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THE CATALYTIC MECHANISM OF α-AMYLASES BASED UPON ENZYME CRYSTAL STRUCTURES AND MODEL BUILDING CALCULATIONS

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Based upon the known crystal structures of Taka-amylase A and the recently refined Porcine pancreatic α-amylase inhibitor complex a mechanism of catalysis in amylase active centers is proposed. The mechanism differs significantly from the well-known lysozyme model of catalysis. The hydrolysis is catalyzed by three carboxyl groups and it starts from a water nucleophilic attack and opening of the glucose ring in the catalytic center rather than from protonation of the glycosidic oxygen. The main supporting experimental observations are briefly discussed.

Since the cornerstone works of Phillips et al. on Hen Egg White Lysozyme (HEWL) (1) the S_N2 mechanism of HEWL with a carbonium ion or a glycosyl-enzyme intermediate has become a textbook example and a paradigm for studying other glycanases. Recently the crystal structures of two α-amylases: Taka Amylase A from *Aspergillus oryzae* (TAA) (2), Porcine Pancreatic α-Amylase (PPA) (3) and the structure of PPA with a carbohydrate inhibitor (4), have been refined. In Fig.1 the inhibitor conformation in the PPA binding region is shown (4). The enzyme catalytic groups are clearly targeted to the position of the substrate glycosidic oxygen, and, therefore, at first it seems that reaction can follow the HEWL-like mechanism. However, a more careful analysis shows that the structure in Fig.1 resembles a later intermediate state of reaction rather than the initial productive enzyme substrate complex and, moreover, that it is unlikely that hydrolysis starts with protonation of the glycosidic oxygen.

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ABBREVIATIONS: HEWL - Hen Egg-White Lysozyme (EC 3.2.1.17); TAA - Taka Amylase A (α-amylase from *Aspergillus orysae*, EC 3.2.1.1); PPA - Porcine Pancreatic α-amylase. (EC 3.2.1.1); NMR -Nuclear Magnetic Resonance.

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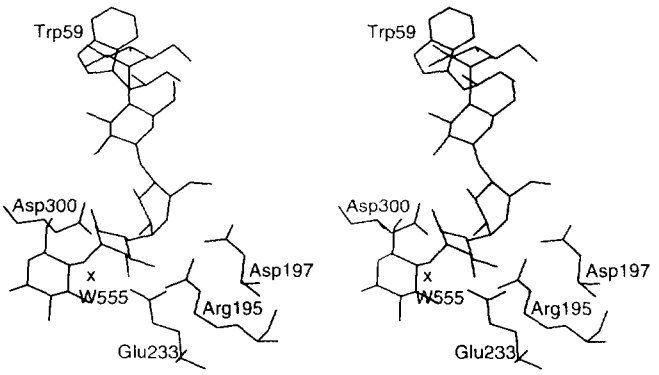

Figure 1. The structure of the carbohydrate inhibitor bound in the PPA active center as refined by X-ray crystallography (4). The enzyme residues involved in catalysis and referred to in the text are also shown. The pentamer inhibitor is produced during soaking of enzyme crystal by acarbose (4,16). The central ring in the chain is a non-sugar cyclohexene residue. It is connected with the next pyranose ring by a secondary amino group which occupies the position of the O-glycosidic bond being attacked in true substrates. In the PPA-inhibitor complex this nitrogen is strongly hydrogen bonded with catalytic carboxyls of Glu233 and Asp300 (4). The inhibitor is viewed from the protein to show the direction of the first nucleophilic attack suggested in the text.

One of the most compelling arguments that has lead us to the above conclusion concerns specific α-amylase substrates - maltocyclodextrins. A striking feature of the inhibitor conformation in Fig. 1 is a flip of the sugar chain at the suggested place of enzyme attack. Calculations show that without such a flip none of the three catalytic side chains can come close to the glycosidic oxygen. It is clear, however, that it must be difficult to flip the sugar chain in a small cycle. Moreover, it is known that cyclohexa- and cycloheptadextrins are strained molecules with the bond angles at glycosidic oxygens increased above the ideal value by 4.0^{o} and 2.7^{o} , respectively (5). In our calculations we found that in both these molecules there are unfavorable Van der Waals interactions even in the relaxed state and any chain rotation encounters energy barriers of tens of kcal/mole.

The alternative α-amylase catalytic mechanism is presented in Fig. 2. The basic idea comes from assigning the function of the third residue of the catalytic triad, Asp197. We postulate that after substrate binding the carboxyl group of Asp197 forms a hydrogen bond with the cyclic oxygen of the glucose residue at the catalytic center. However, the necessary proton probably does not reside on this carboxyl group in the free enzyme state. After the cyclic oxygen of the substrate glucose residue comes close to the charged carboxyl group of Asp197 a low-energy proton state is formed between two oxygen atoms. Due to a conformational fluctuation the side chain of Asp197 rotates around C_{α}-C_{β} bond and the proton from the O_{δ}(Asp197)-N_{η}(Arg195) hydrogen bond moves to the newly formed energy minimum. Simultaneously its previous position is reoccupied by a proton coming from the proton transfer chain which is drawn schematically in Fig. 3.

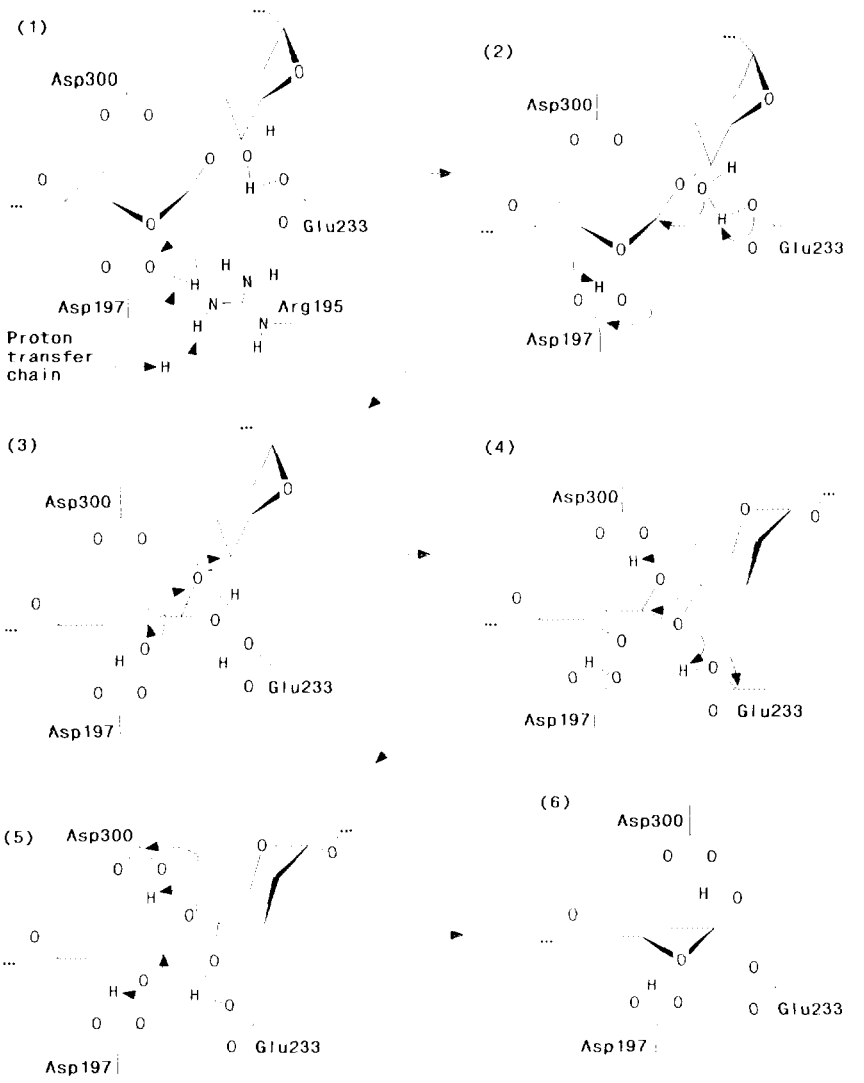

Figure 2. The main stages of catalysis by PPA. Hydrogen bonds are shown by dash lines. Step 1. A proton is supplied from the transfer chain to the new low-energy state formed between the cyclic sugar oxygen and O_{δ}-Asp197. The dotted arrows show the proton movement. Step 2. A nucleophilic attack at C_1 by the water molecule bound in the active center. Reaction is catalyzed by Glu233 and Asp197 and goes via a planar intermediate state. Step 3. Rotation of the newly formed hemiacetal center as shown by the arrows to make the chain oxygen exposed to Glu233. The sugar chain takes the flipped conformation. The flip gives better binding in the aglycone part of the enzyme binding region, which is the driving force of the rotation. Step 4. Cleavage of the chain by protonation of the hemiacetal oxygen catalyzed by Glu233 and Asp300. Step 5. A nucleophilic attack at C_1 catalyzed by Asp197 and Asp300. The fixed orientation of the carbonyl group in the catalytic center leaves only one possible direction for this attack and warrantees an alpha anomeric configuration of the newly formed reducing end.

In TAA the catalytic proton transfer chain is relatively short with an active center histidine acting as an initial proton donor. In PPA the structure of the chain is strikingly more complicated. In this case a complex hydrogen bond network starting at Tyr62 forms a continuous proton

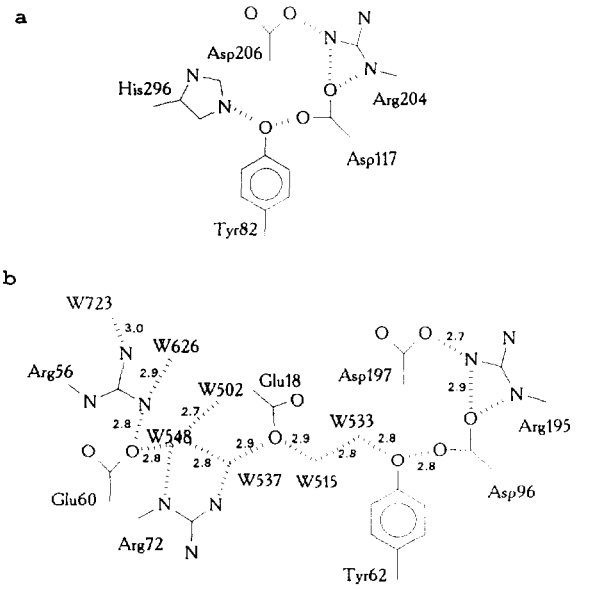

Figure 3. The structures of the catalytic proton transfer chains in TAA (a) and PPA (b). Hydrogen bonds are shown by dash lines. For PPA only a part of the hydrogen bonding network is shown with the distances of the main bonds involved in the proton transfer. The four homologous residues Asp-Arg-Asp-Tyr are conserved in all α-amylase sequences. With the polysaccharide bound in the active center this chain is shielded from the solvent. In PPA water molecules, W533, W515, W537 and W548, are buried in the protein body. The pair of arginine residues, Arg56 and Arg72, are located at the protein surface aside from the substrate binding region, with W723 and W626 belonging to the solvent hydratation shell and W502 also solvent exposed. The lengths of all chain hydrogen bonds are short enough for the proton transfer (17) which can be additionally facilitated by low-dielectric intra-protein environment (18).

pathway trough the protein body to a distant surface site. This difference between TAA and PPA structures and a strikingly long proton pathway in PPA correlates with unique ability of pancreatic amylases of mammals to catalyze several bond cleavages in the same sugar chain without dissociation (6-9). According to Fig.2 Asp197 remains protonated after the catalytic cycle. It is quite likely that this proton has a high probability to be taken by a stronger acceptor, like Glu233, or water molecules which replace the leaving group. In TAA the remaining substrate fragment protects the proton transfer chain from the solvent, and complete substrate dissociation is necessary to "recharge" it. In PPA, however, the proton transfer chain can supply protons with the substrate bound in the active center and probably this is the key structural factor of the multiple attack mechanism (6-9).

According to Fig.2 the hydrolysis starts from a nucleophilic attack by a water molecule hydrogen bonded with Glu233 in the enzyme catalytic center. This place is completely protected from the solvent, therefore, the catalytic water must be bound before the substrate and it is likely to be present in the free enzyme as well. Water 555 shown in Fig.1 satisfies these conditions although it is not directly targeted to C_1. Calculations show that when a substrate chain is docked

at the position of the inhibitor a small free room is left between Arg195, Glu233 and Asp197 close to the ideal position for the nucleophilic attack at C_1. Water 555 is likely to be able to move to this position. Moreover, in the free enzyme structure there is an additional water molecule (W643) approximately at this position which is hydrogen bonded with N_η of Arg195 (3). The inhibitor pushes this water out getting deeper into the cleft because it models the intermediate reaction state where the catalytic water has been already used.

Our mechanism reveals the catalytic function of several repetitive structural motives in α-amylase active centers; for instance, three catalytic carboxyl groups and several conserved residues involved in the proton transfer. Since the HEWL-like mechanism needs nothing but two carboxyl groups the intricate α-amylase machinery seemed quite puzzling before. We believe that the striking correspondence of the enzyme structure to the mechanism proposed is a very important supporting argument for our hypothesis. Earlier there has been considerable discussion of the possibility of ring opening in the acid catalyzed hydrolysis of glycopyranosides (10) and for HEWL catalysis (11), but it is still considered just as an interesting hypothesis. In case of amylases, however, there are rather many experimental observations which better agree with our mechanism than with the HEWL-like models of catalysis. We can mention, for instance, complexity and substrate dependence of pH-profiles (12), pH-dependence of the multiple attack (7), the results of site directed mutagenesis of histidine residues in the active center (13), observation of an intermediate with a new peak in the acetal region of the ^{13}C NMR spectrum (14). Leaving the detailed discussion for a later publication we discuss only one simple and clear result which evidence to our mechanism rather than to the HEWL-like model.

It was reported recently that in the *Saccharomycopsis* α-amylase, which is highly homologous to TAA, mutation Trp84Leu greatly enhances transglycosylation with respect to hydrolysis (15). According to the HEWL mechanism an intermediate carbonium ion (or a sugar covalently linked to the enzyme) is bound in the glycone part of the active center. The C_1 carbon of the intermediate is attacked by a water molecule or a hydroxyl group of an acceptor, which results in hydrolysis or glycosyl transfer, respectively. The mutated tryptophane residue (15) is located in the glycone part of the binding region approximately at position of Trp59 in Fig.1 so it cannot affect the binding of the acceptor. The only possible effect of this mutation can be a displacement of the glycone sugar chain, but the shift of the target carbon must be very small especially in the case of a glycosyl enzyme. However, even if the target carbon atom is shifted significantly this must equally influence the rates of hydrolysis and transfer, and the observed experimental result (15) looks very strange from the viewpoint of any HEWL-like mechanism.

The hydrolysis/transfer branching in our model occurs at state (5) of Fig. 2. The carbonyl carbon can be attacked by O_6 of the opened glucose residue, as in Fig.2, or by an external hydroxyl group, which results in state (4). These reactions are catalyzed by different enzyme groups and go via different transition states. Mutation Trp84Leu in *Saccharomycopsis* α-amylase (15) probably shifts the glycone sugar chain so that the conformation of the opened glucose ring is changed and the free energy barrier of the ring closure is raised. This slows down the rate of the ring closure (transition (5)↦(6) in Fig.2) thus making the glycosyl transfer more probable (transition (5)↦(4)). We can even predict that mutations in the glycone part of the binding region

are generally likely to shift the balance to the glycosyl transfer because any disturbance of the substrate binding here should slow down both opening and closure of the glucose ring.

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