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CONSERVED RESIDUES OF LIQUEFYING α-AMYLASES ARE CONCENTRATED IN THE VICINITY OF ACTIVE SITE

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Three dimensional structure of three liquefying type *Bacillus* α-amylases were modeled based on sequence analyses and refined structure of *Aspergillus oryzae* enzyme. The models suggest that the overall folding motif of α-amylases is conserved. The active site, substrate binding and stabilizing calcium binding residues are conserved and concentrated in a cleft between two domains. They constitute the core of α-amylases to which other, less conserved regions are attached. The bacterial enzymes have a loop of about 45 residues near the active site and Ca^{2+} binding region. The loop may be important for the liquefying function of these enzymes. © 1990 Academic Press, Inc.

α-Amylases can be divided into two classes according to the degree of starch hydrolysis. Saccharifying enzymes hydrolyse 50-60% and liquefying α-amylases 30-40% of the glucosidic linkages of starch [1]. Several α-amylases have been studied in details and their DNA and amino acid sequences are known (for a review see [2]), but the three dimensional structure is known only for Taka-amylase A of *Aspergillus oryzae* [3] and for the porcine pancreatic enzyme [4].

We have been studying production and secretion of the liquefying, thermostable *Bacillus stearothermophilus* α-amylase [5-7] and its structure-function relationships by site-directed mutagenesis (Vihinen et al., submitted). Because knowledge about the structure of the enzyme is important in this kind of studies, three dimensional structures of three liquefying *Bacillus* α-amylases were modeled. Sequence comparisons have shown existence of some highly conserved regions in α-amylases [8-10]. According to sequence analyses and models conserved residues constitute the regions important for the function of α-amylase: the active site and substrate binding residues and the amino acids important for binding the stabilizing Ca^{2+} ion. The model of *Bacillus stearothermophilus* α-amylase has been used to target mutations for protein engineering and to interpret altered properties of mutant enzymes.

Abbreviations: BAA, *Bacillus amyloliquefaciens* α-amylase; BLA, *Bacillus licheniformis* α-amylase; BStA, *Bacillus stearothermophilus* α-amylase; PPA, porcine pancreatic α-amylase; TAA, *Aspergillus oryzae* α-amylase.

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METHODS

The structures of liquefying *B. stearothermophilus* [5], *Bacillus amyloliquefaciens* [11] and *Bacillus licheniformis* [12] α-amylases were modeled based on the structure of TAA taken from Brookhaven databank. The coordinates of porcine pancreatic α-amylase were not available. Sequence comparisons were performed according to Vihinen [9] and sequence alignments according to Needleman and Wunsch [13]. Profound sequence analysis is important to avoid erroneous sequence alignments leading to wrong models [14]. Modeling was performed on Evans & Sutherland PS330 and PS390 terminals with programs Frodo [15] Hydra (Polygen Corp., Waltham, MA), Insight (Biosym Technologies, Ltd., San Diego, CA) and Gromos (Biomos b.v., Groningen, The Netherlands).

MODELS

Sequence comparisons of α-amylases [8], the liquefying α-amylases [9] and several other amylolytic enzymes [10] have revealed conserved regions. Number of these regions has varied in different studies [16], but all the comparisons agree on the amino acids involved in the active site and in the substrate binding regions. Sequence analysis was performed both on sequence and secondary structural level to find the correct alignments [9, 10; Vihinen in preparation]. Generally three dimensional structures and secondary structural elements of proteins are more conserved than primary sequences and thus secondary structural features were compared, too. The sequence similarity of the liquefying α-amylases with TAA, 40-50 %, is high enough for modeling especially when secondary structures are conserved.

α-Amylase is an $(\alpha/\beta)_8$ barrel enzyme, which consists of three domains A, B and C (Fig. 1). The main domain A forms the barrel to which the other domains are linked. The domain B of about 100 residues is located between the third β-strand and the third α-helix of domain A. The C terminal domain is about 110 residues. The active site is situated in a cleft between domains A and B (Fig. 1) and the conserved regions are located near it (Fig 2.). A loop of about 45 residues in domain B is typical only for the liquefying *Bacillus* α-amylases because it has not been found in other α-amylases. This region was not modeled. It could be important for the liquefying function, since it is located near the active site and could interact with long chain substrates. About 20 residues from the C terminal end could not be modeled because of lack of coordinates in TAA.

Replacements were introduced according to alignments. Parameters from energy minimizations cannot be used to determine reliability of the models, since the models are not for complete molecules. However, the relative energies of minimized TAA and the modeled α-amylases are of the same order. The overall fold of α-amylases is highly conserved [4]. Deletions and insertions appeared outside secondary structural elements, thus the core of the models is rather accurate especially in the most conserved areas near the active site. The carboxy terminal domain and altered loops on the surface are the least accurate parts of the models.

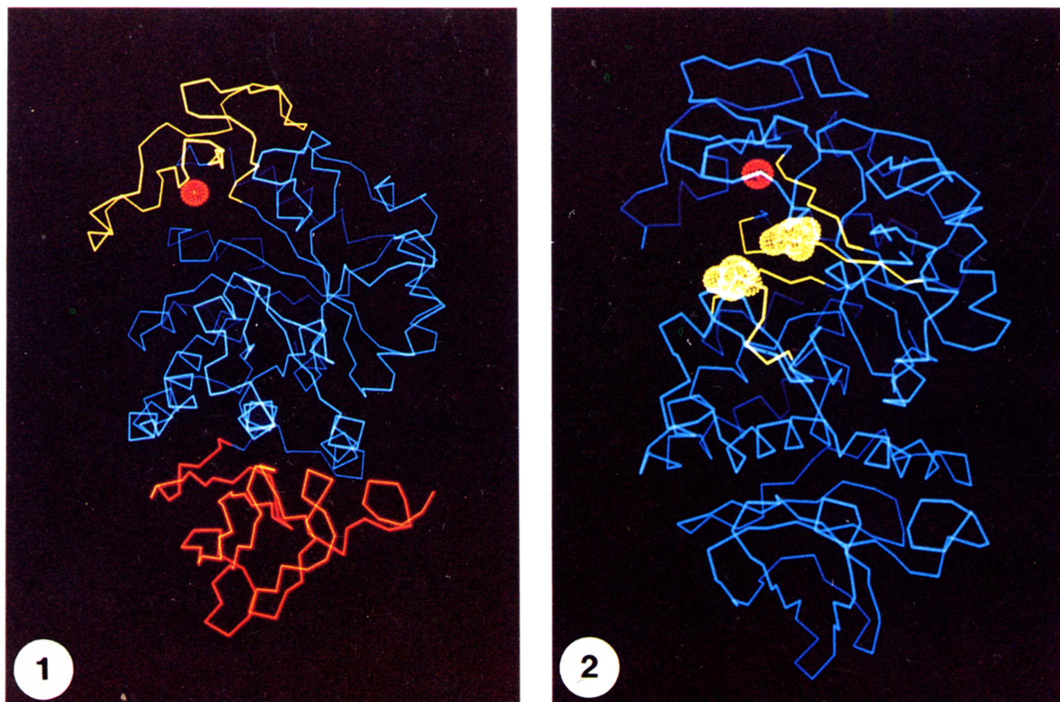

Figure 1. Computer model of the backbone of *Bacillus stearothermophilus* α-amylase. The three domains are indicated with different colours. The upmost domain is B, in the middle domain A and the bottom domain C. Stabilizing Ca^{2+} is shown by dot surface.

Figure 2. Conserved sites are located in the vicinity of the active site in the liquefying *Bacillus* α-amylases. The conserved sites in *B. stearothermophilus* enzyme are indicated with yellow. Dot surfaces show active site Asp 234 and Asp 331. Calcium ion is shown by red dot surface.

Catalytic site and substrate binding residues

The conserved residues constitute the active site and the substrate binding cleft (Fig. 2). The catalytic cleft including the active site and the substrate binding sites is structrually the most restricted region. These residues tend to have similar spatial arrangements based in the models, suggesting similar catalytic action in α-amylases regardless of origin. The substrate is bound to several subsites on the surface of the protein and the active site is one of these subsites [3]. Matsuura et al. [3] propose that Glu 230 and Asp 297 and Buisson et al. [4] that Asp 206 and Asp 297 are catalytic residues in TAA and that Glu 230 is presumably involved in substrate binding. We suggest that Asp 234 and Asp 331 are the catalytic residues in BStA and aspartates 231 and 328 in BAA and BLA. Mutagenesis of Asp 331 to Glu in BStA inactivated the enzyme almost totally (Vihinen et al., submitted). Although the glutamate is conserved in all these sequences the models suggest that the aspartates serve as the active residues. The structure of PPA supports this hypothesis, as well. Mutagenesis will reveal the function of these residues. At least some of the subsites are present also

in the *Bacillus* enzymes, since the most conserved regions constitute the surroundings of the active site. However the exact number of subsites in the enzymes cannot be predicted from the models.

Calcium binding site

α-Amylases are metalloenzymes containing at least one Ca^{2+} ion [2], which stabilizes connection between the domains A and B [4, Vihinen et al., submitted]. Ca^{2+} is a ligand for four residues in TAA; Asn 121, Asp 163, Asp 175 and His 210 [4] and presumably also in the modeled enzymes eg. in the BStA Asp 105, Asp 190, Asp 203 and His 238. His 210 of TAA binds Ca^{2+} presumably by the main chain oxygen [4]. This residue has been suggested to be conserved because it could direct the proper orientation of a flexible substrate in the active site (Vihinen et al., submitted). Because mutation of His 238 to Asp in BStA decreased specific activity to 12%, the side chain has to be very important for the activity. This mutation also somewhat decreased thermostability presumably due to altered backbone dihedral angles, which prevent the mutant protein from binding efectively the Ca^{2+} ion and thus the domains to each other. Also the requirement for calcium in almost all known α-amylases [2] indicates that the folding motif have to be quite similar at least in the vicinity of the active site. In addition to the Ca^{2+} binding residues, only two of which are located in the conserved regions, some residues forming the interface between the domains are conserved. The interface could be important for the integrity and stability of the enzyme.

The models and sequence comparisons indicate that the overall fold of α-amylases is conserved and the residues involved in the structure and function of the enzyme are highly conserved and situated in a cleft between two domains. These residues constitute the core to which other regions, including the extra loop of the liquefying *Bacillus* α-amylases, are attached. Modifications appeared mainly on the surface and in the loops of the enzyme. This data has been used to direct alterations on interesting regions by protein engineering and to interpret results obtained from altered proteins.

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