

Structure and Possible Catalytic Residues of Taka-Amylase A

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A complete molecular model of Taka-amylase A consisting of 478 amino acid residues was built with the aid of amino acid sequence data. Some typical structural features of the molecule are described. A model fitting of an amylose chain in the catalytic site of the enzyme showed a possible productive binding mode between substrate and enzyme. On the basis of the difference Fourier analysis and the model fitting study, glutamic acid (Glu230) and aspartic acid (Asp297), which are located at the bottom of the cleft, were concluded to be the catalytic residues, serving as the general acid and base, respectively.

Taka-amylase A (EC 3.2.1.1, α -1,4-glucan 4-glucanohydrolase), an α -amylase produced by *Aspergillus oryzae* and isolated in high yield (1) from 'Takadiastase Sankyo,' catalyzes the endoamylolytic degradation of starch. The physical and chemical properties of this enzyme (TAA) have been extensively studied (2), and the three-dimensional structure of TAA has been investigated by X-ray structure analysis at 3 Å resolution using the multiple isomorphous replacement method (3, 4). As the complete amino acid sequence determination progressed (5), we constructed a complete molecular model by fitting skeletal models to the electron density results which had been refined by the molecular replacement method (6). We briefly present here the three-dimensional structure and discuss possible catalytic residues and their roles. Details of the refinement of the electron density map and the structural features of the molecule will be described elsewhere.

DESCRIPTION OF THE STRUCTURE

The TAA molecule consists of 478 amino acid residues which are folded in two large domains in the three-dimensional structure (Fig. 1). The first 380 residues from the N-terminus constitute the first domain, A, and the rest make up the second domain, B. Domain A contains a $(\beta\alpha)_8$ supersecondary structure as shown in Fig. 2. The spatial arrangement of α -helices and β -strands in the $(\beta\alpha)_8$ structure is similar to that of triose phosphate isomerase (9), pyruvate kinase (10), or aldolase (11). Domain B is an eight-stranded antiparallel β -sandwich structure. The two domains are linked by a single polypeptide chain with an extensive interface which is mainly composed of hydrophobic residues. A large cleft (Fig. 1) is located at the carboxyl end of the parallel β -barrel in domain A.

Four disulfide bonds have been assigned in the electron density map linking residues 30-38, 150-164, 240-283, and 439-474. A covalently

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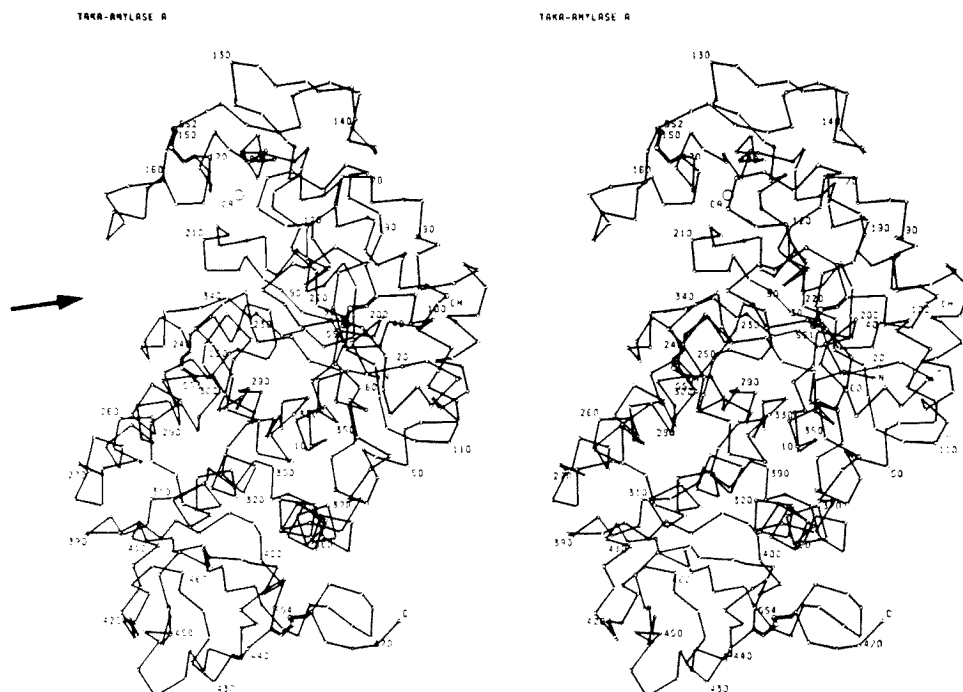


Fig. 1. Polypeptide chain folding of the α -carbon chain shown in stereo. The molecular dimension is about 80 Å (height) in this figure. A large cleft which is the active site of the enzyme is indicated by an arrow. Four disulfide linkages are shown by thick lines labeled SS1-SS4. The carbohydrate binding position (Asn197) is marked CH on the right hand side of the figure. A calcium ion is shown as CA located at the upper part of the figure. The coordinates used for plotting were refined by the use of Levitt's energy refinement program (7).

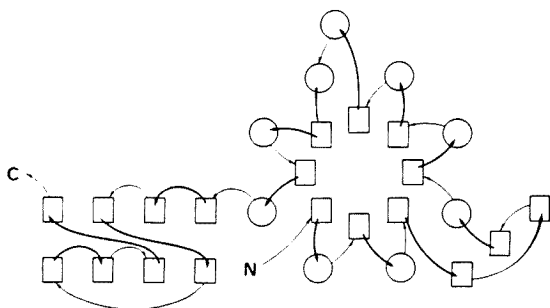


Fig. 2. Topological/packing diagram (8) showing the arrangement of α -helices ($\circ$) and β -strands ($\square$) along the polypeptide chain of TAA. The right-hand half of the figure shows the $(\beta\alpha)_8$ structure of domain A and the left shows the antiparallel β -sandwich structure of domain B.

bound carbohydrate group (12, 13) in the molecule was found in the electron density map as a large branch from Asn197. The diffused electron density of this branch suggests a disordered structure

of the carbohydrate group in the solvent region. An isolated electron density peak buried near the cleft was assigned to an essential calcium ion (14), which appears to be liganded by the surrounding residues, possibly playing a role in stabilizing the cleft architecture.

DISCUSSION

Maltotriose, a poor substrate of α -amylase, was diffused into a TAA crystal in 75% saturated ammonium sulfate solution of pH 5.5 (4). It was found in the difference Fourier map near Glu230 located at the bottom of the cleft. The shape of the difference peak (Fig. 3), however, appears to be a maltose molecule instead of maltotriose, suggesting that one glucose unit has already been removed from maltotriose, leaving maltose bound in the cleft. A binding site of inhibitors, for instance 6'-deoxy-6'-iodo-maltotriose (15), is also found at the same site in the cleft (3). These

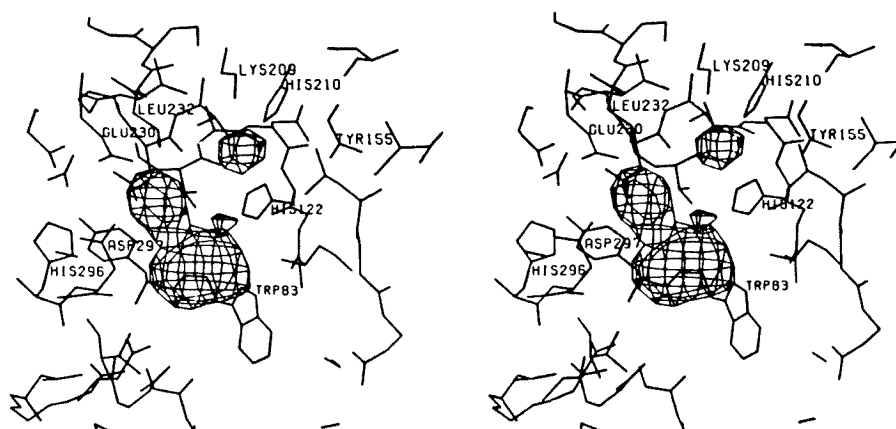


Fig. 3. Stereo view of the difference Fourier map at 3 Å resolution of a TAA crystal soaked in maltotriose solution for 6 days. Contour level at $0.15 \text{ e} \text{ \AA}^{-3}$. The surrounding amino acid residues are also shown. The concentration of maltotriose was 200 mM in 75% saturated ammonium sulfate solution buffered with 0.05 M acetate at pH 5.5.

results strongly suggest that this part of the cleft is the catalytic site of this enzyme.

The apparently small size of one half of the twin peak in Fig. 3 may be the result of exclusion of water present in the native enzyme upon binding of maltotriose. An isolated peak near the side chain of His210 appears to be a bound water molecule introduced with the maltotriose. The model fitting of a maltose molecule to the twin peak in the difference Fourier map as well as to the surrounding amino acid residues has shown that the reducing end of maltose should be pointed toward Glu230. It was also shown that the hydroxyl group at C-6 of the reducing end glucose should be placed for steric reasons at the His122 side instead of the His296 side, thus making a hydrogen bond with N ϵ of His 122. This hydrogen bond may be essential for the catalytic reaction, as suggested by Ikenaka *et al.* (16, 17) since C-6 substituted phenyl- α -maltosides are not hydrolyzed by TAA. In this binding mode, the hydroxyl groups at C-2 and C-3 of the same glucose unit make hydrogen bonds with O γ of Asp297 and N ϵ of His296, respectively.

Because the carboxyl group of Glu230 lies very close to the difference peak which was assigned to the reducing end glucose of the bound maltose, it probably takes part in the catalytic reaction considering that a maltotriose releases the reducing end glucose unit on hydrolysis by

TAA (18). The pK_a value of the carboxyl group of Glu230 could be increased due to the non-polar environment produced by Val231 and Leu232 which lie close to Glu230, so that it might be in an unionized form at pH 5.5, the pH optimum for the hydrolysis reaction by TAA. Thus, Glu230 in TAA may play a role as a general acid (or proton donor) in the catalytic mechanism of α -amylase (19, 20). On the other hand, the carboxyl group of Asp297, which is in a hydrophilic environment at a distance of about 6 Å from that of Glu230, may exist in ionized form, and play a role as a general base in the catalytic pathway. In this position, the negatively charged side chain of Asp297 would stabilize the reaction intermediate carbonium cation (19) and also help to increase the pK_a value of Glu230. The acid-base catalytic mechanism described here is similar to that proposed for lysozyme (21), which breaks a β -1,4-glycosidic linkage by the catalytic action of the same kind of amino acid residues.

The fact that cyclodextrins are also hydrolyzed by TAA is suggestive for understanding the binding mode of amylose to this enzyme. A model of cyclodextrin, for instance β -cyclodextrin which is composed of seven glucose units, was shown to fit well in the model of the TAA molecule at the bottom of the cleft, although the binding with the enzyme may not be fully productive because the rate of hydrolysis of cyclodextrin is much slower

than that of amylose (22), possibly due to the conformational restriction of the cyclic structure of the cyclodextrins. These considerations suggest that the substrate amylose could be bound in a curved configuration for productive binding. A linear amylose chain model was thus located in the cleft with two glucose units fitted to the twin peak of the difference Fourier map and also to make good contacts with the surrounding amino acid residues. A proposed substrate binding model derived from the fitting study involving seven glucose residues is schematically shown in Fig. 4. A curved binding mode could be preferred by the amylose chain which has a tendency to adopt helical configuration (23). Among the amino acid residues at the substrate binding site, His296, Asp297, His122, Glu230, Asp206, Val231, His210, Lys209, and Tyr155 are conserved in hog pancreatic α -amylase and TAA (5). The number of glucose residues located in the binding site in the present study is nearly equal to that deduced from kinetic studies (18, 24, 25). The 4th, 5th, and

6th sites are mainly composed of the conserved residues in the two α -amylases. The possible catalytic residues in TAA, Glu230 and Asp297, are both located at distances of about 3 Å from the glucosidic oxygen between the 4th and 5th site glucoses (Fig. 4) to break the glucosidic bond. This is in agreement with kinetic studies which have shown that the cleavage point lies between the 4th and 5th subsites.

The chemical modification of histidines of TAA showed that the ethoxycarbonylation of histidines decreases amylase activity but increases maltosidase activity (26). The maltosidase activity can be enhanced if the phenyl- α -maltoside occupies the 3rd and 4th sites with the maltosidic group and the 5th site with the phenyl group, which may have hydrophobic interaction with the side chains of Val231, Leu232, and the ethoxy group attached to the side chain of His210, which appears to be the most accessible histidine residue to such reagents around the active site, thus increasing the binding affinity of phenyl- α -maltoside to the en-

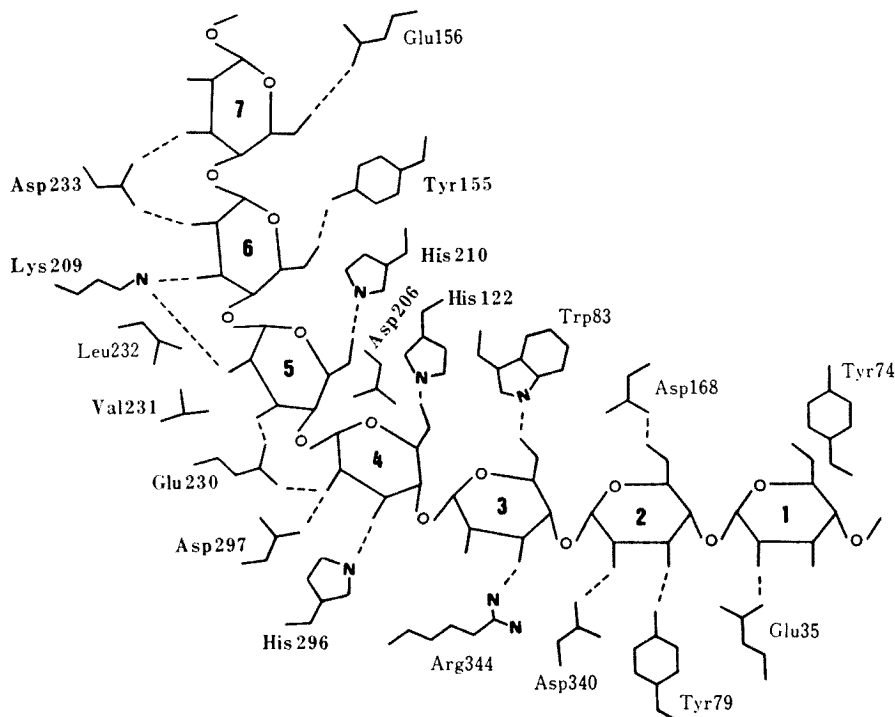


Fig. 4. Proposed substrate binding model of TAA. The numbers specified in the glucose residues indicate the site number assigned on the protein molecule. The dashed lines represent possible hydrogen bonds between amylose and the enzyme. The boldfaced letters indicate the amino acid residues that have been shown to be conserved in hog pancreatic α -amylase and TAA (5).



Fig. 5. Stereo view of the $(\beta a)_8$ structure of the polypeptide backbone together with the outline of the twin peak (guitar shape in the figure) in the difference Fourier map obtained from the crystal soaked in maltotriose solution (see also Fig. 3). The difference peak is located at the C-terminus of a β -strand.

zyme. The ethoxycarbonylation of His210, on the other hand, might cause loss of the hydrogen bond between amylose and the side chain of His210, decreasing the amylase activity of the enzyme. Similar behavior of phenylazobenzoylated TAA has also been reported (27).

The bound maltose is located at the carboxyl end of the parallel β -barrel in the $(\beta a)_8$ structure as depicted in Fig. 5. The substrates of triose phosphate isomerase (9), pyruvate kinase (10), and aldolase (11) are all phosphates and have been shown to bind at the C-terminus of the parallel β -barrel and/or the N-terminus of the α -helices in the $(\beta a)_8$ structure. The substrate binding position in TAA is similar to those in the above glycolytic enzymes, in spite of the fact that the substrate amylose of TAA is not a charged phosphate but a neutral carbohydrate. The principal attractive force of the substrate to the molecule of TAA might be dipole interaction between amylose and the parallel β -barrel, as suggested by Hol *et al.* (28, 29) in an attempt to understand the nature of protein folding and substrate binding. The helically arranged dipole moments of each glucose residue on the amylose chain might interact with that of the parallel β -barrel of TAA, in which the negative end of the dipole is pointed toward the C-terminus of the β -barrel. The surrounding eight strands of α -helix are arranged antiparallel with respect to the central β -strands, producing a large electrostatic field due to the strong helix dipoles. The position of the bound maltose, however, lies

very close to the C-terminus of a central β -strand rather than to the N-termini of the surrounding α -helices. A large electrostatic field produced by the whole $(\beta a)_8$ structure may also serve as a widely extending attractive field to the substrate. The electrostatic field, however, could influence the whole catalytic reaction process, that is, not only the binding of the substrate but also the transition state of the reaction and the release of the products. It is also possible that the similarity in the position of substrate binding between glycolytic enzymes and TAA can be understood on the basis of the overall reaction processes which take place on the surface of the enzymes.

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