Structural basis of the synthesis of large cycloamyloses by amylomaltase

Norbert STRÄTER1*, Ingo PRZYLAS1, Wolfram SAENGER1, Yoshinobu TERADA2, Kazutoshi FUJI2, Takeshi TAKAHA2

1Institut für Chemie - Kristallographie, Freie Universität Berlin, Takustraße 6, 14195 Berlin, Germany; tel.: ++ 49 30 8385 3456, fax: ++ 49 30 8385 6702, e-mail: strater@chemie.fu-berlin.de
2Biochemical Research Laboratory, Ezaki Glico Co. Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502, Japan


Large cycloamyloses probably form hydrophobic channels resembling those of V-type amylose helices. Because these channels can accommodate guest molecules, cycloamyloses may find technical applications similar to the smaller cyclodextrins. Cycloamyloses are synthesized by bacterial amylomaltases and by the related plant D-enzymes. We have analyzed the three-dimensional structures of amylomaltase from Thermus aquaticus and also of a complex of this enzyme with the maltotetraose inhibitor acarbose in order to gain insight into the structural basis of cycloamylose formation. Amylomaltase differs from α-amylase or CGTase in the absence of a C-terminal domain C and in the presence of an additional predominantly α-helical insertion between barrel strands 2 and 3. Two acarbose molecules have bound to the enzyme, one at the active site and a second molecule ~14 Å away from the nonreducing end of the first acarbose. The subdomains B1, B2 and B3, which are located around the rim of the C-terminal side of the barrel, and in particular the two loops around residues 460 and 250, probably influence the product specificity.

Key words: acarbose, α-amylase family, glucanotransferase, protein crystallography.

Abbreviations: CA, cycloamylose; CD, cyclodextrin; CGTase, cyclodextrin glucanotransferase; D-enzyme, starch disproportionating enzyme.

Cyclodextrins and cycloamyloses

Cyclodextrins (CDs) are small cyclic maltooligosaccharides with 6 (α-CD), 7 (β-CD) and 8 (γ-CD) glucose residues. These molecules have an annular structure with a hydrophilic rim, formed by hydroxyl groups, at both sides of the cone. The central hydrophobic cavity with a diameter of 4.7–5.3 Å for α-CD to 7.5–8.3 Å for γ-CD can complex hydrophobic guest molecules (SAENGER et al., 1998). These inclusion complexes can change the solubility, reactivity and stability of the complexed substances and have found many applications in the food, chemical and pharmaceutical industries.

* Corresponding author
These larger cycloamyloses have also been reported to be useful in protein refolding. CA can complex and remove detergents from the refolding buffer and has a chaperone-like activity (MACHIDA et al., 2000).

Production of CDs and CAs by CGTase and amylolamtase

Cyclodextrins are produced by the action of cyclodextrin glucanotransferase (CGTase) that converts starch into a mixture of α-CD, β-CD and γ-CD as well as residual dextrin. However, CGTase initially produces larger cycloamyloses which are subsequently converted to cyclodextrins (TERADA et al., 1997). The product specificity (proportions of α-, β-, and γ-CDs) is different for CGTases from different sources, but it can be influenced by the reaction conditions (RENDLEMAN, 1993; MORI et al., 1995) and by single amino-acid substitutions (NAKAMURA et al., 1993, 1994; PENNINGA et al., 1995; WIND et al., 1998).

Larger cycloamyloses are produced by the action of bacterial amylomaltases or the plant D-enzymes (starch disproportionating enzymes) on amylose. It has been demonstrated that in the early stages of the reaction of potato D-enzyme with amylose AS-320, CAs with several hundred glucoses are produced (TAKAHA et al., 1996). As the reaction progressed, the mean product size tended towards a DP of about 90 and the yield of CA reached more than 95%. The smallest product formed was CA-17. It was later shown that the action of the amylomaltases from E. coli and Thermus aquaticus on amylose also produced cycloamyloses (TERADA et al., 1999). The former enzyme produced CA-17 and the latter CA-22 as the smallest product.

4-α-Glucanotransferases

CGTase (EC 2.4.1.19) as well as amylomaltase (EC 2.4.1.25) are 4-α-glucanotransferases. A third member of this group of enzymes is glycogen debranching enzyme (EC 3.2.1.33 and EC 2.4.1.25). These enzymes break an α-1,4 link and transfer the resulting glucan group to an acceptor molecule through creation of a new α-1,4 link. The intramolecular reaction is often called the “disproportionating reaction”:

\[
(\alpha - 1, 4 - \text{glucan})_m + (\alpha - 1, 4 - \text{glucan})_n = (\alpha - 1, 4 - \text{glucan})_{m-x} + (\alpha - 1, 4 - \text{glucan})_{n+x}
\]
An intramolecular glucan transfer yields cyclic glucan products:

\[(\alpha-1,4-\text{glucan})_m = \text{cyclic}(\alpha-1,4-\text{glucan})_n + (\alpha-1,4-\text{glucan})_{m-n}\]

**D-Enzyme and amylomaltase**

Amylomaltase was first described by Monod & Torriani (1948) as a maltose-inducible enzyme. Since then the amylomaltase gene has been cloned from several bacteria and homologous genes were identified in further bacterial genomes. It is a widely-distributed enzyme, but probably not ubiquitous since some genomes do not contain an obvious amylomaltase gene. A similar enzyme is present in plants and is termed disproportionating enzyme (D-enzyme).

Early studies on the biochemical properties focused on D-enzymes from different plant sources. These studies showed that the different D-enzymes have common reaction characteristics. The smallest donor molecule is maltotriose, the smallest acceptor is glucose and the smallest transferred glucosyl transfer.

Based on the presence of a few conserved regions, in particular the four conserved regions which are characteristic for the \(\alpha\)-amylase family, it has been proposed that these enzymes belong to the \(\alpha\)-amylase family (Heinrich et al., 1994; Svensson, 1994). It was also noted, that amylomaltase appears to be the most distantly-related member of this family (Janecek, 1997) and was therefore classified as family 77, whereas related members such as amylase and CGTase are members of the large family 13.

Amylomaltase appears to have different physiological functions in bacteria. In *E. coli*, amylomaltase is expressed with glucan phosphorylase from the same operon. It is proposed to be a member of the maltooligosaccharide transport and utilization system (Schwartz, 1987) and apparently plays a role in converting short maltooligosaccharides into longer chains upon which glucan phosphorylase can act (Takahashi et al., 1990). However, the genomes of *Haemophilus influenzae* and *Aquifex aeolicus* lack the genes coding for maltose transport proteins. In these organisms, the genes for amylomaltase are part of the glycogen operon, indicating that the enzyme might be involved in glycogen metabolism. It has recently been shown, that the plastidial plant D-enzyme is required for malto-oligosaccharide metabolism during starch degradation (Critchley et al., 2001).

We have crystallized the amylomaltase from *Thermus aquaticus* and analyzed its structure to a resolution of 2.0 Å resolution by X-ray crystallography (Przylas et al., 2000a). On the basis of the good resolution of these crystals an atomic model of all residues of the 57 kDa (500 aa) protein could be built and the conformations of ordered side chains and in particular of the acarbose inhibitor, which was soaked into the crystals could be reliably determined (Przylas et al., 2000b).

**Protein fold and topography**

Amylomaltase contains several insertions between the strands of the central \((\beta\alpha)_{8}\)-barrel (subdomain A). All insertions are present at the C-terminal side of the barrel, where the substrate binding site also is located. These insertions have been subdivided into three subdomains, in order to facilitate comparison to related enzymes (Fig. 2). Subdomain B1 corresponds to domain B in the related family-13 enzymes. The core of this domain is always composed of an insertion between the third and fourth barrel strand. Whereas in related CGTases and \(\alpha\)-amylases two strands from an insertion between the second and third barrel strand complete domain B, in amylomaltase two strands from an insertion between the fourth and fifth barrel strand are part of this subdomain. In fact, amylomaltase also contains an insertion between the second and third barrel strand (subdomain B2). This insertion, however, is considerably larger in amylomaltase and mainly \(\alpha\)-helical. Subdomain B2 is unique to amylomaltase.

The most striking difference between amylomaltase and the other members of the \(\alpha\)-amylase superfamily is the absence of the C-terminal domain C, which is present in all other structures of these enzymes determined so far. The function of this domain is not clear. When the amylomaltase structure is superimposed onto CGTase and amylase, subdomain B3 of amylomaltase is located at approximately the same position as domain C in the other enzymes and might have a similar function.

No calcium-binding site was located in the electron density maps. This finding is in agreement with biochemical data, which indicate that this enzyme is not influenced by metal ions.

**A co-crystal structure with acarbose**

In order to study substrate binding to the enzyme,
crystals of amylomaltase were soaked for 4 hours in a buffer containing 100 mM acarbose. After that, the crystals were shock-frozen in liquid nitrogen and data to 1.9 Å resolution were collected at 100 K.

Acarbose is a maltotetraose derivative that is a potent inhibitor of many enzymes of the α-amylase family. It contains the inhibitory acarviosine group in which a valienamine ring is linked to a 4-amino-4,6-dideoxy-D-glucopyranose via an α-1,4-linkage. The double bond between C5 and C7 (corresponding to O5 of a glucose unit) promotes a \( \tilde{\text{2}}\text{H} \) half-chair conformation, which resembles the presumed transition state anticipated for glycoside hydrolysis. Although it is not an ideal mimic of such a transition state, acarbose is a tight-binding inhibitor.

The active site

A surprising result of the co-crystal structure with acarbose was that the acarviosine moiety was not bound to subsites −1 and +1, as in the related family-13 enzymes. Instead, the inhibitor occupies subsites −3 to +1 of the active center (Fig. 3). No salt bridge is present between the amino group of the valienamine moiety and a protein residue. This interaction has been proposed to contribute significantly to the inhibition of these enzymes by acarbose.

The acarbose inhibitor has also not been processed as in many other cases of reactions of this inhibitor with enzymes of family 13. In the catalytic subsites −1 and +1 an intact maltose group is bound, which is nevertheless not turned over. The high pH of 9.0 of the crystallization conditions, the low reaction temperature compared to the optimum of 75–80°C for this thermophilic enzyme and the absence of a suitable acceptor unit might account for this finding. Indeed, the X-ray structure shows that two catalytically important side chains have unexpected conformations. Firstly, Asp-293, which is the nucleophile that attacks the C1 carbon of the substrate in a putative mechanism involving a covalent intermediate, is not positioned to interact with the substrate. Instead, this Asp-293 is hydrogen-bonded to Arg-291 and Asp-213. Secondly, Glu-340, which protonates the glycosidic oxygen atom of the scissile bond (UITDEHAAG et al., 1999), is, in the amylomaltase structure, bound to the oxygens O1, O2 and O3 of the glucose unit at the reducing end. In structures of α-amylase and CGTase with inhibitor, this residue is bound to O4 of the scissile bond. Thus, the two most important catalytic residues are not well positioned in the crystal with respect to their catalytic function.

However, the observed binding mode of acarbose to subsites +1 to −3 is in agreement with the analysis of the location of the cleavage sites of potato D-enzyme on maltotetraose substrates (JONES & WHELAN, 1969).

The catalytic residues

A structural superposition of the catalytic site of amylomaltase with CGTase and amylase shows that seven residues are conserved and have a similar orientation in these structures: The three catalytic residues Asp-293, Glu-340 and Asp-395 and residues Tyr-59, Asp-213, Arg-291 and His-394. These seven residues build up the core of the cat-
alytic cleft. Except for Tyr-59, they are part of the four conserved motifs of family 13. The sequence homology is too weak to detect if Tyr-59 is conserved in all α-amylase family members, as are the other six residues. As has been previously noticed on the basis of sequence comparisons, the histidine residue of the first conserved region (His122 of Taka-amylase), which is conserved in most other family members, is not present in amylomaltase.

A secondary acarbose binding site

A second acarbose molecule was located in a groove close to the active center. The distance between the reducing end of this maltotetraose and the nonreducing end of the substrate analog bound to the active site is ~14 Å. Hydrophobic contacts of Tyr-54 with glucose unit B and of Tyr-101 with unit C of the inhibitor are probably the most important interactions that determine the conformation and binding of the inhibitor to this site. The acarbose winds around Tyr-54, which is highly solvent-exposed in the unliganded structure. Tyr-101 is involved in a hydrophobic stacking interaction with glucose unit C. Overall, the second acarbose has significantly fewer interactions with the
Fig. 5. Molecular surface of amylomaltase. The two acarbose molecules are shown in addition to two putative binding paths for the smallest cycloamyloses that are formed by this enzyme.

protein compared to the acarbose bound to the active site (Fig. 4).

In contrast to the residues liganding the acarbose bound to the active site, the residues binding to the second acarbose are not strictly conserved among other amylomaltases or D-enzymes. However, the amino-acid sequence around Tyr-54 shows a pattern with several glycines and two conserved prolines (PLGPTYGSP). Tyr-54 is not conserved. It remains unclear if this binding site is conserved among amylomaltases from other species and if this binding site is occupied for the formation of smaller cycloamyloses. An alternative pathway appears possible, which goes around the 250s loop (dotted black line in Figure 5). This pathway includes a cleft which leads from the reducing end of the acarbose molecule at the active site to the outside of the barrel. There are indications that this cleft, which is near to the tip of the 250s loop, is part of the glucan-binding site and possibly needs to be occupied for the glucan-transfer reaction to occur.

Cycloamylose formation

For the formation of cyclic products, the nonreducing end of the glucan chain has to fold back to the active center. In T. aquaticus amylomaltase, the secondary binding site around Tyr-54 might help to form a curved conformation of the amylose chain in this region, which would favour the formation of cyclic products. Two loops are proposed to influence the product specificity of amylomaltase: the 460s loop (the loop around residue 460) and the 250s loop (Fig. 5).

The 460s loop is involved in substrate binding at the active-site cleft. Residues Asn-464, Pro-466, Asn-472 and Trp-473 are strictly conserved and the first and last of these residues are involved in the binding of acarbose at the active site (Fig. 3). In addition, a view of the molecular surface suggests that this loop might also sterically influence the size of the smallest cycloamylose formed (Fig. 5).

Thus, one putative binding pathway for the smallest cycloamylose products of around 20 glucoses is obtained by connecting the two acarbose molecules bound to amylomaltase, as indicated in Figure 5 by a straight white line. As outlined above, it is not clear if the secondary binding site is conserved among amylomaltases from other species and if this binding site is occupied for the formation of smaller cycloamyloses. An alternative pathway appears possible, which goes around the 250s loop (dotted black line in Figure 5). This pathway includes a cleft which leads from the reducing end of the acarbose molecule at the active site to the outside of the barrel. There are indications that this cleft, which is near to the tip of the 250s loop, is part of the glucan-binding site and possibly needs to be occupied for the glucan-transfer reaction to occur.

The 250s loop

The 250s loop (residues 247–255), which is part of subdomain B1, is located on top of a cleft which leads from the +1 binding site out of the active site groove. Two solvent-exposed hydrophobic residues sit at the tip of the loop: Tyr-250 and Phe-251. Both residues show some disorder (flexibility) in the unliganded enzyme structure as well as in the complex with acarbose. In the complex structure a weak density feature, which might correspond to a single glucose residue, is present ~4.5 Å away from the 250s loop. Furthermore, this loop is highly conserved among the amylomaltases. These findings suggest that the 250s loop is involved in substrate binding. Possibly, the loop changes conformation or becomes less flexible when larger substrates bind close to the loop.

A comparison of the molecular surfaces of α-amylase, CGTase and amylomaltase shows that α-amylase has a relatively open active-site cleft, which is formed by residues of domains A and B. In CGTase, domain B also forms part of the active site pocket. A tyrosine or phenylalanine side chain (Tyr-195 in CGTase from Bacillus circulans strain 8), which is derived from this domain, has been proposed to favour the synthesis of cyclodextrins by forming a non-polar core around which the α-glucan could wrap (NAKAMURA et al., 1994; PEN-
NINGA et al., 1995). Trp-258 is near this residue in amylomaltase and indeed also interacts with the acarbose bound to the active site by forming hydrophobic contacts to the glucose residue bound to subsite +1 (Fig. 3). These findings suggest an important role of domain B (subdomain B1 in amylomaltase) in controlling substrate and product specificity.

Acknowledgements

N.S. thanks the Deutsche Forschungsgemeinschaft for financial support.

References


Received October 22, 2001
Accepted April 18, 2002