Biologia, Bratislava, **57/Suppl. 11:** 21—27, 2002 **Review**

A possible mechanism of catalysis involving three essential residues in the enzymes of α-amylase family

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Matsuura, Y., A possible mechanism of catalysis involving three essential residues in the enzymes of α-amylase family. Biologia, Bratislava, **57/Suppl. 11:** 21—27, 2002; ISSN 0006-3088.

The three-dimensional structures of various types of enzymes of the α-amylase family are briefly reviewed. From the substrate-complexed structures so far known, a consensus structure of the active site consisting of conserved residues has been deduced. Possible roles of the three catalytic residues are discussed, and a reaction scheme is presented. In particular, the role of the aspartic acid (Asp297 in Taka-amylase A) is proposed as crucially working to give rise to distortion at the -1 subsite residue of the substrate. Moreover, a characteristic mode of substrate binding with respect to the $(\beta/\alpha)_8$-barrel in the amylase enzymes is addressed for further discussion.

Key words: α-amylase family, catalytic mechanism, substrate binding, $(\beta/\alpha)_8$-barrel, consensus structure.

Introduction

In the α-amylase enzyme family, it has been shown that the three essential catalytic residues Asp206, Glu230, and Asp297 (TAA numbering) are strictly conserved in both the amino acid sequence and the three-dimensional structure. The mutagenesis of any of these residues to other amino acids results in almost complete loss of activity. In contrast to lysozyme where degradation of bacterial cell walls consisting of β-1,4 linked GlcNAc – MurNAc chains involves the action of two essential carboxylates, glutamic (acid catalyst) and aspartate (base catalyst) acids, the presence and the role of the third residue in α-amylases has been a focus of attention. In α-amylases, the functional roles of the three catalytic residues have been discussed in terms of the structures of the active site and the substrate (analogue)-complexed structures, together with the results of mutagenesis. The roles of Glu230 and Asp206 seem to have been generally accepted as working in catalysis as acid (proton donor) and base (nucleophile), respectively. However, the crucial role of the third residue Asp297 in the reaction pathway has not been clearly described, except the fact that it is generally involved in substrate binding.

In the present article, the three-dimensional structures of enzymes of the α-amylase family are briefly summarized, mainly based on those structures for which the author himself has been concerned with the analysis. Moreover, the possible roles of the catalytic residues are described, with emphasis on that of the third catalytic residue.

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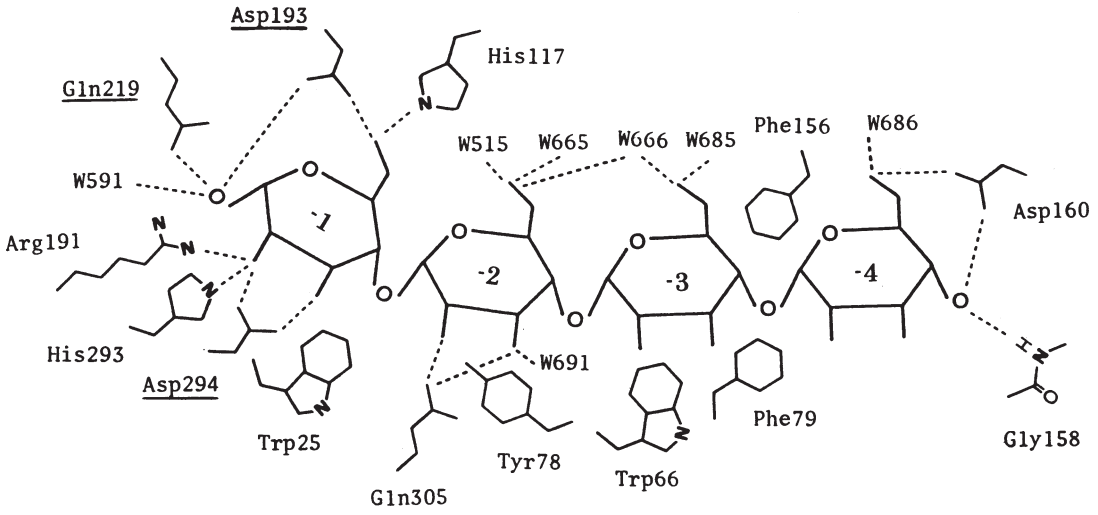

Fig. 1. A schematic drawing of the environment of the bound maltotetraose in G4-amylase. Bound water molecules are shown with W followed by a number. Hydrogen bonds are indicated by a dotted line. The catalytic residues are underlined, where Glu219 is replaced by glutamine through mutagenesis.

Structures of the active site and of bound substrate

Taka-amylase A (TAA)
The first reported structure in the α-amylase family (MATSUURA et al., 1979; 1984) was that of this enzyme. The structure of the monoclinic crystal form has been refined (KUSUNOKI et al., 1990), as has that of the orthorhombic form for both the native (BOEL et al., 1990) and for the acarbose-complexed forms (BRZOZOWSKI et al., 1997). In the monoclinic structure analysis, bound maltose was found at the active site and the hypothetical substrate-binding mode was presented, based on a molecular modeling study. Later, the acarbose complexed structure showed the essential binding mode of the substrate. The inhibitor acarbose has also been used to visualize the substrate-binding mode in other α-amylase structure analyses (QIAN et al., 1994; FUJIMOTO et al.; 1998, KADZIOLA et al.; 1998, BRAYER et al., 2000). These structures showed possible interactions between substrate and enzyme. The hydrogen bonds between the catalytic residues and the substrate are Asp206 – O1(-1), O6(-1), Glu230 – O1(-1), and Asp297 – O2, O3(-1). The structure of oligosaccharide binding at the four subsites (-1 to -4) on the non-reducing end of the cleavage point has been demonstrated, whereas the structure of the binding at the reducing-end subsites is not fully known.

Maltotetraose-forming α-amylase (G4A)
The structure of this enzyme has been determined for wild-type (MORISHITA et al., 1997) and five maltotetraose-complexed mutants (E219Q, E219G, D193N, D193G, D294) (YOSHIOKA et al., 1997; HASEGAWA et al., 1999) of the catalytic domain (G4-2). The structure of the full-length enzyme (G4-1) has also been determined (MEZAKI et al., 2001), and shows a disordered C-terminal starch-binding domain even in the crystal. The complexed structures showed detailed features of substrate binding at the non-reducing end subsites. The schematic binding mode is shown in Figure 1. This enzyme is an exo-type α-amylase which forms maltotetraose as a major product. For this reason, the mode of recognition of the non-reducing end of the substrate has been a point of interest. As shown in Figure 1, the non-reducing end is bound by the side chain of Asp160 and the main chain nitrogen of Gly158 via hydrogen bonds. Surprisingly this structure of recognition is far simpler than expected, which might explain the non-rigorous (ca 80 %) preference of its exo-type degradation. The substrate binding is stabilized by hydrogen bonds involving water molecules and van der Waals contacts with the polypeptide chain, which is characteristic of carbohydrate-protein interactions. The glucose residue at subsite -1 is especially firmly bonded to the surroundings. This firm binding is necessary for cleaving the glycosidic bond of the substrate at this site. As a result

of this firm binding, in the complexed structures except that involving mutant D294N, the glucose residue ring at the subsite −1 is deformed, adopting a half-chair conformation.

Cyclodextrin glucosyltransferase (CGTase)
This enzyme is multi-functional and catalyses cyclization of amylose fragments, coupling, disproportionation, and hydrolysis. These functions are derived from the ability of this enzyme to both cleave and form the glucosidic bond. This class of enzyme is known for the unique property of synthesizing cyclodextrins, and is named α-, β-, and γ-CGTase depending on the size of the major cyclodextrin product. The structure of this enzyme has been determined for *Bacillus circulans* (Klein & Schulz, 1991); *B. stearothermophilus* (Kubota et al., 1994; Kubota, 2001), alkalophilic *Bacillus* (Harata et al., 1996), and *Thermoanaerobacterium thermosulfurigenes* (Knegtel et al., 1996). The substrate (analogue)-complexed structures have also been determined for these enzymes, and showed a binding mode similar to that of α-amylases, but gave more information on binding at the reducing end subsite (Strokopytov et al., 1996). The polypeptide structure is different from ordinary α-amylases, specifically at the reducing end subsites reflecting the functionality of cyclization and transglycosylation. A characteristic feature is the existence of phenylalanine or tyrosine that is located at the center of the product cyclodextrin so as to help the cyclization of substrate. The enzyme has a raw-starch-binding domain at the C-terminal end of the polypeptide, but this domain is not flexible as in glucoamylase (Aleshin et al., 1992) or G4-1 (Mezaki et al., 2001). The mechanism of catalysis is considered similar to that of α-amylases with the corresponding three catalytic residues, Asp225, Glu253 and Asp324 in *B. stearothermophilus* CGTase. The pattern of hydrogen bonds at the catalytic center between substrate and amino acids (Kubota, 2001) is similar to that in α-amylases.

Isoamylase (IAM)
This enzyme catalyzes the debranching of starch by hydrolyzing α-1,6-glucosidic linkages. The structure of the enzyme isolated from *P. amyloderamosa* has been determined (Katsuya et al., 1998). It has also the same corresponding catalytic residues: Asp375, Glu435 and Asp510. The structure has been further refined and a maltotetraose-complexed structure has been obtained (Katsuya & Matsuura, to be published). These structures showed a similar binding structure at the catalytic center, and some indications specific for binding a 1,6-branched substrate.

Neopullulanase (NPL)
This enzyme catalyses hydrolysis not only of α-1,4-glucosidic but also of α-1,6-glucosidic linkages; for example, the reaction on pullulan produces panose, maltose and glucose (Imanaka et al., 1989). It is one of the cyclomaltodextrinase subgroup of enzymes, being similar to maltogenic amylase (Park et al., 2000) and TVAII (Kamitori et al., 1999). The structure determination of this enzyme has recently been performed in the native form (Hondoh et al., 2002) and that of the panose-complexed form is underway. It also has the common catalytic residues Asp328, Glu357 and Asp424 found in the α-amylase family. The preliminary structure of the complexed form has shown some characteristic features which allow binding of α-1,6-linked substrate.

Trehalose synthase (MTS)
This enzyme catalyses the intramolecular transglycosylation of maltooligosaccharide at the reducing end glycosidic linkage, converting an α-1,4 to an α,α-1,1 linkage and producing maltooligosyltrehalose. The structure of this enzyme originating from *Sulfolobus acidocaldarius* has been determined (Kobayashi et al., 2001). The catalytic center also contains the common catalytic residues Asp228, Glu255 and Asp443. The most significant feature of the catalytic site is that it forms a deep pocket at the presumed substrate reducing-end binding site at the catalytic center. This feature is considered to be responsible for the intramolecular transglycosylation ability of the enzyme, enabling enclosure of the once-released glucose unit of the reducing end inside the pocket to reform an α,α-1,1-glucosidic bond. A modeling study involving substrate and product molecules in the pocket has shown that the polypeptide structure is constructed so as to favor the binding of an α,α-1,1-linked trehalose moiety over that of an α-1,4-linked substrate.

Consensus structure in the catalytic center of the α-amylase family

All enzymes in the α-amylase family contain an $(\beta/\alpha)_8$-barrel as the catalytic domain, and the active center is located at the C-terminal end of the β-barrel in the $(\beta/\alpha)_8$-structure. The three catalytic residues Asp206, Glu230 and Asp297 lie on the C-terminal end loops of the 4th, 5th and 7th β-strands of the barrel, respectively. These three

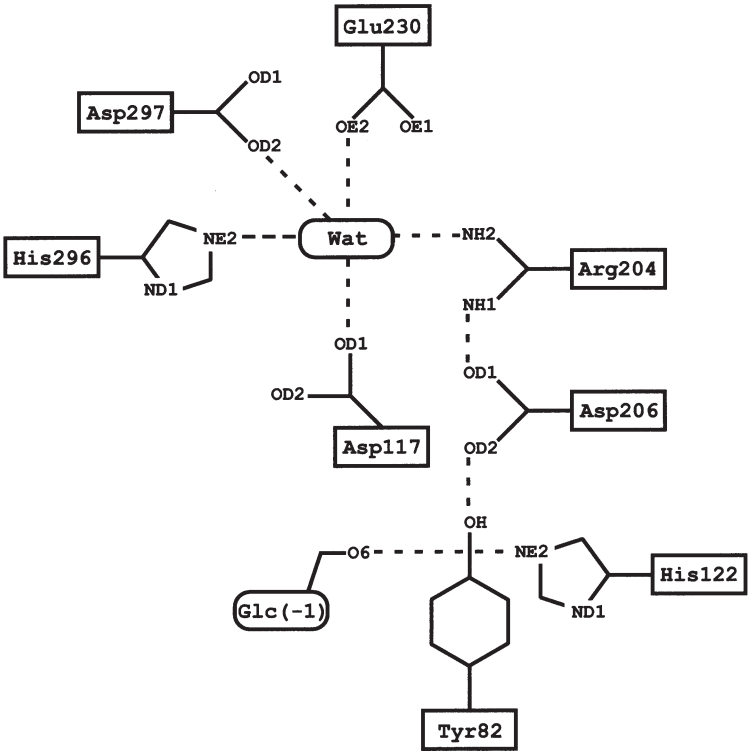

Fig. 2. Consensus hydrogen-bond network in the active site of α-amylase family enzymes. The residue numbers are based on Taka-amylase A.

catalytic residues form a triad with distances between carboxylate groups ranging from 5 to 7 Å, with no direct hydrogen bond to each other. For all enzymes in the α-amylase family such as described in the previous section, a conserved spatial arrangement of these residues including one water molecule exists (KATSUYA et al., 1998). The schematic picture of the conserved hydrogen-bond network is shown in Figure 2 with the residue numbers based on Taka-amylase A. These residues are involved in the highly conserved regions of amino acid sequence in the family. The conserved bound water molecule seems to play a key role in maintaining the constant spatial arrangement of these residues at the catalytic center. This water molecule lies very close to the point of cleavage and is replaced upon binding of substrate. It is speculated that this water molecule might be utilized for the hydrolysis reaction. Among these residues, Tyr82 is working to stack onto the plane of the glucose residue at subsite -1, and His122 hydrogen bonds to O6 of the glucose at the -1 subsite. Asp206 is working to bridge Tyr82 and Arg204 by hydrogen-bonds, probably to maintain their spatial arrangement, helping their important roles in the activity. His296 is working to keep the right orientation of the Asp297 side chain by stacking, thus helping Asp297 to exhibit its essential role in catalysis.

Possible roles of the three essential residues

In the conserved structure at the catalytic center (Fig. 2), the Arg204 imino group is always hydrogen-bonded (or salt-bridged) to the side chain of the essential carboxylate Asp206. Since the arginine imino group carries a cation, the side chain of Asp206 must be anionized. This event causes a resistance to deprotonation of the Glu230 side chain which lies close to Asp206. The side chain of Glu230 is also considered to be in a hydrophobic environment, as a hydrophobic residue is normally found just after this residue in the amino acid sequence, and overhangs the side chain of Glu230 in the three-dimensional structure. The hydrophobic environment enhances the Coulombic interaction between the Asp206 and Glu230 side chains. This leads to a rise in the pKa value of Glu 230, probably to near neutral pH as seen in the pH-activity profile (ONO et al., 1958). The side chain of Asp297 is in a more exposed and hydrophilic environment, and thus is more likely to

Table 1. Hydrogen-bonds and close contacts surrounding the reducing end glucose unit (Glc-1) in the product complexed mutants.[a]

	E219Q	E219G	D193N	D193G	D294N
O1	Q219(NE2: 3.4)	D193(OD1: 2.8)	E219(OE1,2: 3.0, 3.1)	E219(OE1: 2.5)	E219(OE2: 3.3)
	D193(OD1: 2.9)	Wat733(2.6)	Wat731(2.7)	R191(NH1: 3.2)	Wat755(3.2)
	Wat591(2.5)				
O2	D294(OD1: 2.6)	D294(OD1: 2.7)	E219(OE2: 3.1)	D294(OD1: 2.6)	N294(OD1: 2.7)
	R191(NH2: 3.2)	R191(NH2: 3.3)	D294(OD1: 3.3)	R191(NH2: 3.0)	R191(NH2: 3.2)
	H293(NE2: 3.2)	H293(NE2: 3.0)		H293(NE2: 3.1)	H293(NE2: 3.1)
O3	D294(OD2: 2.8)	D294(OD2: 2.8)	D294(OD2: 2.7)	D294(OD2: 2.6)	N294(ND2: 2.9)
	H293(NE2: 3.1)	H293(NE2: 2.9)	Wat725(2.3)	H293(NE2: 3.2)	H293(NE2: 3.1)
O6[b]	D193(OD2: 3.0)	D193(OD2: 3.0)	N193(OD1: 3.1)	H117(NE2: 3.2)	D193(OD2: 2.7)
	H117(NE2: 3.3)	H117(NE2: 3.1)	H117(NE2: 3.5)	Wat730(3.1)	H117(NE2: 3.6)
O5[c]	D193(OD1,2: 3.4, 3.5)	D193(OD1,2: 3.4, 3.7)	N193(OD1,ND2: 3.8, 3.0)	Wat730(3.0)	D193(OD1,2: 3.3, 3.5)
C1[d]	D193(OD1: 2.6)	D193(OD1: 2.8)	N193(OD1: 2.9)	–	D193(OD1: 3.1)

[a] The atom names and distances (in Å) are indicated in parentheses. Residue numbers as in G4-amylase.
[b] Long distance pairs between H117NE2 are also listed for comparison.
[c] This atom posesses potential hydrogen bond ability, and may form weak hydrogen bonds between the atoms listed.
[d] This atom has no possiblity to form hydrogen bond, but is listed here because of unusually short interatomic distances to side chain atoms at the position 193.

be ionized. As a result of these events, the side chain of the non-ionized carboxylate of Glu230 is ready to work as an acid catalyst giving a proton to the glucosidic bond of the substrate, leading to cleavage.

In the structure analyses of the complexed mutants in the G4-amylase as described earlier, we investigated the detailed structure of contacts between bound substrate and amino acids. The results are shown in Table 1 (Hasegawa et al., 1999) for interatomic distances between substrate and amino acids. The side chain atoms of Glu230 (Glu219 in Table 1) is shown always in a position close to O1 of Glc (-1). This supports the suggested role of the residue as the acid catalyst.

The side chain atom of Asp206 (193 in Table 1) is shown to be at a very short distance from the C1-atom of Glc (-1) despite the fact that they are not a potentially hydrogen-bonding pair. Since the sugar ring of Glc (-1) is deformed in the complexed structures, except in the case of the D294N enzyme, the C1-atom might also be electronically distorted, leading to a partial positive charge on the atom. The unusually close contacts (<3 Å) might be a result of strong interaction between these atoms. These facts suggest the possibility of Asp206 as base catalyst (nucleophile) in the reaction pathway, probably getting involved in forming a reaction intermediate with the substrate.

Another fact worth noting is that in the D297N (D294N in G4A) complexed mutant, deformation of the glucose ring at subsite −1 was not observed. Side chain atoms of Asp297 are always involved in hydrogen bonds between the O2 and O3 atoms of Glc (-1). In this mutant moreover they are apparently within hydrogen-bonding distance. However the fact that the Glc (-1) ring is not deformed in this complex indicates that the strength of the hydrogen bonds between the side chain atoms of Asp297 and the hydroxyl groups of the substrate are not sufficiently strong to deform the ring. This also leads to the poorer affinity for substrate binding of this mutant, which has been shown crystallographically by reduced occupancy of the bound maltotetraose at the active site in this complexed mutant. Thus we may draw the conclusion that Asp297 is centrally working to establish binding of the substrate, resulting in a deformation of the substrate ring at the cleavage point, which is indispensable for catalysis. Thus we may describe the role of this residue as "fixer" in the catalysis of α-amylase enzymes. A summary of the discussions above gives a probable scheme of reaction as depicted in Figure 3.

Substrate binding with respect to the $(\beta/\alpha)_8$-barrel

From a global point of view of the association of substrate and amylase enzymes, a characteristic mode of binding has been observed. In the substrate (analogue) complexed structure in α-

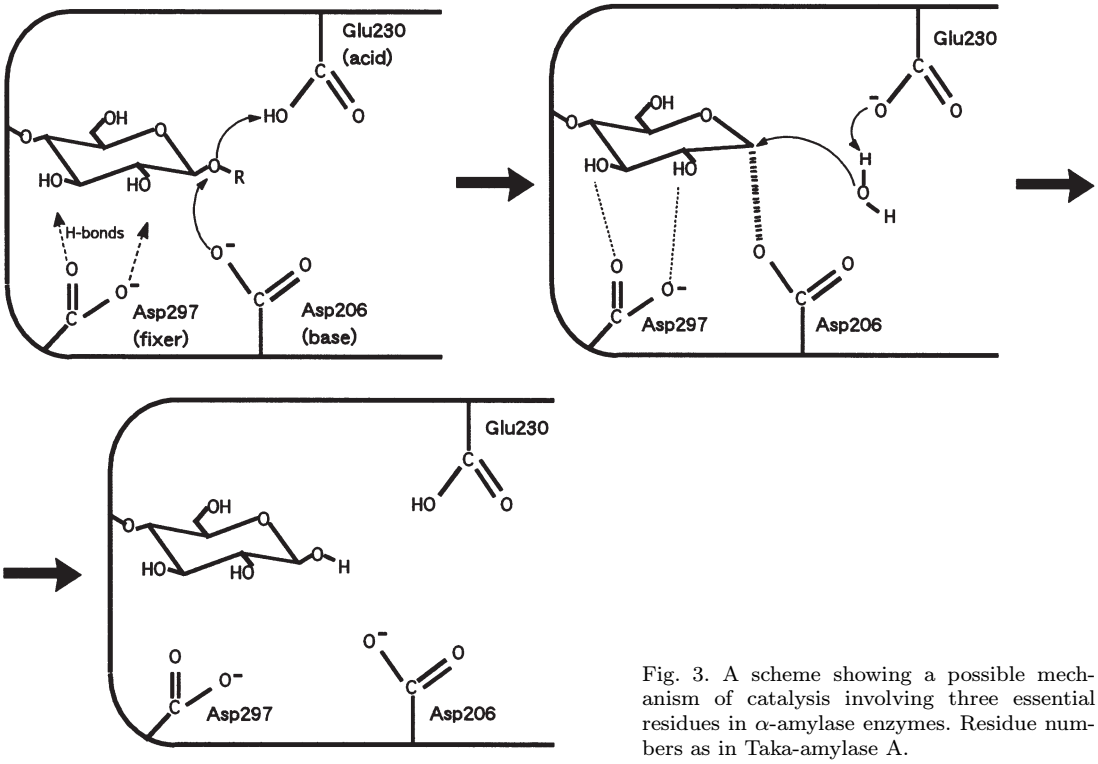

Fig. 3. A scheme showing a possible mechanism of catalysis involving three essential residues in α-amylase enzymes. Residue numbers as in Taka-amylase A.

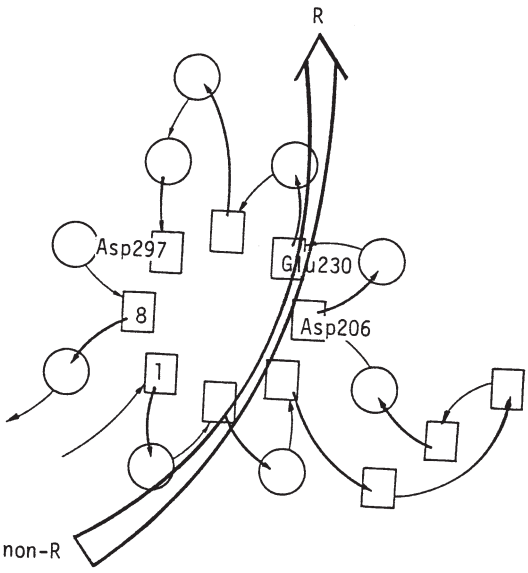

Fig. 4. Direction of binding substrate (arrow) with respect to $(\beta/\alpha)_8$-barrel in amylases. R denotes reducing and non-R non-reducing end of amylose chain.

amylases and β-amylase (MIKAMI et al., 1994), to my knowledge, the fragments of substrate amylose have been shown to bind on the $(\beta/\alpha)_8$-barrel such that the direction of the reducing/non-reducing end of the chain is as shown in Figure 4 (MATSUURA et al., 1991). The exo-wise recognition always takes place at the non-reducing end of the substrate. The catalytic site is without exception located at the C-terminal end of the 4th and 5th β-strands of the central β-barrel with the catalytic residues on the 4th, 5th and 7th (4th and 7th in β-amylase) loops. This mode of association may be the result of the interaction of the amylose chain and the electrostatic field provided by the large scale dipoles of the $(\beta/\alpha)_8$-barrel, or by the local interaction forces contributed by hydrogen bonds and other interactions. The amylose chain, composed of neutral glucose units, has only a small permanent dipole. However, the glucosidic bonds of the α-1,4-linkages may bring about, in total, a significant strength of permanent dipole. The amylose chain is thought to adopt a loose helical conformation in solution, and upon binding onto the enzyme it may adjust the conformation to what fits best to the enzyme structure. These

interaction forces may be weak, and at the same time must be greatly affected by the existing ordered/disordered water molecules.

The latter local interactions may be stronger than those of distant dipole interactions, but, in this case the residues to be involved in the binding must be arranged in such a way as to align the amylose chain with the direction of binding always as in Figure 4. The quantitative estimation of the interactive forces is important in this kind of intermolecular interactions, though it still seems difficult in such a complex system.

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Received October 4, 2001
Accepted February 12, 2002