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Review

Engineering of cyclodextrin glycosyltransferase reaction and product specificity[1]

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1. Introduction

Many plants produce starch, a high molecular weight polymer of glucose, for storage as a carbon and energy source. These starch molecules are mostly found in seeds (e.g., wheats) or roots (e.g., potato) in the form of granules consisting of two types of glucan polymers: highly branched amylopectin and linear amylose. Many bacteria are able to use starch as a carbon and energy source for growth. For this purpose these micro-organisms convert starch molecules extracellularly into molecules suitable for uptake and further conversion by the cells. A whole range of starch-degrading enzymes with different reaction specificities has evolved in these organisms yielding a wide variety of products. A number of these enzymes find application in the industrial processing of starch, either for modification of starch molecules or for the production of specific degradation products. These enzymes are therefore studied extensively, leading to increasing knowledge of their reaction mechanisms and factors determining substrate and product specificity. A particularly interesting enzyme is cyclodextrin glycosyltransferase (CGTase), which has the unique capability of forming cyclodextrins from starch. This enzyme is a member of the α-amylase family (family 13) of glycosyl hydrolases. Current insights in the catalytic mechanism employed by these enzymes is discussed. Emphasis in this review is on structural and mechanistic features of CGTase determining cyclodextrin product specificity.

2. Industrial applications of CGTase

CGTase enzymes are able to produce cyclodextrins from starch via the cyclization reaction (see Section 4.1), which is the basis of their industrial application. Recent developments, however, also concentrate on the use of the CGTase catalysed coupling and disproportionation reactions (see Section 4.1) for the

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synthesis of modified oligosaccharides by using alternative acceptor substrates. Furthermore, applications of CGTase limit dextrins are being explored.

2.1. Applications of cyclodextrins

Cyclodextrins are cyclic α(1–4) linked oligosaccharides mainly consisting of 6, 7, or 8 glucose residues (α-, β, or γ-cyclodextrin, respectively (Fig. 1a)). The glucose residues in the cyclodextrin rings are arranged in such manner that the secondary hydroxyl-groups (C2 and C3) are located on one edge of the ring and the primary hydroxyl-groups (C6) on the other edge, resulting in torus shaped molecules (Fig. 1b). The apolar C3 and C5 hydrogens and ether-like oxygens are at the inside and the hydroxyl-groups at the outside of these molecules. This results in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix, enabling cyclodextrins to form inclusion complexes with a wide variety of hydrophobic guest molecules (Fig. 1c). Their three-dimensional form and size (Table 1) provide an important parameter for complex formation with hydrophobic compounds or functional groups. Thus specific (α-, β-, or γ-) cyclodextrins are required for complexation of specific guest molecules. The driving force of inclusion complex formation is the entropic effect of displacement of water molecules from the hydrophobic environment of the cavity, probably combined with the fact that this water causes strain on the cyclodextrin ring, which is released after complexation, producing a more stable, lower energy state [1,2]. Because the inclusion complexes are quite stable they can be separated from the medium by crystallization [3].

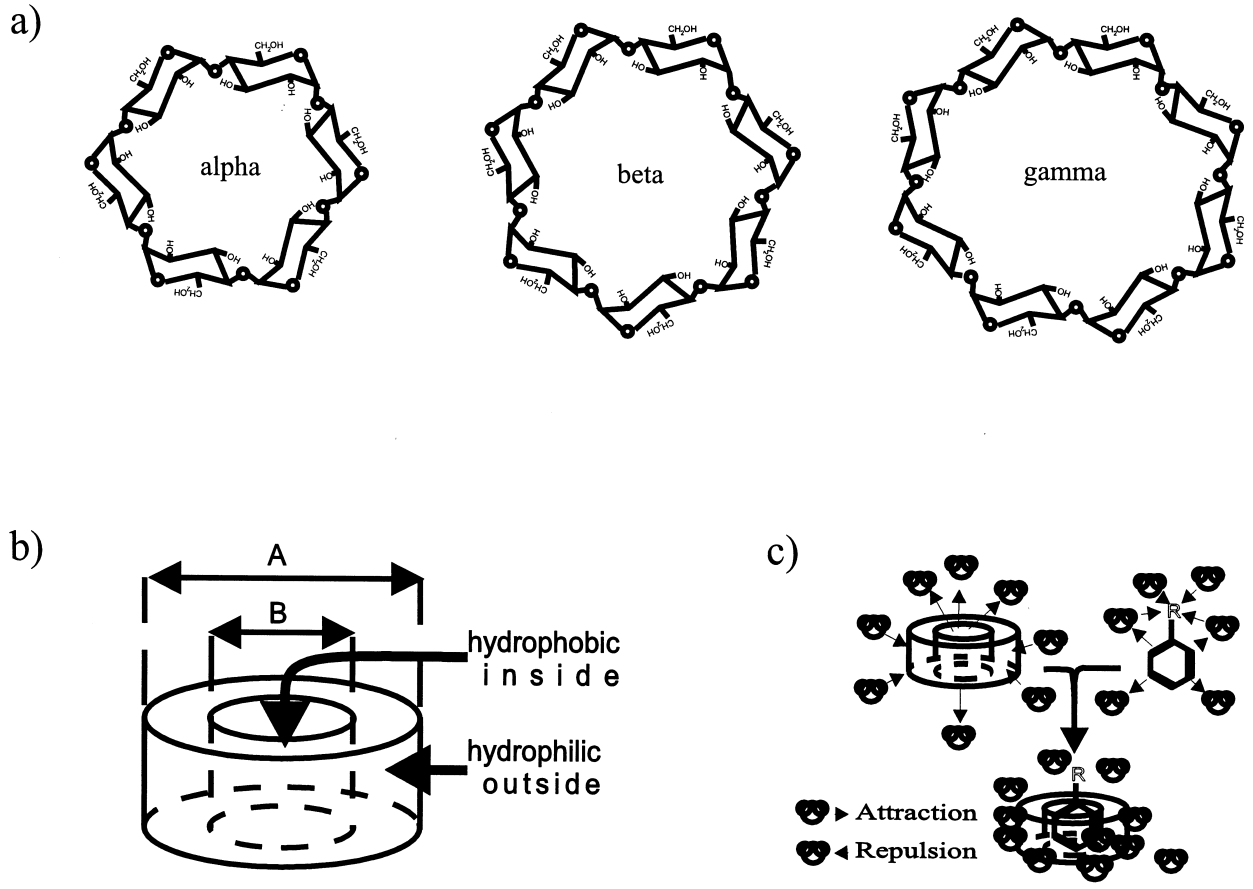

Fig. 1. Structure and properties of cyclodextrins. (a) α-, β-, and γ-cyclodextrins; (b) three-dimensional form and properties of cyclodextrins (for sizes of A and B, see Table 1); (c) formation of inclusion complex of a cyclodextrin with a hydrophobic molecule. (Reproduced from Penninga [139].)

Table 1
Cyclodextrin properties

	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25°C (%w/v)	14.5	1.85	23.2
Outer diameter (A) (Å)	14.6	15.4	17.5
Inner diameter (B) (Å)	4.7–5.3	6.0–6.5	7.5–8.3
Height of torus (Å)	7.9	7.9	7.9
Approx. cavity volume ($Å^3$)	174	262	427

Values from Uekama and Irie [120] and Szejtli [121]. Outer (A) and inner (B) diameter are indicated in Fig. 1.

The formation of inclusion complexes leads to changes in the chemical and physical properties of the guest molecules (Table 2). These altered characteristics of encapsulated compounds have led to various applications of cyclodextrins (or their derivatives) in analytical chemistry [4,5], agriculture [1,6], biotechnology [7,8], pharmacy [9,10], food [7,11], and cosmetics [7].

2.2. Other CGTase applications

In addition to production of cyclodextrins through the cyclization reaction, CGTase can be used for it's coupling and disproportionation reactions (see Section 4.1) for the transfer of oligosaccharides from donor substrates such as cyclodextrins or starch to various acceptor molecules. Increasingly, the use of alternative acceptors is reported, resulting in novel glycosylated compounds [12–14]. A commercial application of this method is found in glycosylation of the intense sweetener, stevioside. This bitter compound is isolated from the leaves of the plant *Stevia rebaudiana* and has a low solubility. Glycosylation decreases bitterness and increases solubility [15].

Other applications are found for the CGTase limit dextrins. Due to the inability of CGTase to bypass α(1–6) bonds in gelatinized starches, degradation of these substrates leads to a reduction in viscosity without a corresponding decrease of the high-molecular character of starch. This CGTase limit dextrin is applied in processes for surface sizing or coating of paper, to improve the writing quality of the paper and to obtain a glossy and well printable surface. The coating or sizing liquid contains converted starch which has been obtained by treating gelatinized starch with a starch-converting enzyme selected from the group of the CGTases and the branching enzymes [16].

CGTases can also be used in the preparation of doughs for baked products which comprises incorporation of the CGTase into the dough to increase the volume of the baked product [17].

Table 2
Possible effects of the formation of inclusion complexes on properties of the guest molecules

Stabilization of light- or oxygen-sensitive compounds
Stabilization of volatile compounds
Alteration of chemical reactivity
Improvement of solubility
Improvement of smell and taste
Modification of liquid compounds to powders

2.3. CGTase properties limiting its industrial application

Like most starch-degrading enzymes, the CGTase from *Bacillus macerans*, which is used for the commercial production of cyclodextrins [18], is poorly active on native starch due to the well organized structure of the granules held together by internal hydrogen bonds. Heating in water (jet cooking) weakens these hydrogen bonds and causes swelling and gelatinization [19], resulting in a very viscous starch solution when performed at starch concentrations of industrial interest. Therefore, in this initial processing step, operating at temperatures up to 105–110°C, an α-amylase is added in order to liquefy the starch to make it suitable for incubation at the lower temperatures (55°C) required for the CGTase catalysed production of cyclodextrins. Unfortunately, the α-amylase used for liquefaction produces maltodex-

trins, which will act as acceptor molecules in the coupling reaction catalysed by CGTase, severely reducing cyclodextrin yields [15]. More recently, very thermostable CGTases have been characterized from thermophilic anaerobic bacteria belonging to the genera *Thermoanaerobacter* [3,20] and *Thermoanaerobacterium* [21]. These CGTases are active and stable at high temperatures and low pH values, and are able to solubilize starch, thereby eliminating the need for α-amylase pretreatment, without any traces of low molecular weight oligosaccharides produced in the initial stages of the reaction [22]. The use of these thermostable CGTases has the added advantage that the total cyclodextrin production time can be shortened [15]. The *Thermoanaerobacter* CGTase (maximal activities at 90°C and pH 5.8) has found commercial application in 1996. Still there are high energy costs for solubilization of starch, together with viscosity problems. These can be overcome by using enzymes active on raw starch granules [23]. Screening efforts thus far has yielded two different mesophilic micro-organisms capable of growth on native potato starch granules, a *Microbacterium* species and a *Bacillus firmus/lentus* strain, producing native starch-degrading amylases [23]. More recently a CGTase active on native wheat starch has been isolated from a *B. firmus* strain [24].

A major disadvantage of cyclodextrin production by CGTases is that all known wild-type CGTase enzymes produce a mixture of α-, β-, and γ-cyclodextrin and are sensitive to product inhibition by these cyclic compounds. The *Thermoanaerobacter* CGTase for instance produces an approximately equal mixture of α- and β-cyclodextrin with a small amount of γ-cyclodextrin. Two different industrial approaches are used to purify the produced cyclodextrins: selective crystallization of β-cyclodextrin (which is relatively poorly water-soluble) and selective complexation with organic solvents. These processes not only serve to purify the cyclodextrins, but also result in decreased product inhibition, enhancing the total conversion of starch from 40% to 60% [25]. Toluene and cyclohexane are commercially used for the complexation and selective precipitation of β-cyclodextrin. For α-cyclodextrin 1-decanol can be used, but this compound is difficult to remove from aqueous solutions because of its high boiling point (229°C). Cyclododecanone can be used for complexation and selective precipitation of γ-cyclodextrin, but this solvent is too expensive for commercial use. Further disadvantages of the use of organic solvents are their toxicity, their flammability, and the need for a solvent recovery process [15]. The availability of α- and γ-cyclodextrins is thus rather limited at present; consequently, there is a great demand for a process that could produce these cyclodextrins economically. Also the processes used for β-cyclodextrin production are not ideal, since they make the production of cyclodextrin too costly for many applications, and the use of organic solvents limits applications involving human consumption.

Clearly, the availability of CGTase enzymes capable of producing an increased ratio of one particular type of cyclodextrin and with reduced product inhibition would help to avoid the above described expensive and environmentally harmful procedures involving organic solvents. This situation has strongly stimulated studies of CGTase structure–function relationships, with the mechanisms of the CGTase catalysed reactions and inhibition by the cyclodextrin products as important research topics. In recent years detailed knowledge has become available, allowing rational design of mutant CGTase biocatalysts with improved cyclodextrin product specificity and reduced product inhibition [15,26,27].

3. The α-amylase family (family 13) of glycosyl hydrolases

CGTase is a member of the α-amylase family of glycosyl hydrolases (family 13), an important group of starch converting enzymes. Enzymes belonging to this group show a wide diversity in reaction specificities, and many of these enzymes are active on starch. Whereas amylases generally hydrolyse glycosidic bonds in the starch molecules, CGTases mainly catalyse transglycosylation reactions, with hydrolysis being a minor activity [28]. Structure/function relationships in the α-amylase family have been studied extensively and may help to clarify the mechanistic basis of the unique activities of CGTase.

3.1. Starch-degrading enzymes

Fig. 2 shows an overview of enzymes active on

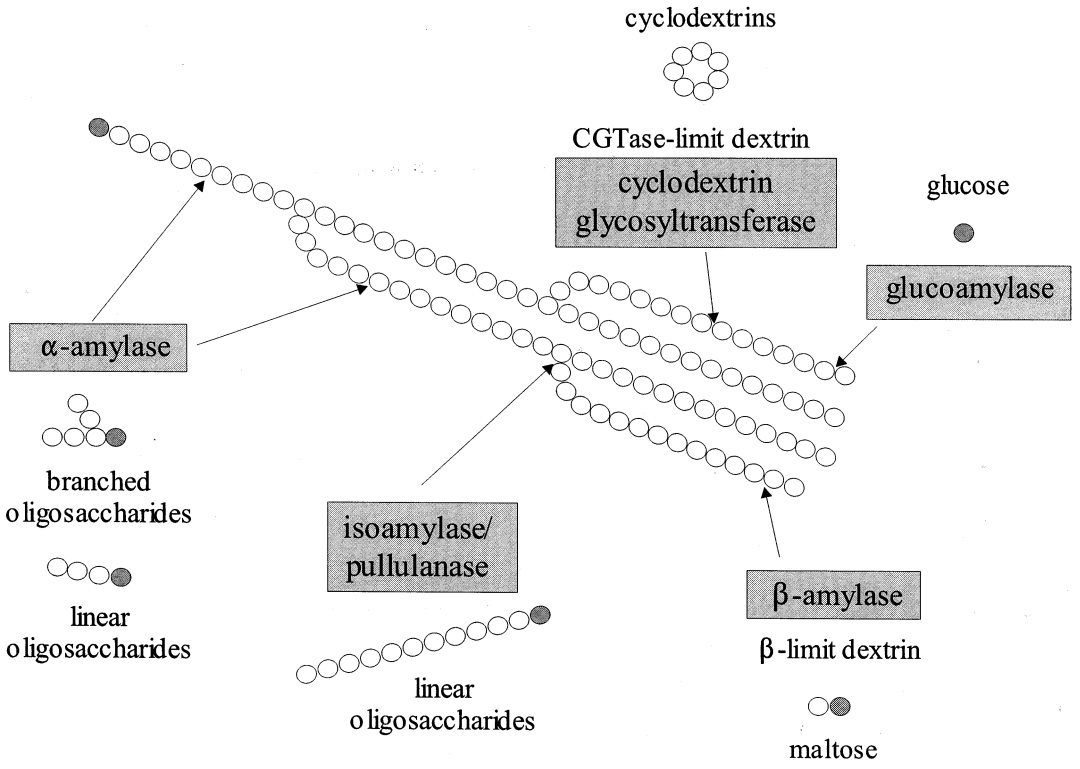

Fig. 2. Action of enzymes involved in the degradation of starch. (●) Glucose molecule with a reducing end; (○) glucose molecule without a reducing end. Arrows indicate preferred cleaving points in the starch molecule. (Reproduced from Wind [140], with modifications.)

starch (amylopectin in this case). Many starch-degrading enzymes are hydrolytic, cleaving the linkages in the starch molecule followed by the reaction of the cleavage product with water, resulting in a new reducing end. These can be roughly divided into amylases, hydrolysing α(1–4) linkages, and debranching enzymes, hydrolysing α(1–6) linkages.

Examples of debranching enzymes are isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41). Isoamylase specifically cleaves α(1–6) linkages in amylopectin and branched dextrins. Pullulanase hydrolyses α(1–6) linkages in pullulan, which is a linear α-glucan consisting of maltotriose units joined by α(1–6) glycosidic linkages, but is also capable of cleaving the α(1–6) linkages in amylopectin. Amylopullulanase (pullulanase type II) hydrolyses both α(1–4) and α(1–6) linkages. These different activities can be catalysed by one active site, as has been shown for the amylopullulanase from *Thermoanaerobacter ethanolicus* 39E, where individual replacements of two catalytic carboxylic amino acids by their amide forms resulted in loss of both activities [29]. The amylopullulanase from alkalophilic *Bacillus* sp. KSM-1378, however, contains two independent active sites [30,31] and the specific hydrolytic activities can be separated by limited proteolysis with papain, yielding two protein fragments of which one has the amylase and the other the pullulanase characteristics (including pH and temperature profiles) of the parental enzyme [32].

Amylases can be further subdivided into endo- and exo-acting enzymes. A typical endo-acting enzyme is α-amylase (EC 3.2.1.1), cleaving α(1–4) bonds randomly in the starch molecule, producing (branched) oligosaccharides of various lengths. Exo-acting amylases such as β-amylase (EC 3.2.1.2, family 15 of glycosyl hydrolases) cleave α(1–4) bonds at the non-reducing end of the starch molecule and hence produce only low molecular weight products from starch (mostly glucose or maltose). Most of these enzymes are incapable of bypassing α(1–6) linkages; degradation of branched substrates therefore remains

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          132        140   223          233   253        260   324        332
CGT         VIIDFAPNH       IDGIRMDAVKH       FTFGEWFL       IDNHDMERF
TAA         LMVDVVANH       IDGLRIDTVKH       YCIGEVLD       VENHDNPRF
CD          VMLDAVFNH       IDGWRLDVANE       YILGEIWH       LESHDTSRL
PUL         VIMDVVYNH       IDGFRFDLMGY       YFFGEGWD       VSKHDNQTL
ISO         VYMDVVYNH       VDGFRFDLASV       DLFAEPWA       IDVHDGMTL
            ...* . **       .**.*.*..           ..* .        .. **   :
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Fig. 3. Amino acid sequence alignment of the four conserved regions for diverse members of the α-amylase family. TAA, α-amylase from *Aspergillus oryzae* (Taka-amylase A) [39]; CGT, CGTase from *Bacillus circulans* strain 251 [44]; CD, cyclodextrinase from *Klebsiella oxytoca* [141]; PUL, pullulanase from *Klebsiella aerogenes* [142]; ISO, isoamylase from *Pseudomonas amyloderamosa* [143]. The residues are numbered according to the CGTase from *B. circulans* strain 251. An asterisk indicates amino acid identity, a dot indicates amino acid similarity.

incomplete, leaving high molecular weight compounds (limit dextrins). Some, however, are also able to cleave α(1–6) linkages, for instance glucoamylase (EC 3.2.1.3, family 14 of glycosyl hydrolases) and α-glucosidase (EC 3.2.1.20), but this reaction is slow compared to the hydrolysis of α(1–4) bonds.

Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) is a unique member of the α-amylase family of glycosyl hydrolases with a low hydrolytic activity. CGTase mainly catalyses transglycosylation reactions and is usually considered to be an exo-acting enzyme, unable to bypass branching points. Its main products when acting on starch are cyclodextrins and highly branched high molecular weight dextrins (CGTase limit dextrins).

Other members of the α-family catalysing mainly transglycosylation reactions are branching enzyme (EC 2.4.1.18) [33], cleaving α(1–4) bonds and forming α(1–6) bonds, amylomaltase (EC 2.4.1.25) [34,35], cleaving α(1–4) bonds and forming α(1–4) bonds, and amylosucrase (EC 2.4.1.4) [36,37], cleaving the bond between glucose and fructose in sucrose molecules and coupling the glucose moiety via an α(1–4) linkage to a growing oligosaccharide chain. These enzymes, however, are not directly involved in starch degradation, and show less homology to CGTase than α-amylase does. They will therefore not be fully discussed here.

3.2. Sequence similarities in the α-amylase family

Although the overall sequence similarity within the α-amylase family of glycosyl hydrolases (family 13) is relatively low ($< 30\%$), four highly conserved regions have been identified in α-amylases by Nakajima et al. [38]. These regions were found to be also present in other members of the α-amylase family; α-glucosidases, pullulanases, isoamylases and CGTases [34]. An amino acid sequence alignment showing these four conserved regions for diverse members of the α-amylase family is presented in Fig. 3. All four regions contain completely invariant amino acid residues within the α-amylase family and the functions of most of these have been elucidated by X-ray crystallography, site-directed mutagenesis, and chemical modification of various members of this family. These residues are directly involved in catalysis, either through substrate binding, bond cleavage, transition state stabilization, or as ligands of a calcium binding site present near the active site. Three carboxylic acid groups, one glutamic acid and two aspartic acid residues, were found to be essential for catalytic activity in α-amylases and CGTases. The amino acids are equivalent to Asp206, Glu230, and Asp297 in α-amylase from *Aspergillus oryzae* [39] and Asp229, Glu257 and Asp328 in CGTase from *Bacillus circulans* [40,41]. Two conserved histidine residues, His140 and His327 (CGTase numbering), are involved in substrate binding and transition state stabilization [42,43]. A third histidine, present only in some α-amylases and CGTase (His233, CGTase numbering), is involved in substrate binding and acts as a calcium-ligand with its carbonyl oxygen [44,45]. Arg227 is important for the orientation of the nucleophile (Asp229, see below) [43]. The role of Asp135 is not clear, but it is in close proximity of the catalytic site. Asn139 again is a calcium-ligand. The importance of the calcium binding site is illustrated by the identification of a fifth conserved region in α-amylases [46] and, more recently, in several other members of the α-amylase family [47]. This region consists of the stretch 197-LADLN in CGTase from *B. circulans* strain 251 (173-LPDLD

in the α-amylase from *A. oryzae*) and contains the calcium-ligand Asp199.

3.3. Catalytic mechanism of the α-amylase family

The reactions catalysed by the enzymes belonging to the α-amylase family proceed with retention of the substrate's anomeric (α-) configuration. Since each substitution at a chiral centre results in inversion of configuration, catalysis must proceed through a double displacement reaction (Fig. 4) [48]. The first step involves a protonation of the glycosidic oxygen by a general acid catalyst, creating an oxo-carbonium transition state which subsequently collapses into an intermediate [49,50]. This intermediate is attacked by a water nucleophile (or the C4-OH at the non-reducing end of another oligosaccharide in case of transglycosylases (e.g., CGTase)) in the second step, assisted by the base form of the acid catalyst. The roles of the three carboxylic amino acids in this mechanism has been clarified by X-ray crystallographic studies on α-amylase [51] and CGTase [41] with acarbose, a potent pseudotetraose inhibitor, bound in the active site. Glu257 (CGTase numbering) is the general acid catalyst, acting as proton donor; Asp229 serves as the nucleophile, stabilizing

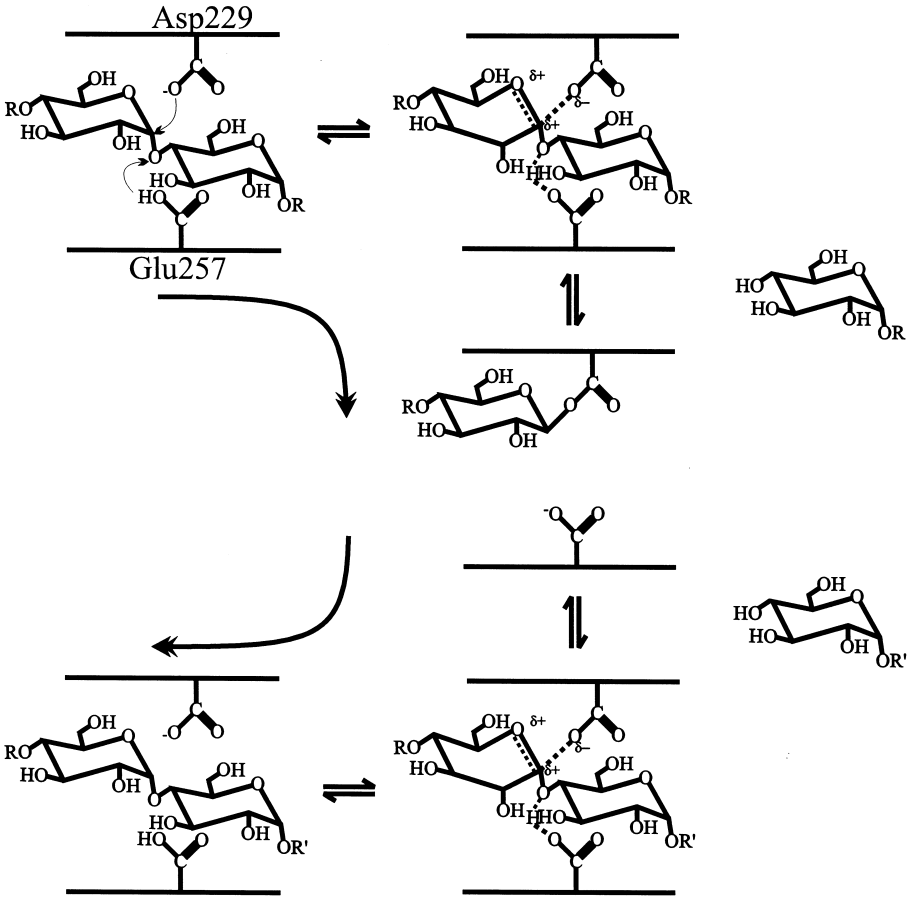

Fig. 4. Reaction mechanism of family 13 glycosyl hydrolases as revealed by X-ray crystallographic studies of CGTase. Retaining enzymes act via a double displacement mechanism. The first step involves a protonation of the glycosidic oxygen by a general acid catalyst (Glu257 in CGTase), creating an oxo-carbonium transition state which subsequently collapses into an intermediate covalently linked to the nucleophile (Asp229 in CGTase). This intermediate is attacked by the C4-OH at the non-reducing end of another oligosaccharide (or a water nucleophile in case of hydrolysis) in the second step, assisted by the base form of the acid catalyst. (Reproduced from Uitdehaag et al. [43].)

the intermediate, and Asp328 has an important role in substrate binding. For retaining enzymes the intermediate could either be an oxo-carbonium ion which is electrostatically stabilized by a carboxylate, or involves formation of a covalent bond, in which one of the catalytic aspartates is presumed to act as a nucleophile (see Fig. 4). Although initially the nature of the intermediate was disputed, it is now generally accepted that the reaction proceeds via a covalent intermediate. Clear evidence for a covalent glycosyl–enzyme intermediate in family 13 has been obtained from rapid trapping studies with natural substrates. Low-temperature ^{13}C NMR experiments have provided evidence for the formation of a β-carboxylacetal ester covalent adduct between maltotetraose and porcine pancreas α-amylase [52]. Conclusive evidence recently came from experiments involving trapping of a covalent intermediate with 4-deoxymaltotriosyl α-fluoride as a substrate in the virtually inactive Glu257Gln mutant of *B. circulans* 251 CGTase [53] and elucidation of the X-ray crystallographic structure of the enzyme with the covalently linked intermediate [43].

3.4. Three-dimensional structure similarities in the α-amylase family

In contrast to a limited similarity in primary structure (< 30%), the three-dimensional structures of α-amylases [39,51,54–59] and CGTases [44,60–63] are quite similar. α-Amylases generally consist of three structural domains, A, B, and C, while CGTases show a similar domain organization with two additional domains, D and E (see Fig. 5). Domain A contains a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight α-helices. This so-called $(\beta/\alpha)_8$- or TIM-barrel catalytic domain [64] of 300–400 residues is present in all enzymes of the α-amylase family. The $(\beta/\alpha)_8$- barrel was first found in the structure of chicken muscle triose-phosphate isomerase (TIM) [65], but it has been shown to be very wide-spread in functionally diverse enzymes [66]. Several prolines and glycines flanking loops connecting the β-strands and α-helices have been found to be highly conserved in these enzymes [67]. The catalytic and substrate binding residues conserved in the α-amylase family are located in loops at the C-termini of β-strands in domain A. The

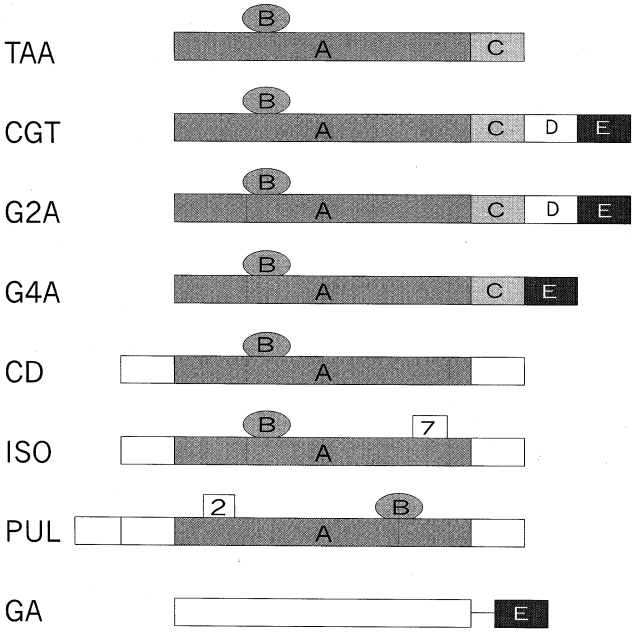

Fig. 5. Domain level organization of starch-degrading enzymes. CGT, CGTase from *B. circulans*; G2A, maltogenic α-amylase from *Bacillus stearothermophilus*; G4A, maltotetraose forming α-amylase from *Pseudomonas stutzeri*; TAA, α-amylase from *A. oryzae* (Taka-amylase A); CD, cyclodextrinase from *Klebsiella oxytoca*; ISO, isoamylase from *P. amyloderamosa*; PUL, pullulanase from *K. aerogenes*; GA, glucoamylase (family 15 of glycosyl hydrolases) from *Aspergillus niger*. (Reproduced from Jespersen et al. [69] with modifications.)

loop between β-strand 3 and α-helix 3 of the catalytic domain is rather large and is regarded as a separate structural domain. This B-domain consists of 44–133 amino acid residues and contributes to substrate binding. The C-domain is approximately 100 amino acids long and has an antiparallel β-sandwich fold. Domain C of the CGTase from *B. circulans* strain 251 contains one of the maltose binding sites observed in the structure derived from maltose dependent crystals [44]. This maltose binding site was found to be involved in raw starch binding [68], suggesting a role of the C-domain in substrate binding. Some authors suggest that this domain is involved in bond specificity, since in enzymes hydrolysing or forming α-1,6-bonds (e.g., pullulanase, isoamylase, branching enzyme) the A-domain is followed by a different domain (see Fig. 5)[69]. The D-domain, consisting of approximately 90 amino acids with an immunoglobulin fold, is almost exclusively found in CGTases and has an unknown function. The E-do-

main, following the D-domain in CGTases is more widespread in starch-degrading enzymes. Besides in the α-amylase family, where it is found as the C-terminal domain when present, it is also found in glucoamylases (family 15 of glycosyl hydrolases), where it is attached to the C- or N-terminus of the catalytic domain via a glycosylated linker (see Fig. 5). The E-domain consists of approximately 110 amino acids and was found to be responsible for the adsorption onto granular starch (see below).

3.5. Substrate binding of (α-)amylases and CGTases

The first important step in enzyme catalysis is binding of the substrate. In several starch-degrading enzymes a separate domain responsible for absorption unto raw starch has been found. For *Aspergillus niger* two forms of glucoamylase (GA) have been described. GAI is 114 amino acids longer than GAII and was found to contain an additional C-terminal domain required for binding raw starch [70]. Fusion of the corresponding domain of the *Aspergillus awamori* glucoamylase to the C-terminus of β-galactosidase resulted in a protein with binding affinity for corn starch and cross-linked amylose [71] Sequence comparisons between the E-domain of several amylases and CGTases and the raw starch binding domain from glucoamylases revealed the presence of this domain in various starch-degrading enzymes [72]. Later, evidence for a starch binding site in CGTases separate from the active site was presented [73] and fusion of the E-domain of the *B. macerans* CGTase to β-galactosidase demonstrated that it can indeed function as a starch binding domain [71]. Studies on the CGTase from *B. circulans* strain 251 have revealed the function of the E-domain in more detail. High concentrations of maltose are required for crystallization of this CGTase [74]. Three maltose binding sites (MBS) were observed at the protein surface (Fig. 6), two of which (MBS1 and MBS3) contribute to intermolecular crystal contacts. MBS1 and MBS2 are both located on the E-domain, suggesting a role in the raw starch binding function of this domain [44]. Indeed, mutational studies revealed that maltose binding site 1 is important for (raw) starch binding, while maltose binding site 2 assists

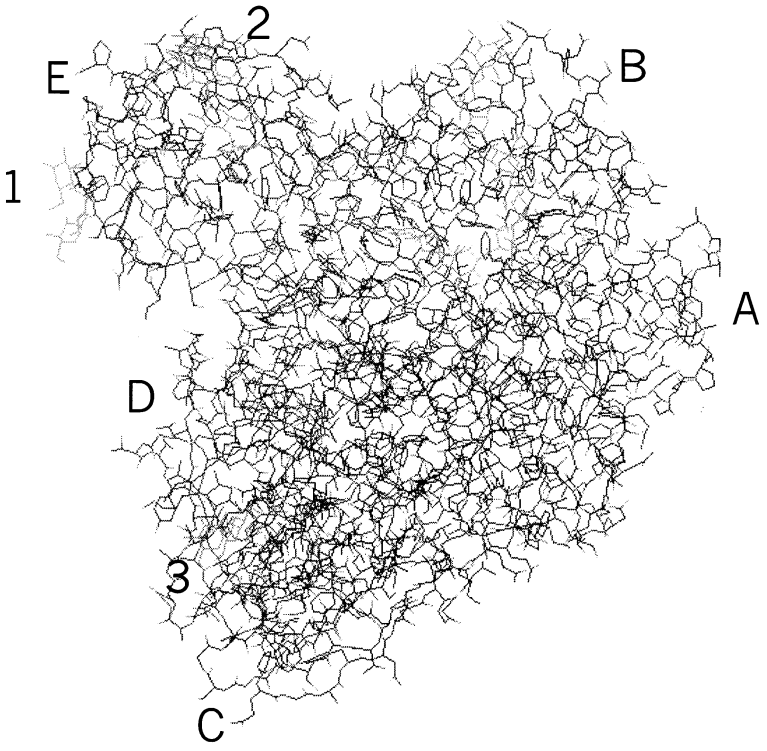

Fig. 6. Structure of the *B. circulans* strain 251 CGTase. The different domains (A–E) and maltose binding sites (1–3) are indicated.

in guiding the linear starch chains into the active site via a groove at the surface of the CGTase protein (see below) [44,68]. The maltose binding sites on the CGTase E-domain were found to interact strongly with cyclodextrins and oligosaccharides [75]. Also in the raw starch binding domain of glucoamylase from *A. niger* two sites interacting with maltoheptaose and β-cyclodextrin, similar to the maltose binding sites in the E-domain of CGTase, have been identified [76,77]. Further experiments showed that also the roles of these binding sites are similar to those of CGTase [78,79]. Recent studies revealed that the raw starch binding domain has an additional function in the disruption of the structure of granular starch [80].

As mentioned above, the A-domain contains the catalytic residues of α-amylases and CGTases, while domain B is involved in substrate binding. X-ray crystallographic studies have revealed a groove on the surface of these enzymes formed on one side by loops of the A-domain and on the other side by the B-domain. In crystal structures from pig pancreatic α-amylase (PPA) [81] and CGTase from *B. circulans* strain 251 [44], where maltose molecules serve as contact points between the enzyme molecules in the crystals, the functionality of this groove in substrate binding has been nicely shown. In PPA the maltose is bound at one end of the groove and the contact point in the crystal is formed by interactions of this maltose with the other end of the groove of the neighbouring enzyme molecule [81]. Soaking of these crystals with α-cyclodextrin revealed three binding sites for this cyclic compound. The first α-cyclodextrin replaces the maltose serving as the contact point between the amylase molecules. The second binding site was found in the middle of the groove, in close proximity to the catalytic residues. The third α-cyclodextrin is further removed from the substrate binding groove and bound in a slight depression formed by an edge of the B-domain and the first turn of α-helix 3 of the A-domain. Interestingly, the depression in which the third α-cyclodextrin binds in PPA [81] corresponds to a region in CGTase which is involved in interactions between the catalytic domain (A) and the starch binding domain (E) [63]. From soaking experiments with the CGTase from *B. circulans* strain 251 the structure of this enzyme with a maltononaose inhibitor was obtained (Fig. 7), revealing in more detail the mode of substrate binding in the groove [45]. The substrate binding sites are numbered +2 to −7 (numbering according to Davies et al. [82]), with the catalytic site between subsites +1 and −1. The non-reducing end of the oligosaccharide is bound at subsite −7, which agrees with the formation of mainly β-cyclodextrin from starch by the enzyme. The glucose residue at subsite −7 is located at the end of the substrate binding groove, interacting with amino acid residues of the B-domain. These residues correspond to the region in PPA where the first α-cyclodextrin, bound to the beginning of the groove in one enzyme molecule, interacts with the end of the groove in the neighbouring molecule. The glucose residues bound at subsites +1 and −1

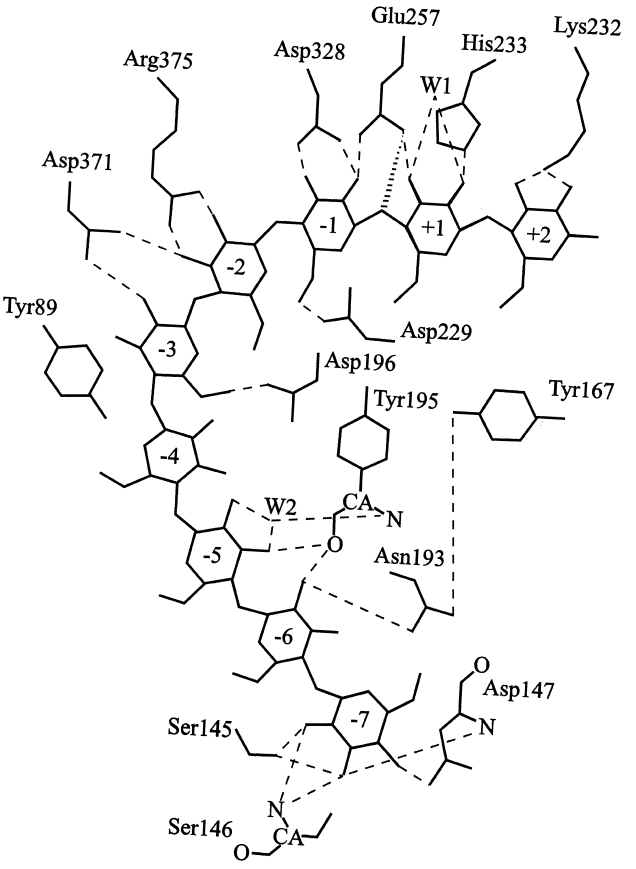

Fig. 7. Schematic representation of the hydrogen bonds between the *B. circulans* strain 251 CGTase and a maltononaose inhibitor bound at the active site. The subsites are numbered according to the general subsite labelling scheme recently proposed for all glycosyl hydrolases [82]. (Reproduced from Strokopytov et al. [45], with modifications.)

only interact with amino acid residues conserved in the whole α-amylase family. Thus beside the catalytic mechanism and the global 3-D structure also the substrate binding groove appears to be well conserved in α-amylases and CGTases.

3.6. Product specificity of α-amylases and CGTases

Individual glycosyl residues of an oligosaccharide bind at highly specific subsites in the active site cleft of the protein. At each subsite, binding energy is generated by hydrogen-bonds with the OH-groups of the carbohydrates, or Van der Waals interactions with aromatic residues, or also by the hydrophobic effect from displacement of bound water molecules [83–85]. To determine the contribution to the Gibbs free energy (ΔG) of the different subsites, or the amino acid residues which interact with the glycosyl residues at these subsites, two methods are being applied: firstly, the hydroxyl groups of the ligands can be exchanged with hydrogen atoms to measure the individual contribution of these hydroxyl groups [86], and secondly, site-directed mutants can be compared with the wild-type protein [42]. Furthermore, a kinetic and product formation analysis involving substrates of different length can be used to calculate the number and positions of the subsites. For CGTase this latter method indicated a total of nine subsites for both the CGTase of *Klebsiella pneumoniae*, forming mostly α-cyclodextrin (with 6 glucose residues in the ring), and the CGTase from *B. circulans* strain 8, forming mostly β-cyclodextrin (with 7 glucose residues in the ring) [87]. Analysis of the preference of the products formed suggested that for the CGTase of *B. circulans* strain 8, also producing mostly β-cyclodextrin, these 9 subsites range from +2 to −7, as confirmed by the structure of the maltononaose inhibitor bound in the active site of the β-CGTase from *B. circulans* strain 251 (Fig. 7) [45]. For the α-CGTase of *K. pneumoniae* the 9 subsites were predicted to range from +3 to −6. Binding up to subsite +3 has also been observed in the structure of the α-CGTase from *Thermoanaerobacterium thermosulfurigenes* complexed with a maltohexaose inhibitor [88]. A similar method, using 4-nitrophenyl-α-maltooligosaccharides of different lengths as substrates, revealed five high-affinity subsites in PPA, ranging from +2 to −3 [89]. From these experiments it appears likely that the number and positions of sugar binding subsites determine the differences in product specificity between individual α-amylases and CGTases. For example PPA, with an active site consisting of five subsites, produces mainly maltose and maltotriose, whereas α-amylase from barley contains at least ten subsites and yields mainly maltose, maltohexaose and maltoheptaose. For TAKA-amylase a substrate binding model was proposed involving six or seven glucose residues [39,90], which has recently been confirmed by the structure of *A. oryzae* α-amylase complexed with an acarbose derived maltohexaose inhibitor binding from subsites +3 to −3 [91]. Increasingly, X-ray crystallographic studies of protein–carbohydrate complexes result in the identification of the protein–ligand interactions, also providing information about factors determining the carbohydrate substrate and product specificities of different enzymes. Amino acids on loops in the A-domain (including the B-domain), linking the C-terminal end of a β-strand to the N-terminal end of the adjacent α-helix, form the subsites of the active site. The number of subsites can be changed by changing the length and folding of the loops of the $(\beta/\alpha)_8$-barrel [92], or by changing specific amino acids using site directed mutagenesis [93–96].

4. Cyclodextrin glycosyltransferases

4.1. CGTase catalysed reactions

Whereas α-amylases generally hydrolyse α(1–4) glucosidic bonds (Fig. 8A), CGTases mainly catalyse transglycosylation reactions. Such reactions can be described as: $G(n)+G(m) \leftrightarrow G(n-x)+G(m+x)$ in which $G(n)$ is the donor and $G(m)$ the acceptor oligosaccharide consisting of n and m glucose residues, respectively. Disproportionation (Fig. 8B) can be regarded as the default reaction, and is also catalysed by several other members of the α-amylase family (e.g., 4-α-glucanotransferase, EC 2.4.1.25 (amylomaltase, disproportionating enzyme)). The specific CGTase reaction is the cyclization reaction (Fig. 8C) in which the part of the donor that has been cleaved off also acts as the acceptor, resulting in formation of a cyclodextrin, described as: $G(n) \leftrightarrow$ cyclic$G(x)+G(n-x)$. The reverse reaction is also cata-

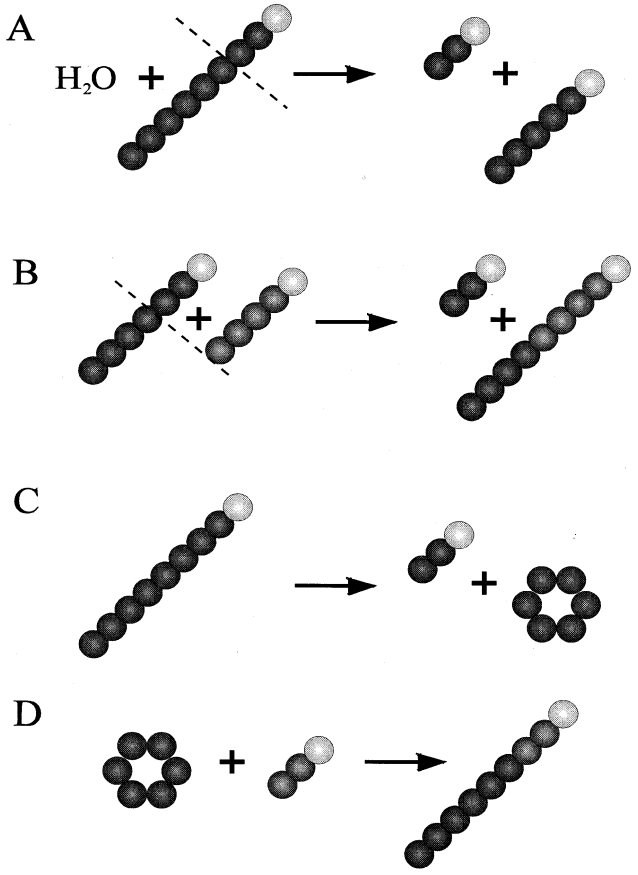

Fig. 8. Schematic representation of the CGTase-catalysed reactions. The circles represent glucose residues; the white circles indicate the reducing end sugars. (A) hydrolysis; (B) disproportionation; (C) cyclization; (D) coupling. The k_{cat} values (s^{-1}) of the wild-type enzyme for these reactions are: (A) 3 (hydrolysis of soluble starch) [115]; (B) 1200 (disproportionation using 4-nitrophenyl-α-D-maltoheptaoside-4-6-*O*-ethylidene (EPS) as donor and maltose as acceptor) [28]; (C) 25 (α-cyclization), 345 (β-cyclization), 65 (γ-cyclization) [28]; (D) 240 (α-CD coupling), 370 (β-CD coupling), 190 (γ-CD coupling) [28].

lysed by the enzyme and is referred to as the coupling reaction (Fig. 8D).

CGTases and some α-amylases (e.g., maltotetraose forming α-amylase from *Pseudomonas stuzeri* (EC 3.2.1.60, G4α, [97]) and maltogenic α-amylase from *Bacillus stearothermophilus* (EC 3.2.1.133, G2α, novamyl, [98])) have been described as exo-acting enzymes degrading starch molecules from their non-reducing ends, whereas most α-amylases are endo-acting enzymes cleaving α(1–4) glucosidic bonds more randomly in the starch molecules. True exo-acting enzymes like β-amylase and glucoamylase (family 14 and 15 of glycosyl hydrolases, respectively) have substrate binding clefts which are closed to one side by specific loops, forcing binding of the non-reducing end of the substrate in the active site. In the crystal structure of G4α a similar feature has been observed [99], however, the open groove found in CGTases and other α-amylases should allow binding of extended substrates in the active site, consequently resulting in an endo-type activity. Analysis of the action on amylose by CGTase, forming large cyclic α(1–4) glucans [100], and G2α [101] indeed showed that these enzymes attack this high molecular weight substrate in an endo-like manner. The seemingly exo-type action reported for these enzymes must therefore result from the preferred use of low molecular weight and/or highly branched substrates in laboratory activity assays, which are easier to work with than high molecular weight amylose. Interestingly, CGTases and also G4α and G2α all possess the E-domain, which is absent in most α-amylases (see Fig. 5). The strong interaction of this domain with starch and various oligosaccharides [45,68,75] may cause some physical constraints in the degradation of these compounds, which also could lead to the seemingly exo-type of attack. Furthermore, binding of amylopectin to the E-domain, or the mere presence of this domain, may limit the accessibility of the regions between branching points leading to incomplete degradation of starch, which can also be interpreted as an exo-type of attack. No experiments supporting or rejecting this hypothesis have been described so far.

The formation of large cyclic α(1–4) glucans from amylose by CGTase was also interpreted as opposing generally held views on cyclodextrin product specificity of CGTases (see below). The same study, however, also revealed significantly higher peaks for α-, and β-cyclodextrin in the reaction with α- and β-CGTase, respectively, present even in the early stages of the reaction [100]. Furthermore, the action of potato disproportionating enzyme (DE) [102] and *B. stearothermophilus* branching enzyme (BE) [103] on amylose indicate that the CGTase specific cyclization reaction is not required for the formation of the large cyclic products. DE catalyses intermolecular transglycosylation reactions similar to the disproportionation reaction of CGTase, while BE cleaves an α(1–4) bond in one oligosaccharide molecule or starch chain and links the cleaved off part via an α(1–6) linkage

to another molecule or chain. Although for neither enzyme intramolecular transglycosylation reactions had been reported before, both enzymes were found to produce large cyclic α(1–4) glucans similar to those formed by CGTase (with one α(1–6) bond in the ring for BE) [102,103]. In these experiments low concentrations of high molecular weight amylose (0.4, 0.2, and 0.3% for CGTase, DE, and BE, respectively) were used, amounting to concentrations in the μM range. Therefore the 'cyclization of amylose molecules' is not necessarily a novel reaction catalysed by the enzymes, but is a direct effect of the limited availability of acceptor molecules. For DE and BE the smallest cyclic glucans formed consisted of 17 and 18 glucose residues, respectively, indicating that the specific CGTase cyclization reaction is only required for production of smaller cyclic oligosaccharides (cyclodextrins: 6–8 glucose residues mainly). Although the preferred use of low molecular weight and/or highly branched substrates for the determination of cyclodextrin formation and the rather simple HPLC methods generally used for the detection of the produced cyclodextrins have probably limited observations of large cyclodextrins, production of δ-, ε-, ζ-, and η-cyclodextrins (consisting of 9, 10, 11, and 12 glucose residues, respectively) from starch has been reported [104,105].

4.2. CGTase versus α-amylase action

Since the first description of a *B. macerans* strain capable of producing cyclodextrins from starch [106], numerous CGTase enzymes, mostly from gram positive bacteria, have been purified and characterized. The question what precisely determines the difference in reaction specificity between α-amylases and CGTases (see Fig. 8) has received much attention. When retrieving CGTase amino acid sequences from the SWISS-PROT/EMBL protein data base the following description of CGTases is kindly provided: "CGTase may consist of two protein domains: the one in the amino-terminal side cleaves the alpha-1,4-glucosidic bond in starch, and the other in the C-terminal side catalyses other activities, including the reconstitution of an alpha-1,4-glucosidic linkage for cyclizing the maltooligosaccharide produced."

Indeed, the major difference between CGTases and α-amylases is the presence of additional C-terminal domains in the former enzyme. It has been hypothesized that these additional domains are involved in catalysing the formation of cyclodextrins [107]. Experiments with CGTase from alkalophilic *Bacillus* sp. 1011 of which 10 or 13 amino acid residues were deleted from the C-terminus were considered to support this hypothesis [108]. Later experiments, however, failed to confirm these findings [87,109] and indicated that the observed effects may have been caused by interference of the deletions with the structural integrity of the enzyme [109]. As explained above, the C-terminal domain (E-domain) is responsible for binding to (raw) starch. The differences in reaction specificity between CGTases and α-amylases, therefore, appear to be based on specific differences in the active centres.

4.3. Sequence similarities in CGTases

In general, CGTases show a clear similarity in amino acid sequence, ranging from 47% to 99%, which should be sufficient to allow identification of residues responsible for the differences in α-amylase and CGTase action. Fig. 9 shows the amino acid sequence of the *B. circulans* strain 251 CGTase and the conserved residues deduced from a sequence alignment of the 24 CGTases listed in Table 3. The structural features of the enzyme are indicated to allow a thorough comparison with α-amylases, which are the members of the family 13 of glycosyl hydrolases most closely related to CGTases. For this comparison an alignment including 30 amylases from various sources (fungi, plants, bacteria) performed by Finn Drablos (personal communication) was used. The first specific CGTase residues (unique and completely conserved) are found between β-strand 1 and α-helix 1 and consist of the stretches 27DG and 32NNPXG and the single residues 46L and 53D. Of these residues only Pro34 is not completely conserved; it is absent in the CGTase of *K. pneumoniae*, the most dissimilar of the CGTases included in the alignment. Asp27, Asn32, Asn33, and Asp53 are ligands of a calcium binding site observed in CGTases [44,61], but not in α-amylases. Gly28, Pro34 and Gly36 probably serve as structural support for this calcium binding site. Leu46 is not involved in calcium binding, but the neighbouring residue 47 is in-

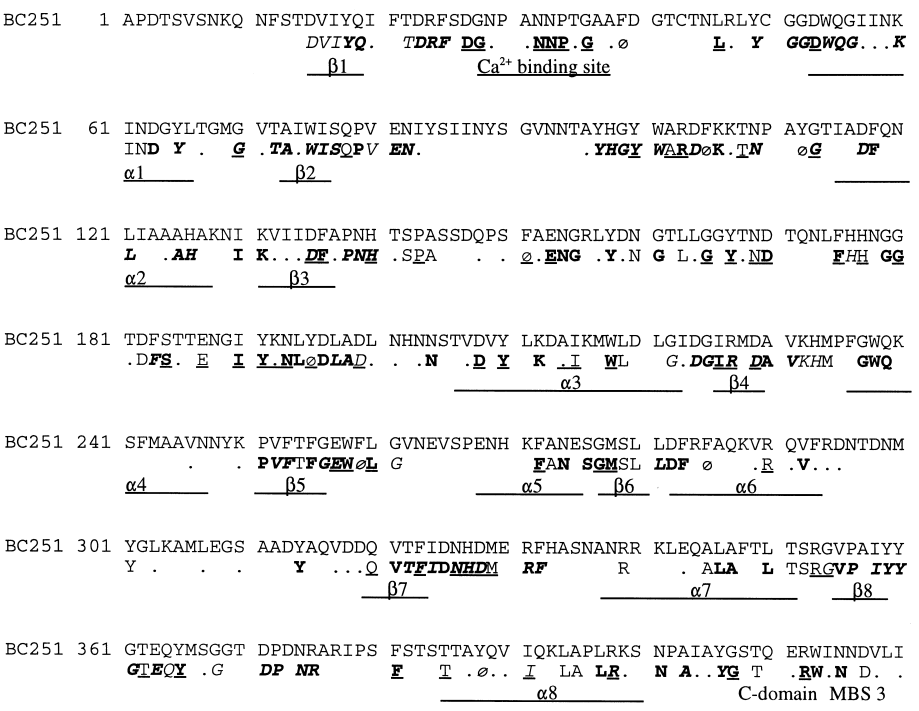

Fig. 9. Amino acid sequence of the catalytic domain of *B. circulans* strain 251 CGTase. Residues indicated below the sequence are conserved in CGTases as determined by an alignment of the CGTases listed in Table 3; (∅) Tyr or Phe; (.) conserved replacements in other CGTases. **Boldface** residues are completely conserved, underlined residues are unique for CGTases, *italic* residues are ***(completely)*** conserved in CGTases and at least one group of α-amylases, *italic underlined* residues are ***(completely)*** conserved in all CGTases and α-amylases.

volved in binding (semi)cyclic oligosaccharides (see below) and is typically an Arg, Lys, or His in CGTases. More unique CGTase residues are found in the B-domain: Phe136 (Tyr in *K. pneumoniae*), Phe/Tyr151, Glu153, Gly165 (Thr in *K. pneumoniae*, but typically an aromatic amino acid in α-amylases), Tyr167, Phe175 (Tyr in *K. pneumoniae*), His177, Gly180, and the stretch 192-K/R,N,L,F/Y,D-196, of which only Leu194 is observed also in α-amylases. In *K. pneumoniae* this stretch starts with His and ends with Asn, but these are conservative modifications, which are quite different from the corresponding residues in α-amylases. Also residues Ile190 and Tyr191 are conserved in all CGTases, except the enzyme from *K. pneumoniae*. Noticeably, Tyr191 forms a contact between domains B and D [63] and domain D is partially deleted in the CGTase from *K. pneumoniae*. Apart from amino acid residue 195, usually a smaller amino acid (Gly, Ser or Val) in α-amylases, the functions of none of the conserved residues in the B-domain has been studied thus far. The phenyl group of residue 195 is located at the centre of the CGTase active site and thus might be involved, by hydrophobic interaction with the carbohydrate residues, in bending the non-reducing end towards the reducing end of the bound oligosaccharide, resulting in cyclodextrin formation. Mutations at this position [105,110–112] indeed showed that it is important for the cyclization process. However, even Tyr195Gly mutations retained 10–25% of cyclization activity, indicating that the aromatic character of this residue is not crucial for this reaction. An alternative role for Tyr195 might be keeping water from the active site, thus preventing hydrolysis [105]. Mutating Tyr-(Phe)195 into residues found at the corresponding position in α-amylases, however, did not result in drastically increased hydrolysing activity [105,112]. Relatively few unique residues are found in the second part of the A-domain: Tyr210, Trp218, Ile226, and Trp258. The last residue is positioned directly next to the catalytic Glu257 and is usually a small hydrophobic residue (A, V, I, or L) in α-amylases. Mutation of this residue in the CGTase from *B. stearothermophilus* (W254V) resulted in a fourfold

Table 3
CGTases used in the amino acid sequence alignments

	Source	Product	Ref.
KLEPN	*K. pneumoniae* strain M5a1	α	[122]
BMAC2	*B. macerans*	α	[123]
BMACE	*B. macerans* strain NRRL B388	α	[124]
TBNOVO	*Thermoanaerobacter* sp. ATCC53.627	α/β	Patent DK 96/00179
TABIUM	*T. thermosulfurigenes* EM1	β/α	[125]
BSTEA	*B. stearothermophylus* strain NO2	α/β	[124]
BLICH	*B. licheniformis*	α/β	[126]
BCIR8	*B. circulans* strain 8	β	[127]
BC192	*B. circulans* strain E192	β	[128][b]
B663	*Bacillus* sp. strain 6.6.3	β	[a]
BF2	*B. circulans* strain F2	β	[129]
BC251	*B. circulans* strain 251	β	[44]
B1018	*Bacillus* sp. strain B1018	β	[130]
B1011	Alkalophilic *B.* sp. strain 1011	β	[107]
B382	Alkalophilic *B.* sp. strain 38.2	β	[131,132]
B171	Alkalophilic *B.* sp. strain 17.1	β	[133]
BKC201	*Bacillus* sp. strain KC201	β	[134]
BSP11	Alkalophilic *B.* sp. strain 1.1	β (no α)	[135]
BOHB	*B. ohbensis* (strain C-1400)	β (no α)	[136]
BREV	*Brevibacillus brevis* strain CD162	γ/β	[137]
BF290	*B. firmus/lentus* strain 290-3	γ/β	[138][b]

Indicated are the sources, the main product formed by the enzyme and the reference where the enzyme characteristics are described. Sequences were obtained from the SWISS-PROT/EMBL protein data base.
[a]A.A. Akhmetzjanov, ENTREZ-NCBI sequence ID: 39839 (1992).
[b]Sequence obtained from the Roquette Company.

decrease in cyclization activity, while the hydrolysing activity was hardly affected [110].

Summarizing, about 25 unique or uniquely conserved residues have been identified for CGTases. Of these conserved residues only two (Tyr or Phe195 and Trp258) have been shown to be involved in the CGTase catalysed cyclization reaction. However, the mechanistic basis of the involvement of these hydrophobic residues has not been elucidated yet.

4.4. CGTase three-dimensional structures

The increasing availability of X-ray crystallographic structures of CGTase proteins, especially with inhibitors, substrates, or products bound at the active site, can provide more insights in factors determining the unique cyclodextrin producing activity of these enzymes. The first requirement for the formation of cyclodextrins is binding of a substrate of sufficient length in the active site. As described above, this requirement is well met in CGTases and the structure of the maltononaose inhibitor bound in the active site has allowed determination of the subsite architecture in the substrate binding groove (see Fig. 7) [45]. Especially subsites +1, −1 and −2, where the bond-cleavage process takes place, have an architecture identical to *A. oryzae* α-amylase [39,92], except for the presence of Tyr195, located at the centre of the active site. Of the amino acid residues forming subsites −3 to −7 Asp196, hydrogen bonding to the glucose residue at subsite −3, and Asn193, hydrogen bonding to the glucose residue at subsite −6, have been identified as specific CGTase residues. These amino acids thus may specifically contribute to the cyclization reaction, but no mutants clarifying their roles have been reported, and their involvement therefore is unclear. The amino acid residues interacting with the sugar residue at subsite −7 (Ser145, Ser146, Asp147) are not specifically conserved in CGTases. The loop of the B-domain in which they are located, however, contains proline

143, which is present in most CGTases, but not in α-amylases (see Figs. 9 and 10). At subsite +2 a special binding mode of the glucose residue is observed. Besides hydrogen bonding to Lys232 (almost completely conserved in CGTase, but also at least functionally conserved in α-amylases) hydrophobic interactions with both Phe183 and Phe259 are observed. Although these residues have been reported to be typical for CGTases [111], in most α-amylases at least one and many times both of the corresponding residues is highly hydrophobic (Phe, Tyr, or Trp). For instance in PPA the residue corresponding to Phe183 is Tyr151, and this residue interacts with the α-cyclodextrin bound near the catalytic residues [81]. In *A. oryzae* α-amylase, complexed with an acarbose-derived maltohexaose inhibitor bound in the active site, the hydrophobic moiety of Leu232 (equivalent to Phe259 in CGTases) has stacking interactions with the glucose residue at subsite +2 [91]. This indicates that also at subsite +2 substrate binding of CGTase is similar to that of α-amylase. Mutation Phe183Leu in the CGTase from alkalophilic *Bacillus* sp. 1011 resulted in fourfold and sixfold decreases in cyclization and starch-degrading activities, respectively [111]. Mutation Phe259Leu resulted in a similar decrease in cyclization activity and a threefold decrease in starch-degrading activity, while a similar mutation in the CGTase from *B. stearothermophilus* (Phe255Ile) resulted in complete removal of the cyclization activity, with a fourfold reduction in the starch-degrading activity and a doubling of the saccharifying activity (hydrolysis) [110]. A double mutant Phe183Leu/Phe259Leu in the CGTase from alkalophilic *Bacillus* sp. 1011 displayed a cyclization activity which was 0.5% of that of the wild type, which is a much larger decrease in activity than would be predicted from the combination of the two single mutations [111]. Probably these residues play a cooperative role in binding the non-reducing end of the linear chain when it assumes the circular conformation required for cyclodextrin production.

More recently two structures of CGTases with cyclodextrin products bound in the active site have been elucidated [113,114]. A γ-cyclodextrin bound in the active site of the CGTase from *B. circulans* strain 251 revealed a binding mode similar to that of a linear maltooligosaccharide at subsites +1 to −2, resulting in distortion of the cyclodextrin ring. At subsite +2 the hydrophobic interactions of the glucose residue with Phe183 and Phe259 are modified; whereas the linear substrate has better stacking interactions with Phe183, the cyclodextrin product stacks better with Phe259. Furthermore, the hydrogen bonding interaction with Lys232 is absent in the cyclodextrin structure. These findings support a specific role of the residues at subsite +2. At subsite −3 the glucose residue of the γ-cyclodextrin hydrogen bonds with Arg47, an interaction not observed with linear oligosaccharides [114]. As mentioned above, residue 47 is functionally conserved in CGTases (Arg, Lys, or His) and may therefore play a role in the specific CGTase catalysed reactions. A similar interaction between Lys47 and a glucose at subsite −3 was found in the structures of mutant Glu257Ala of the CGTase from *B. circulans* strain 8 with a β-cyclodextrin bound in the active site [113] and of the *T. thermosulfurigenes* strain EM1 CGTase with a maltohexaose inhibitor bound in a semicyclic conformation in the active site [88]. Site-directed mutagenesis experiments have revealed the involvement of Arg47 in the conformational change of the oligosaccharide chain during the cyclization and coupling reactions catalysed by CGTase [115].

4.5. Cyclodextrin product specificity of CGTases

It has been suggested that the size of the aromatic amino acid (Phe or Tyr), present in a dominant position in the centre of the active site cleft of CGTases (see above), influences the preferred cyclodextrin size. Sin et al. [116] proposed a mechanism in which the starch chain folds around this residue. Substitution of this central amino acid by a tryptophan, Tyr188Trp in the *B. ohbensis* CGTase [117] and Tyr195Trp in the *B. circulans* strain 8 CGTase [118] indeed doubled the relative production of γ-cyclodextrin. However, several other Tyr188 mutations [117], as well as the substitution of Tyr195 of the *B. circulans* 251 CGTase by other amino acids [105], and the mutation F191Y at the similar position in the CGTase of *B. stearothermophilus* NO2 [110] do not support this proposed mechanism. Furthermore, natural α-, β-, and γ-CGTases all have Tyr or Phe at this position, indicating that this residue is not involved in the differences in product specificity observed for these naturally occurring enzymes.

As explained above, product specificity of α-amylases and CGTase may depend largely on the number of subsites available for binding glucose units in the active site. The structure of the CGTase from *B. circulans* 251 complexed with a maltononaose inhibitor [45] (Fig. 7) has revealed several amino acid residues involved in binding of the maltononaose. Most of these amino acids have been shown to be conserved in all CGTases (Phe183, Phe259, Asn193, Asp196), all CGTases and several α-amylases (Lys232, His233, Asp371, Arg375), or in the whole α-amylase family of glycosyl hydrolases (Asp229, Glu257, His327, Asp328) (see above). These residues are therefore not considered to be involved in product specificity of natural CGTases, although mutations can result in altered product specificity as has been shown for the histidine [42] and the phenylalanines [111]. Less conserved are the residues involved in the strong hydrogen bonding network between the enzyme and the glucose bound at subsite −7 (Fig. 10). Mutation Ser146Pro, aimed at disturbing this hydrogen bonding network, resulted in a decreased preference for β-cyclization [96], confirming that the inhibitor binding mode resembles the mode of substrate binding required for the formation of β-cyclodextrin. The residues involved in this hydrogen bonding network are located in a loop at the start of the B-domain, between the completely conserved residues His140 (conserved in the whole α-amylase family) and Glu153 (unique for CGTases) (Fig. 9). Pro143 is highly conserved in this loop and only present in CGTases, indicating that it may have an important role in preserving a suitable loop conformation. The absence of this proline and the much shorter loop in the CGTase of *K. pneumoniae* again indicate structural differences of this enzyme and do not allow a functional comparison with the other CGTases. The differences in product specificity of the CGTases are reflected in subtle differences in the amino acids following Pro143. In CGTases producing predominantly β-cyclodextrin these are either ASSD or AMET, while in CGTases producing more equal amounts of α- and β-cyclodextrin the stretch ASET is found, and in CGTases producing little or no α-cyclodextrin ALET. The primarily γ-cyclodextrin forming CGTase from *B. firmus/lentus* strain 290-3 completely lacks the residues in this region involved in substrate binding. This situation has recently been copied in the β-CGTase from *B. circulans* strain 8 by replacing residues 145–151 by a single aspartate (Δ(145–151)D mutant) [118], resulting in the stretch HTSPADAE (HTSPVDIE in the *B. firmus/lentus* strain 290-3 CGTase, see Fig. 10), which indeed resulted in increased γ-cyclodextrin production. The higher γ-cyclization activity of this mutant has been explained by inducing a further opening of the active site cleft to produce more space for the bound glucosyl chain [118]. However, from the structure of the maltonaose inhibitor and the comparison with α-amylases (see above), it is evident that the loop region 145–151 flanks the end of the substrate binding cleft, which opens up to the medium, indicating that there is no space needed to accommodate additional sugar residues. An alternative explanation for the increased production of γ-cyclodextrin by this mutant can be deduced from the action of CGTases on amylose (see above). Although large cyclic α(1–4)glucans were formed, the preference for formation of α- or β-cyclodextrin was clearly visible, even in the early stages of the reaction [100]. For the *B. firmus/lentus* strain 290-3 wild-type and the *B. circulans* strain 8 Δ(145–151)D mutant CGTases, missing the residues involved in product specificity in loop region 145–151, cyclodextrin production will therefore automatically shift to formation of the larger cyclodextrins; not by the creation of more space for the

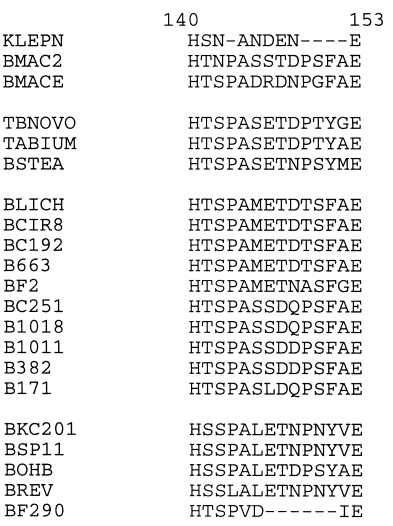

Fig. 10. Sequence alignment of the region 140–153 in CGTases. Included are the CGTases listed in Table 3. The CGTases are ordered according to their cyclodextrin product specificity; α-CGTases on the top, γ-CGTase at the bottom.

bound glucosyl chain, but due to the lack of specific interactions. These findings support the involvement of this loop region in cyclodextrin product specificity. However, the small differences in this loop between enzymes with different specificities and the fact that the Ser146Pro mutation in the *B. circulans* strain 251 CGTase did not result in a shift in product specificity comparable to the naturally occurring variation, although the hydrogen bonding network at subsite −7 was effectively disturbed [96], suggest that there is more to cyclodextrin product specificity than modified subsite specificities.

A second region which may be involved in product specificity is found at subsite −3. Tyr89 in the maltononaose structure (Fig. 7) has hydrophobic interactions with the glucose bound at this subsite, an interaction also observed between Tyr75 and the glucose bound at subsite −3 in the structure of *A. oryzae* α-amylase complexed with an acarbose derived maltohexaose inhibitor [91]. In the more thermostable CGTases this residue is typically an aspartate, which has been shown to create a novel salt bridge with Lys47 in the structure of the *T. thermosulfurigenes* EM1 CGTase [62]. Actually the whole loop containing residue 89 is remarkably different in these enzymes (Fig. 11) and has been proposed to contribute with novel hydrogen bonds and apolar contacts

```
              78          89         97
KLEPN          PPIENVNNT--DAAG---NTGY
BMAC2          QPVENITAVINY-SGVN-NTAY
BMACE          QPVENITSVIKY-SGVN-NTSY

TBNOVO         QPVENIYAVLPD-STFGGSTSY
TABIUM         QPVENIYAVLPD-STFGGSTSY
BSTEA          QPVENVFSVMNDASG---SASY

BLICH          QPVENIFATINY-SGVT-NTAY
BCIR8          QPVENIFATINY-SGVT-NTAY
BC192          QPVENIFATINY-GGVI-NTAY
B663           QPVENIFATINY-SGVT-NTAY
BF2            QPVENIYSVINY-SGVN-NTAY
BC251          QPVENIYSIINY-SGVN-NTAY
B1018          QPVENIYSIINY-SGVN-NTAY
B1011          QPVENIYSVINY-SGVN-NTAY
B382           QPVENIYSVINY-SGVH-NTAY
B171           QPVENIYSVINY-SGVN-NTAY

BKC201         QPVENVYALHP--SGY---TSY
BSP11          QPVENVYALHP--SGY---TSY
BOHB           QPVENVYALHP--SGY---TSY
BREV           QPVENVYALHP--SGY---TSY
BF290          PPIENVMELHP--GGF---ASY
```

Fig. 11. Sequence alignment of the region around residue 89 in CGTases. Included are the CGTases listed in Table 3. The CGTases are ordered according to their cyclodextrin product specificity; α-CGTases on the top, γ-CGTase at the bottom.

to the stabilization of the *T. thermosulfurigenes* EM1 CGTase [62]. That these differences may also inflict changes in product specificity has been shown in the CGTase from *B. circulans* strain 251, where mutation Tyr89Asp resulted in a slight shift towards α-cyclodextrin production [96]. A Tyr89Ser mutation in the CGTase from alkalophilic *Bacillus* strain I-5, however, did not affect production profiles. Mutant Tyr89Phe of the same enzyme showed enhanced β-cyclodextrin specificity, but also resulted in a decreased conversion of starch into cyclodextrins, while an Asn94Ser mutation in the same loop (resembling the situation in the thermophilic enzymes) enhanced α-cyclodextrin specificity, and resulted in an increased conversion of starch into cyclodextrins [119]. The α-CGTases from the *B. macerans* strains, however, are very similar to the β-CGTases in this loop region. The only remarkable difference is the substitution of the aromatic residue 84, which is also replaced in the α-CGTase of *K. pneumoniae* and the γ-CGTase from *B. firmus/lentus* strain 290-3 (see Fig. 11), but otherwise a residue unique for CGTases. This residue may therefore be specifically involved in β-cyclization. Another characteristic of this loop is the stretch 78-QPV-80 immediately following β-strand 2, of which Gln78 is a residue unique for CGTases. Again the *K. pneumoniae* α-CGTase and the *B. firmus/lentus* strain 290-3 γ-CGTase are exceptions, specifically containing the sequence PPI for this stretch in both enzymes. Another similarity between these two enzymes is the fact that the loop is shorter than in most other CGTases. The α-CGTase of *K. pneumoniae*, however, shows clear homology to the α/β-CGTase of *B. stearothermophilus*, whereas the γ-CGTase from *B. firmus/lentus* strain 290-3 is homologous to the CGTases producing virtually no α-cyclodextrin, which are the only other CGTase enzymes showing the same reduction in loop size (Fig. 11).

New insights in factors determining CGTase cyclodextrin product specificity came from crystal soaking experiments with the α-CGTase from *T. thermosulfurigenes* EM1 which resulted in the structure of the enzyme complexed with a maltohexaose inhibitor bound in the active site. The conformation of this maltohexaose was more bent compared to the maltononaose conformation and it was suggested to represent a specific intermediate in cyclization for the

formation of α-cyclodextrin [88]. Also in the *B. circulans* strain 251 CGTase double mutant Y89D/S146P a maltohexaose bound in a similar conformation in the active site cleft was observed. Although this double mutant produced significantly more α-cyclodextrin compared to the wild-type enzyme, with the cyclodextrin product ratio changing from 14:66:20 (α:β:γ) for the wild-type enzyme to 30:51:19 for the mutant enzyme, this mutant still favours β-cyclodextrin production [96]. The bent conformation of the ligands in these enzymes thus probably represent an intermediate in (α-, β-, and γ-) cyclization. The correlation of (increased) α-cyclodextrin specificity and the preference of binding a linear oligosaccharide in a bent conformation can be related to the rate limiting step in the cyclization reaction, which is probably the conformational change from linear substrate to circular product, especially for the smallest (α-)cyclodextrin [28]. Stabilization of an intermediate conformation may therefore result in a generally increased cyclization activity, which is most significant for α-cyclization [96]. Mutations Asp371Arg and Asp197His of *T. thermosulfurigenes* EM1, aiming to hinder and to stabilize, respectively, the maltohexaose bent conformation [88], support this hypothesis. Mutation Asp371Arg resulted in enhanced levels of β- and γ-cyclodextrins produced: from 25:58:14 (α:β:γ) for the wild type to 6:68:26 for the mutant enzyme, however, with drastically decreased specific activities, supporting the role of the bent intermediate in formation of all cyclodextrins. Mutant Asp197His produced enhanced levels of α-cyclodextrin: from 28:58:14 (α:β:γ) for the wild type to 35:49:16 for the mutant. This also explains the effects of mutating Tyr195, which leads to increased production of larger cyclodextrins when changed into Trp [105,117,118], but even more so when changed to Leu [105]. All these mutants resulted in decreased production of cyclodextrins, indicating that the efficiency of the cyclization reaction was negatively affected. Combination with the studies described above indicates that the increased ratio of larger cyclodextrins directly results from interference with the role of Ty195 in cyclization. This role can be partially taken over by Trp, resulting in a rather slight shift towards larger cyclodextrins. In the Tyr195Leu mutant, however, the function of residue 195 is lost, resulting in complete absence of α-cyclodextrin production.

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

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

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

BKC201          CI-DLHKYC
BSP11           CI-DLHKYC
BOHB            CS-DLHKYC
BREV            CS-DLHKYC
BF290           CL-DLTKYC
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Fig. 12. Sequence alignment of the region around residue 47 in CGTases. Included are the CGTases listed in Table 3. The CGTases are ordered according to their cyclodextrin product specificity; α-CGTases on the top, γ-CGTase at the bottom.

Another interesting amino acid found near subsite −3 is residue 47, which interacts with (semi)cyclic compounds, but not with linear oligosaccharides [115]. It is found next to the unique CGTase residue Leu46 in the loop between β-strand 1 and α-helix 1 which has ligands of the CGTase specific Ca^{2+} binding site on both ends (see above, Fig. 9). Except in the α-CGTases and the thermostable CGTases from *Thermoanaerobacter* sp. and *T. thermosulfurigenes* EM1, its position is even more defined by the sulfide bridge formed by Cys43 and Cys50. The nature of residue 47 shows a clear discrimination between the different groups of CGTases as defined in Fig. 12. In α- and α/β-CGTases it is a lysine. In β-CGTases it is either a lysine or an arginine. In the CGTases producing virtually no α-cyclodextrin it is a histidine. Finally, in the γ-CGTase from *B. firmus/lentus* strain 290-3 a threonine is found at this position. Interestingly, in the maltohexaose structure of the α/β-CGTase from *T. thermosulfurigenes* EM1 Lys47 hydrogen bonds to the glucose residue at subsite −3, an interaction which is not observed for Arg47 in the maltohexaose structure of the double mutant Y89D/S146P of the β-CGTase from *B. circulans* strain 251. These differences may be induced by the specific residues (Lys or Arg, respectively) or by the absence or

presence of the sulfide bridge, respectively. In either case the observations again support the hypothesis that stabilization of (the) intermediate conformation(s) of the oligosaccharide chain during the cyclization reactions have the most stimulating effect on α-cyclization. This is also shown by mutations Arg47Leu and Arg47Gln in the CGTase from *B. circulans* strain 251, supporting the involvement of this residue in the change of the oligosaccharide conformation. Both mutants showed a generally decreased cyclization activity, but also a shift towards the production of larger cyclodextrins [115].

5. Conclusions

Summarizing, the size of the cyclodextrin product depends directly upon the number of glucose units which are bound in the active site up to subsites −6, −7, or −8 before the cleavage between subsite −1 and 1 takes place. Mutations (substitution, insertion and/or deletion) in one or more amino acid residues positioned close to the substrate will thus change the α:β:γ product ratio of CGTases. However, the efficiency of the subsequent circularization of the thus formed intermediate to actually produce the cyclodextrin appears to be of equal importance at least. Many factors involved in product specificity of CGTases have been identified, including the identity, position, and interactions of several amino acid residues that play an important role. The detailed insights provided here are necessary for straightforward approaches for rational construction of mutant CGTase enzymes with desired cyclodextrin product specificity.

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