oxocarbonium ion might be sufficiently long-lived in the catalytic site to function as intermediate [33]. This view became widespread in textbooks [1], and was readily extrapolated to the α-amylase family enzymes [3,8,11,34].

The first unambiguous experimental evidence for the existence of a covalent intermediate in the α-amylase family came from NMR studies [35], and trapping experiments using radiolabels [36]. Furthermore, experiments to trap the intermediate with inhibitors and slow substrates were successful [37–41]. However, the most convincing evidence for the covalent character of the intermediate was obtained from a 3D structure of a trisaccharide covalently bound to the nucleophile Asp229 in CGTase [42]. Owing to this evidence, covalent intermediates are at present widely believed to occur in the α-amylase family, thereby confirming Koshland's original hypothesis. Very recently unambiguous evidence has been provided that also hen egg white lysozyme makes use of covalent catalysis [23–29].

7. Stereo-electronic contributions to catalysis

A second important field of debate concerns the predictions of the stereo-electronic theory about the conformations
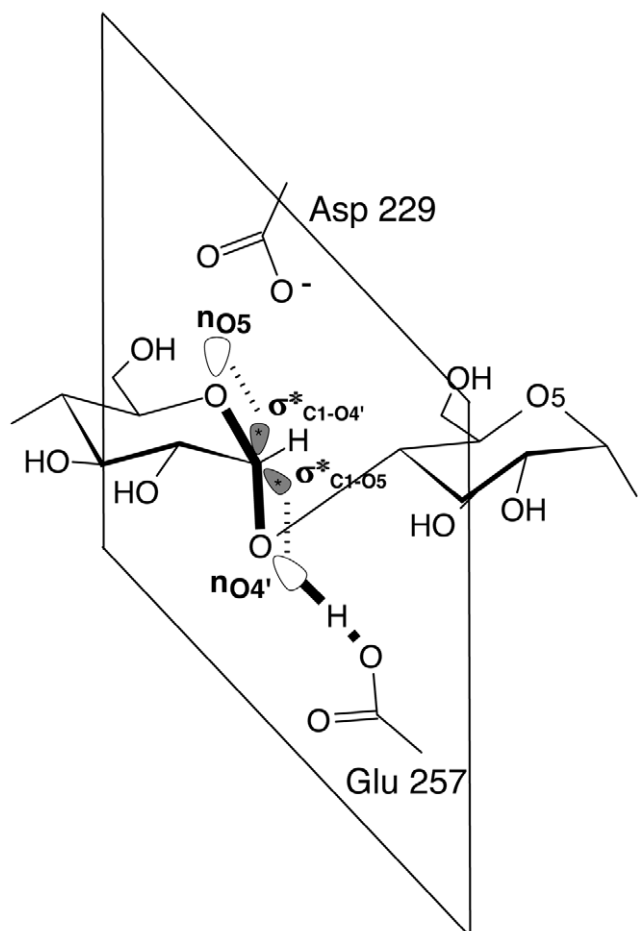
Fig. 3. The $\alpha(1,4)$ glycosidic bond orientation in a substrate bound in the active site of an α-amylase family enzyme. Lone pair electron orbitals are shown in white, anti-bonding orbitals in gray. Dashes indicate orbital overlap. Also depicted is the hydrogen bond that Glu257 donates to the lone pair electron orbital of the scissile bond oxygen ($n_{O4'}$). The bonds that are involved in the reaction (black) are oriented in one plane, which is represented by the frame (see text). The glucose on the left has a $^4C_1$ chair conformation, in which case the α-glycosidic bond assumes an axial orientation.

of substrate and intermediate when bound to the enzyme [23,43]. The stereo-electronic theory is based on the (undebated) anomeric effect, which explains the unexpectedly high preference of a free glucose molecule for having its C1–O1 bond in an axial, instead of an equatorial orientation [44]. The anomeric effect proposes a stabilizing interaction between a non-bonding lone pair orbital of the O5 atom (n_{O5}), and the anti-bonding orbital of a C1–O1 bond or a C1–O4′ bond ($\sigma^*_{C1-O4'}$), when this bond has an axial orientation [23,45] (Fig. 3). This orbital overlap leads to a shortening and strengthening of the O5–C1 bond [23,46], and an elongation and weakening of the C1–O4′ bond. This might be advantageous for enzymes, and therefore it has been stated that glycosidic bonds are preferentially cleaved when they are in an axial position.

For α-glycosidically linked sugars, the glycosidic bond is in an axial position when the sugar is in a $^4C_1$ chair-like

conformation (Fig. 3). Indeed, in X-ray structures of enzymes in complex with an α-glycosidically linked substrate [16], or an α-glycosidically linked intermediate [47], these ligands are observed in a $^4C_1$ chair-like conformation. The stereo-electronic theory further predicts that β-glycosidically linked substrates and intermediates are distorted towards a $^{1,4}B$ boat conformation to impose an axial orientation of the scissile bond. This distortion is indeed observed in a complex of an enzyme with its β-glycosidically linked substrate [48]. However, in a β-glycosidically linked covalent intermediate, as occurs in the α-amylase family, such distortion is not observed [42].

Therefore, not all X-ray structures confirm the stereo-electronic theory. Furthermore, an alternative explanation has been put forward for the distortion of a β-glycosidically linked substrate towards a $^{1,4}B$ boat conformation, stating that this boat conformation might be required to minimize steric hindrance during the enzymatic reaction [49]. Moreover, the $^4C_1$ chair-like conformations in α-glycosidically linked sugars have low energy, and might thus simply be thermodynamically favored. For these reasons, the stereo-electronic theory has fallen out of favor recently [49].

8. The contribution of lateral protonation to catalysis

A third interesting issue in the field of catalysis by glycosyl hydrolases is the function of distortions of glycosidic bond torsion angles. Often the binding of a substrate in the active site of a glycosyl hydrolase results in rotations of the torsion angles around the C1–O4′ and O4′–C4′ bonds, and the disruption of an interglycosidic hydrogen bond between the "intermediate" and "leaving group" parts of the substrate. Therefore, it was suggested that these torsion angle rotations facilitate departure of the leaving group after formation of the intermediate [50,51]. However, the rotations also position a lone pair orbital of the O4′ atom in the direction of the proton donor Glu257 (Fig. 3). This allows lateral protonation of the glycosidic oxygen atom, in which the O5–C1 bond, the C1–O4′ bond, and the "line of protonation" between O4′ and Glu257 occur in one plane (Fig. 3) [27,52]. Fulfilling a requirement for lateral protonation might thus also be a function for the bond torsion angle distortions, although the reason for lateral protonation remains unknown. An analogous requirement for an in-plane orientation of the reactive system is seen in E2-elimination reactions [44]. In both cases it might serve to suitably orient the reactive orbitals for a bond cleavage reaction.

9. The roles of the conserved residues in catalysis

Asp229 is the first absolutely conserved residue in the α-amylase family, and the proposed catalytic nucleophile (Fig. 2). Mutations in Asp229 reduce the activity about $\sim$25,000 times [3,16,18]. In structures of ligand complexes
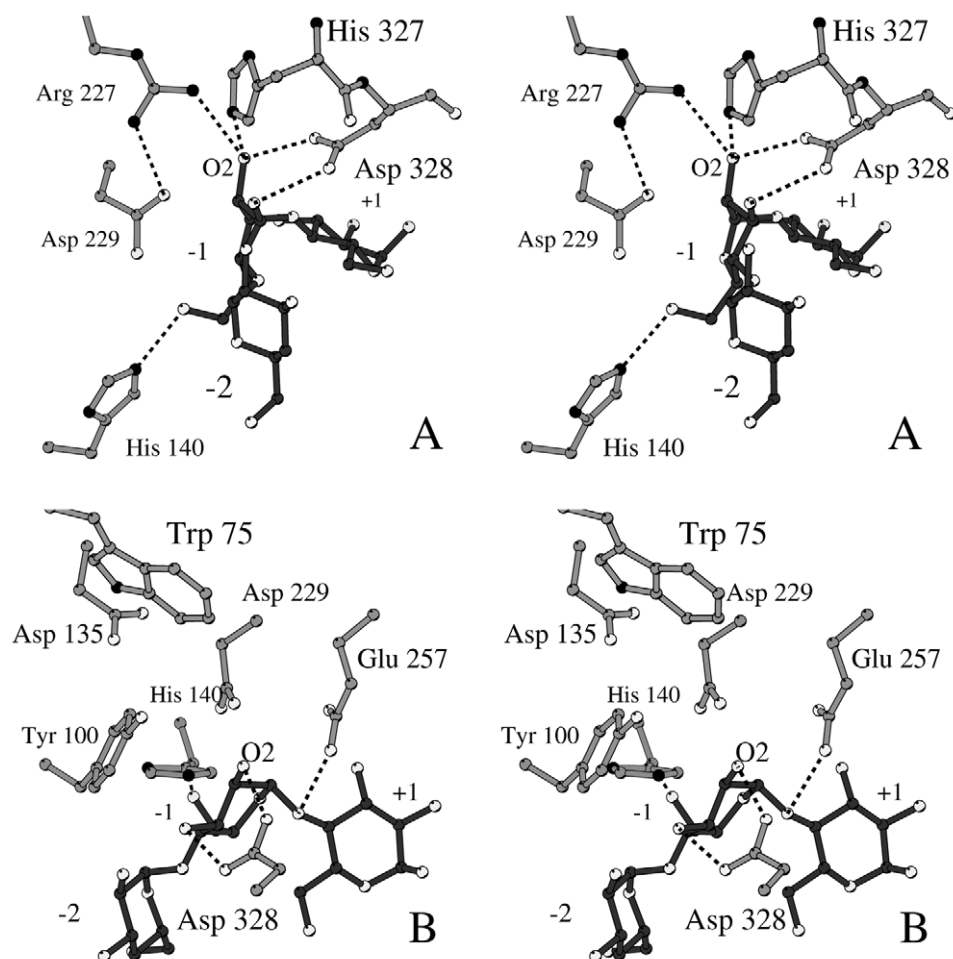
Fig. 4. Stereo-view of the conserved residues in the catalytic site (gray) of the α-amylase family in two orientations (A and B). For clarity, a substrate molecule (black) spanning subsites −2, −1 and +1 is also shown. Dashed lines indicate hydrogen bonding interactions. Asp328 makes hydrogen bonds with the O2 and O3 atoms of the −1 glucose, His140 is hydrogen bonded to the −1 O6 atom, and the Glu257 side chain is at hydrogen bonding distance from the oxygen atom in the scissile glycosidic bond. The figure is based on a BC251 CGTase-substrate complex described in reference [42], and is essentially similar to substrate complexes of other α-amylase family enzymes.

(Fig. 4), the Oδ1 atom of Asp229 is situated close to the glucose C1 atom, and its reactive *syn* lone pair orbital is positioned ideally for nucleophilic attack [53,54].

Glu257 is the second absolutely conserved residue in the α-amylase family, and the proposed catalytic acid/base (Fig. 2). Mutation of Glu257 reduces the enzymatic activity ~5,000 times [3,16,18]. In structures of unliganded α-amylase family enzymes, Glu257 receives a hydrogen bond from Asp328. After sugar binding, the side chain of Glu257 rearranges (Fig. 4), and forms a hydrogen bond to the O4′ atom in the scissile bond [53], as expected for the catalytic acid/base residue [8].

Asp328 is the third absolutely conserved residue. Mutants in Asp328 can reduce the activity ~50,000 times [3,16]. Upon substrate binding Asp328 interacts with the OH-2 and OH-3 groups of the substrate [8,53,54] (Fig. 4). It has been speculated that the hydrogen bond to the OH-2 group could become a strong low-barrier hydrogen bond that stabilizes the transition state of the reaction [54]. It has

also been suggested that Asp328 plays a role in maintaining the proper protonation state of Glu257 [53]. Finally, Uitdehaag et al. demonstrated that Asp328 has an important role in substrate distortion [42].

His327 and Arg227 are two basic residues in the catalytic site. Mutants of His327 typically show a ~50 times reduced k_{cat}/K_M [3,28,55,56]. Mutants of Arg227 have not been reported. His327 and Arg227 both bind to the OH-2 group of the substrate [57] (Fig. 4). These hydrogen bonds serve two purposes. One is the distortion of the sugar ring towards the half-chair conformation. The other is a reduction of the electronegativity of the 2-hydroxyl group, which otherwise would strongly disfavor formation of a positively charged transition state [42].

His140 is located further down in the catalytic site. Mutants of this residue have a ~200 times reduced k_{cat}/K_M [3,28,55,56]. X-ray structures show that His140 binds to the OH-6 group of the −1 sugar of a substrate bound in the catalytic site [42] (Fig. 4). However, in the CGTase-cova-

lent intermediate and the CGTase-γ-cyclodextrin product complex this hydrogen bond is absent, and His140 interacts with the Asn139 side chain [15,42]. Thus, the nature of the His140–OH-6 hydrogen bond most likely changes during catalysis [15,58].

Asp135 is also strongly conserved in α-amylases. However, this residue is not located in the catalytic site. Instead, it binds to the catalytic site residue Tyr100, which is less strongly conserved (Fig. 4). The role of Asp135 is unknown, but Tyr100 has a function in sugar binding. Its aromatic ring interacts with the glucose ring in the catalytic subsite (glucose −1) through hydrophobic stacking contacts [53, 57].

10. Cyclization activity of cyclodextrin glycosyltransferase

A fascinating feature of cyclodextrin glycosyltransferase is its capability to produce cyclodextrins from linear starch oligosaccharide chains. Cyclization activity is a special form of acceptor specificity, in which the non-reducing end of a donor chain is used as acceptor, leading to cyclic products (see Fig. 5). In *Bacillus circulans* strain 251 CGTase, which produces predominantly β-cyclodextrin, seven sugar binding subsites were characterized that bind the covalent intermediate (donor subsites) [14,42]. Surprisingly, in the crystal structure, the 4-OH group at the non-reducing end of the substrate, bound at subsite −7, was about 23 Å away from the catalytic site, and not nearby, as originally expected [59]. Subsequent site-directed mutagenesis studies confirmed the remote location of subsite −7, assuring that its identification is not a crystallographic artifact [60,61]. Thus, cyclization requires a step in which a linearly bound covalent intermediate, reaching subsite −7, changes conformation into a circularly bound intermediate, reaching the acceptor subsite +1. Using stochastic path calculations, Uitdehaag et al. analyzed the pathway along which the non-reducing end of the covalent intermediate moves from subsite −7 to the +1 acceptor subsite [62]. The input data for these calculations were the structure of a linearly bound malto-octaose saccharide, derived from the crystal structures of CGTase with bound maltononaose and with the covalently bound maltotriose [42], and the structure of γ-cyclodextrin, bound in the active site of CGTase [15]. The result is one of the first successful applications of the method. Comparison of the results with site-directed mutagenesis data show that CGTase actively catalyzes the circularization process. The −6, −7, −8 sugar residues successively bind at subsite −6, where they interact with Gly179, Gly180, Tyr167, and Asn193. Further movement of the oligosaccharide is facilitated by interactions with the aromatic side chains of Tyr195, Phe183, and Phe259. Next, residues Leu194, Leu197 and Ala230, assisted by Tyr195 and Phe183, form a hydrophobic cavity that traps the sugar chain before its final transfer to the +1 acceptor subsite.

During the circularization process the sugar binding mode at subsite −3 changes; the initial interactions with Asp196 and Tyr89 weaken, and the saccharide binds instead to Arg47 and Asp371. An animation of this circularization process can be found at http://www.interscience.wiley.com/jpages/0887-3585/suppmat/43_3/v43_3.html.

11. Protein engineering of the product specificity of CGTase

In the past it has been suggested that the size of the aromatic amino acid (Tyr195), which is present in a central position in the active site, is an important determinant for the size of the produced cyclodextrins. Indeed, a Tyr195Trp substitution doubled the amount of γ-cyclodextrin [60,63]. However, several other mutations of this residue did not support these results [63–65]. In the light of the recent findings of Uitdehaag et al. on the circularization mechanism (see above, [62]) it is now clear that the outcome of the cyclization reaction is determined by many more factors than only residue 195. Instead, interactions at subsites −7, −6, and −3 may all contribute to the product specificity of CGTase.

The importance of subsites −7 and −3 for cyclodextrin product specificity is supported by a large body of evidence. The primarily γ-cyclodextrin producing CGTase from *B. firmus/lentus* strain 290-3 completely lacks the residues around residue 146 (subsite −7). This situation was recently copied into the CGTase from *B. circulans* strain 8, by replacing residues 145–151 by a single aspartate residue. The mutant had an increased γ-cyclodextrin production [60]. Similarly, a Ser146Pro mutation in *Bacillus circulans* strain 251 CGTase (subsite −7) resulted in a lower β-cyclization, but a higher α-cyclization activity [61].

At subsite −3 Tyr89 plays an important role. The Tyr89Asp substitution in *Bacillus circulans* strain 251 CGTase showed a slight increase in α-cyclization activity, with no effect on the β- and γ-cyclization activity [61]. Combination of this mutation with the subsite −7 Ser146Pro substitution gave an enzyme that produced significantly more α-cyclodextrin than wild type enzyme [61]. Mutation of Arg47 near subsite −3 into a Leu or Gln resulted in a generally decreased cyclization activity, but also a shift towards the production of larger cyclodextrins [66]. Finally, structural investigations of the CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1 [12,67] revealed the importance of Asp371 and Asp197 at subsite −3 for product specificity. Mutant Asp371Arg produced enhanced levels of β- and γ-cyclodextrin, while mutant Asp197His yielded more α-cyclodextrin [67]. Subsite −3 mutations in the alkalophilic *Bacillus* sp. I-5 CGTase also changed the product specificity [68].

From the above it is clear that subsites −3 and −7 are key sites for the cyclodextrin product specificity. The identity, position and interactions of the amino acid residues
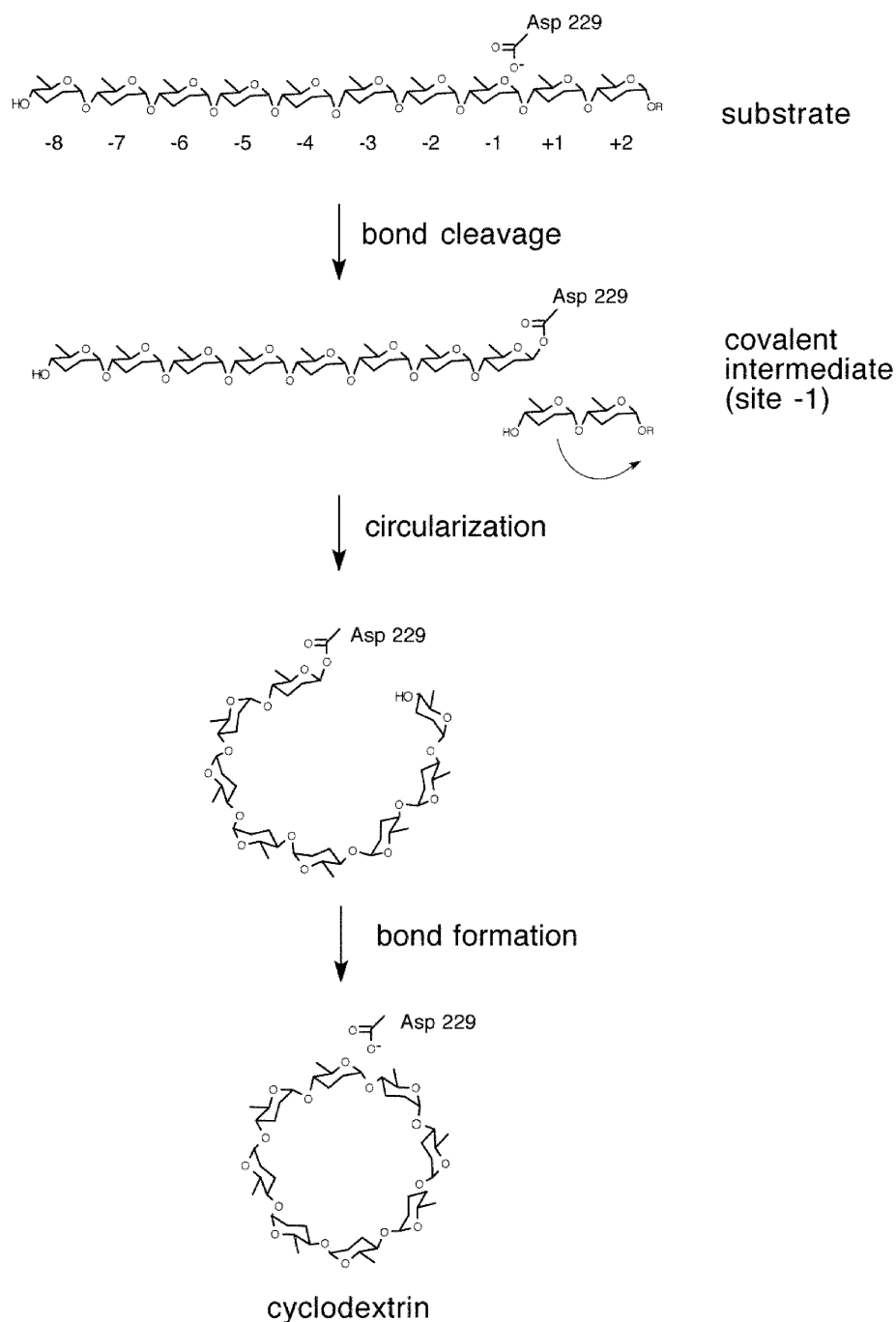
THE CGTASE CYCLIZATION REACTION

Fig. 5. Scheme of the cyclization reaction of CGTase. After the first, bond-cleavage step, a covalent intermediate is formed. In the second step the linear chain first assumes a cyclic conformation, which is the circularization step. Subsequently, a product bond is formed with the terminal 4-OH group of the intermediate. The catalytic residues involved in bond cleavage are Asp229 and Glu257 (the latter is not shown for clarity).

responsible for this product specificity have been identified. This detailed insight now allows the rational construction of mutant CGTase enzymes with desired cyclodextrin product specificity [69].

12. Future prospects

The results from a worldwide research effort to understand the mechanism of action of cyclodextrin glycosyl-

transferase have now yielded insights that can be used in a rational way to tailor CGTases for specific applications [69–71]. Nevertheless, there is much more to understand. Uitdehaag et al. [15,58] obtained structural evidence for three distinctly different conformational states of the CGTase active site in its interaction with substrates, which suggests an induced fit mechanism that could explain the high transglycosylation activity of the enzyme. However, how substrate binding induces these conformational changes, and to what extent and via which mechanism the conformational rearrangements affect activity, is far from understood. Furthermore, the cause of the low hydrolytic activity of CGTase has remained obscure so far, as well as the role of the raw starch binding-sites in the processivity of starch degradation and the regulation of the enzyme's activity [7]. The combined use of in depth enzyme kinetics and mutation studies, together with structural studies and careful reaction pathways analyses, and possibly supplemented by directed evolution approaches [72,73] may be a valuable approach to better understand the catalytic properties of cyclodextrin glycosyltransferase.

References

[1] Stryer L. Biochemistry. 3rd ed. New York: WH Freeman, 1988.

[2] Robyt JF. Essentials of carbohydrate chemistry. In: Cantor CR, editor. Springer advanced texts in chemistry. New York: Springer, 1998.

[3] Svensson B. Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity, and stability. Plant Mol Biol 1994; 25:141–57.

[4] Henrissat B, Davies G. Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 1997;7:637–44.

[5] Pócsi I. Physiological and ecological evaluation of bacterial cyclodextrin glycosyltransferases (CGTases). Biologia 1999;54:603–16.

[6] Pedersen S, Dijkhuizen L, Dijkstra BW, Jensen BF, Jørgensen ST. A better enzyme for cyclodextrins. Chemtech 1995:19–25.

[7] Penninga D, van der Veen BA, Knegtel RMA, van Hijum SAFT, Rozeboom HJ, Kalk KH, Dijkstra BW, Dijkhuizen L. The raw starch binding domain of cyclodextrin glycosyltransferase from Bacillus circulans strain 251. J Biol Chem 1996;271:32777–84.

[8] Matsuura Y, Kusunoki M, Harada W, Kakudo M. Structure and possible catalytic residues of Taka-amylase. J Biochem 1984;95:697–702.

[9] Klein C, Schulz GE. Structure of cyclodextrin glycosyltransferase refined at 2.0 Å resolution. J Mol Biol 1991;217:737–50.

[10] Kubota M, Matsuura Y, Sakai S, Katsube Y. Molecular structure of B. stearothermophilus cyclodextrin glucanotransferase and analysis of substrate binding site. Denpun Kagaku 1991;38:141–6.

[11] Lawson CL, van Montfort R, Strokopytov B, Rozeboom HJ, Kalk KH, de Vries GE, Penninga D, Dijkhuizen L, Dijkstra BW. Nucleotide sequence and X-ray structure of cyclodextrin glycosyltransferase from Bacillus circulans strain 251 in a maltose-dependent crystal form. J Mol Biol 1994;236:590–600.

[12] Knegtel RMA, Wind RD, Rozeboom HJ, Kalk KH, Buitelaar RM, Dijkhuizen L, Dijkstra BW. Crystal structure at 2.3 Å resolution and revised nucleotide sequence of the thermostable cyclodextrin glycosyltransferase from Thermoanaerobacterium thermosulfurigenes EM1. J Mol Biol 1996;256:611–22.

[13] Harata K, Haga K, Nakamura A, Aoyagi M, Yamane K. X-ray structure of cyclodextrin glucanotransferase from alkalophilic Bacillus sp. 1011. Comparison of two independent molecules at 1.8 Å resolution. Acta Crystallogr 1996;D52:1136–45.

[14] Strokopytov B, Knegtel RMA, Penninga D, Rozeboom HJ, Kalk KH, Dijkhuizen L, Dijkstra BW. Structure of cyclodextrin glycosyltransferase complexed with a maltononaose inhibitor at 2.6 Å resolution. Implications for product specificity. Biochemistry 1996;35:4241–9.

[15] Uitdehaag JCM, Kalk KH, van der Veen BA, Dijkhuizen L, Dijkstra BW. The cyclization mechanism of cyclodextrin glycosyltransferase as revealed by a γ-cyclodextrin-CGTase complex at 1.8 Å resolution. J Biol Chem 1999;274:34868–76.

[16] Knegtel RMA, Strokopytov B, Penninga D, Faber OG, Rozeboom HJ, Kalk KH, Dijkhuizen L, Dijkstra BW. Crystallographic studies of the interaction of cyclodextrin glycosyltransferase from Bacillus circulans strain 251 with natural substrates and products. J Biol Chem 1995;270:29256–64.

[17] Søgaard M, Abe J, Martin-Eauclaire MF, Svensson B. α-Amylases: structure and function. Carbohydr Polymers 1993;21:137–46.

[18] Janecek S. α-Amylase family: molecular biology and evolution. Prog Biophys Molec Biol 1997;67:67–97.

[19] Wolfenden R, Lu X, Young G. Spontaneous hydrolysis of glycosides. J Am Chem Soc 1998;120:6814–5.

[20] van der Veen BA, van Alebeek G-JWM, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L. The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from Bacillus circulans (strain 251) proceed via different kinetic mechanisms. Eur J Biochem 2000; 267:658–65.

[21] Davies GJ, Wilson KS, Henrissat B. Nomenclature for sugar-binding subsites in glycosyl hydrolases. Biochem J 1997;321:557–9.

[22] Koshland DE. Stereochemistry and the mechanism of enzymatic reactions. Biol Rev Camb Philos Soc 1953;28:416–36.

[23] Kirby AJ. Mechanism and stereoelectronic effects in the lysozyme reaction. CRC Crit Rev Biochem 1987;22:283–315.

[24] Sinnott ML. Catalytic mechanisms of enzymic glycosyl transfer. Chem Reviews 1990;90:1171–202.

[25] McCarter JD, Adam MJ, Withers SG. Binding energy and catalysis. Biochem J 1992;286:721–7.

[26] White A, Rose DR. Mechanism of catalysis by retaining β-glycosyl hydrolases. Curr Opin Struct Biol 1997;7:645–51.

[27] Heightman TD, Vasella AT. Recent insights into inhibition, structure and mechanism of configuration-retaining glycosidases. Angew Chemie Int Ed 1999;38:750–70.

[28] Ly HD, Withers SG. Mutagenesis of glycosidases. Ann Rev Biochem 1999;68:487–522.

[29] Vocadlo DJ, Davies GJ, Laine R, Withers SG. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. Nature 2001;412:835–8.

[30] Tanaka Y, Wen T, Blanchard JS, Hehre EJ. Transition state structures for the hydrolysis of α-D-glucopyranosyl fluoride by retaining and inverting reactions of glycosylases. J Biol Chem 1994;269:32306–12.

[31] Mosi R, Sham H, Uitdehaag JCM, Ruiterkamp R, Dijkstra BW, Withers SG. Reassessment of acarbose as a transition state analogue inhibitor of cyclodextrin glycosyltransferase. Biochemistry 1998;37: 17192–8.

[32] Kirby AJ. Illuminating the ancient retainer. Nature Struct Biol 1996; 3:107–8.

[33] Blake CCF, Johnson LN, Mair GA, North ACT, Phillips DC, Sarma VR. Crystallographic studies of the activity of hen egg-white lysozyme. Proc R Soc London Ser B 1967;167:378–88.

[34] Kuriki T, Imanaka T. The concept of the α-amylase family: structural similarity and common catalytic mechanism. J Biosci Bioeng 1999; 87:557–65.

[35] Tao BY, Reilly PJ, Robyt JF. Detection of a covalent intermediate in the mechanism of action of porcine pancreatic α-amylase by using ^{13}C nuclear magnetic resonance. Biochim Biophys Acta 1989;995: 214–20.

[36] Mooser G, Hefta SA, Paxton RJ, Shively JE, Lee TD. Isolation and sequence of an active-site peptide containing a catalytic aspartic acid

from two *Streptococcus sobrinus* α-glucosyltransferases. J Biol Chem 1991;266:8916–22.

[37] McCarter JD, Withers SG. 5-Fluoro glycosides: a new class of mechanism-based inhibitors of both α- and β-glucosidases. J Am Chem Soc 1996;118:241–2.

[38] McCarter JD, Withers SG. Unequivocal identification of Asp214 as the catalytic nucleophile of *Saccharomyces cerevisae* α-glucosidase using 5-fluoro glycosyl fluorides. J Biol Chem 1996;271:6889–94.

[39] Braun C, Brayer GD, Withers SG. Mechanism-based inhibition of yeast α-glucosidase and human pancreatic α-amylase by a new class of inhibitors. J Biol Chem 1995;270:26778–81.

[40] Braun C, Lindhorst T, Madsen NB, Withers SG. Identification of Asp549 as the catalytic nucleophile of glycogen-debranching enzyme via trapping of the glycosyl-enzyme intermediate. Biochemistry 1996;35:5458–63.

[41] Mosi R, He S, Uitdehaag J, Dijkstra BW, Withers SG. Trapping and characterization of the reaction intermediate in cyclodextrin glycosyltransferase by use of activated substrates and a mutant enzyme. Biochemistry 1997;36:9927–34.

[42] Uitdehaag JCM, Mosi R, Kalk KH, van der Veen BA, Dijkhuizen L, Withers SG, Dijkstra BW. X-ray structures along the reaction coordinate of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family. Nature Struct Biol 1999;6:432–6.

[43] Sinnott ML. On the antiperiplanar lone pair hypothesis and its application to catalysis by glycosidases. Biochem J 1984;224:817–21.

[44] Carey FA, Sundberg RJ. Advanced organic chemistry part A: structure and mechanism. 3rd ed. New York: Plenum Press, 1990.

[45] Tvaroska I, Carver JP. The anomeric and *exo*-anomeric effects of a hydroxyl group and the stereochemistry of the hemiacetal linkage. Carbohydr Res 1998;309:1–9.

[46] Briggs AJ, Glenn R, Jones PG, Kirby AJ, Ramaswamy P. Bond length and reactivity. Stereoelectronic effects on bonding in acetals and glucosides. J Am Chem Soc 1984;106:6200–6.

[47] White A, Tull D, Johns K, Withers SG, Rose DR. Crystallographic observation of a covalent catalytic intermediate in a β-glycosidase. Nature Struct Biol 1996;3:149–54.

[48] Davies GJ, Tolley SP, Henrissat B, Hjort C, Schülein M. Structures of oligosaccharide-bound forms of the endoglucanase V from *Humicola insolens* at 1.9 Å resolution. Biochemistry 1995;34:16210–20.

[49] Davies G, Mackenzie LF, Varrot A, Dauter M, Brzozowski AM, Schülein M, Withers SG. Snapshots along an enzymatic reaction coordinate: analysis of a retaining β-glycoside hydrolase. Biochemistry 1998;37:11707–13.

[50] Tews I, Terwisscha van Scheltinga AC, Perrakis A, Wilson KS, Dijkstra BW. Substrate assisted catalysis unifies two families of chitinolytic enzymes. J Am Chem Soc 1997;119:7954–9.

[51] O'Reilly M, Watson KA, Johnson LN. The crystal structure of the *Escherichia coli* maltodextrin phosphorylase-acarbose complex. Biochemistry 1999;38:5337–45.

[52] Varrot A, Schülein M, Pipelier M, Vasella A, Davies GJ. Lateral protonation of a glycosidase inhibitor. Structure of the *Bacillus agaradhaerens* Cel5A in complex with a cellobiose-derived imidazole at 0.97 Å resolution. J Am Chem Soc 1999;121:2621–2.

[53] Strokopytov B, Penninga D, Rozeboom HJ, Kalk KH, Dijkhuizen L, Dijkstra BW. X-ray structure of cyclodextrin glycosyltransferase complexed with acarbose. Implications for the catalytic mechanism of glycosidases. Biochemistry 1995;34:2234–40.

[54] Brzozowski AM, Davies GJ. Structure of the *Aspergillus oryzae* α-amylase complexed with the inhibitor acarbose at 2.0 Å resolution. Biochemistry 1997;36:10837–45.

[55] Søgaard M, Kadziola A, Haser R, Svensson B. Site-directed mutagenesis of histidine 93, aspartic acid 180, glutamic acid 205, histidine 290, and aspartic acid 291 at the active site and tryptophan 279 at the raw starch binding site in barley α-amylase. J Biol Chem 1993;268:22480–4.

[56] Nakamura A, Haga K, Yamane K. Three histidine residues in the active center of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011: effects of the replacement on pH dependence and transition-state stabilization. Biochemistry 1993;32:6624–31.

[57] Qian M, Haser R, Buisson G, Duée E, Payan F. The active center of a mammalian α-amylase. Structure of the complex of pancreatic α-amylase with a carbohydrate inhibitor refined to 2.2-Å resolution. Biochemistry 1994;33:6284–94.

[58] Uitdehaag JCM, van Alebeek GJWM, van der Veen BA, Dijkhuizen L, Dijkstra BW. Structures of maltohexaose and maltoheptaose bound at the donor sites of cyclodextrin glycosyltransferase give insight into the mechanisms of transglycosylation activity and cyclodextrin size specificity. Biochemistry 2000;39:7772–80.

[59] Bender H. Studies of the mechanism of the cyclization reaction catalysed by the wildtype and a truncated α-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* strain M 5 al, and the β-cyclodextrin glycosyltransferase from *Bacillus circulans* strain 8. Carbohydr Res 1990;206:257–67.

[60] Parsiegla G, Schmidt AK, Schulz GE. Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the γ-cyclodextrin production. Eur J Biochem 1998;255:710–7.

[61] van der Veen BA, Uitdehaag JCM, Penninga D, van Alebeek G-JWM, Smith LM, Dijkstra BW, Dijkhuizen L. Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase α-cyclodextrin production. J Mol Biol 2000;296:1027–38.

[62] Uitdehaag JCM, van der Veen BA, Dijkhuizen L, Elber R, Dijkstra BW. Enzymatic circularization of a malto-octaose linear chain studied by stochastic path calculations on cyclodextrin glycosyltransferase. Proteins: Struct Funct Genet 2001;43:327–35.

[63] Sin K-A, Nakamura A, Masaki H, Matsuura Y, Uozumi T. Replacement of an amino acid residue of cyclodextrin glucanotransferase of *Bacillus ohbensis* doubles the production of γ-cyclodextrin. J Biotech 1994;32:283–8.

[64] Fujiwara S, Kakihara H, Sakaguchi K, Imanaka T. Analysis of mutations in cyclodextrin glucanotransferase from *Bacillus stearothermophilus* which affect cyclization characteristics and thermostability. J Bacteriol 1992;174:7478–81.

[65] Penninga D, Strokopytov B, Rozeboom HJ, Lawson CL, Dijkstra BW, Bergsma J, Dijkhuizen L. Site directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 affect activity and product specificity. Biochemistry 1995;34:3368–76.

[66] van der Veen BA, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L. 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. Eur J Biochem 2000;267:3432–41.

[67] Wind RD, Uitdehaag JCM, Buitelaar RM, Dijkstra BW, Dijkhuizen L. Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. J Biol Chem 1998;273:5771–9.

[68] Kim YH, Bae KH, Kim TJ, Park KH, Lee HS, Byun SM. Effect on product specificity of cyclodextrin glycosyltransferase by site-directed mutagenesis. Biochem and Mol Biol Int 1997;41:227–34.

[69] Dijkhuizen L, Dijkstra BW, Andersen C, von der Osten C. Cyclomaltodextrin glucanotransferase variants, in PCT international application PCT/DK96/00179. 1996.

[70] van der Veen BA, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L. Engineering of cyclodextrin glycosyltransferase reaction and product specificity. Biochim Biophys Acta 2000;1543:336–60.

[71] van der Veen BA, Leemhuis H, Kralj S, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L. Hydrophobic amino acid residues in the acceptor binding site are main determinants for reaction mechanism and specificity of cyclodextrin glycosyltransferase. J Biol Chem 2001;276 (in press).

[72] Reetz MT, Jaeger K-E. Superior biocatalysts by directed evolution. Topics Curr Chem 1999;200:32–54.

[73] Liebeton K, Zonta A, Schimossek K, Nardini M, Lang D, Dijkstra BW, Reetz MT, Jaeger K-E. Directed evolution of an enantioselective lipase. Chem & Biol 2000;7:709–18.

[74] Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 1991;24:946–50.