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MULTI-FUNCTIONAL ROLES OF A HISTIDINE RESIDUE IN HUMAN PANCREATIC α -AMYLASE

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SUMMARY: Functional roles of histidine residues at the active site in human pancreatic α -amylase were examined by protein engineering. Three histidine residues at 101, 201, and 299 were converted to asparagine residues, respectively. It was found that His201 played multi-functional roles concerning so many functions; substrate binding, control of optimum pH, change in substrate specificity, activation by chloride ion, and inhibition by a proteinaceous inhibitor.

Mammalian pancreatic α -amylase is a principal enzyme for digestion of starch in small intestine (1,2). The function and the subsite pattern have been studied with porcine pancreatic α -amylase (PPA) (3). From the sequence homology in the active site of α -amylases among animal, plant, and microorganism (4), and from X ray analysis of PPA, catalytic residues of mammalian α -amylases were regarded to be two carboxyl residues which are identical with those of a mold α -amylase, Taka-amylase A (TAA) (5-7). There are many functional differences, however, between PPA and TAA in optimum pH (8,9), regulation with an activator (9-11) and a proteinaceous inhibitor (12), and effects of chemical modifications (13-15) etc.. These differences may be related to physiological significance of mammalian α -amylases for the catalytic action in small intestine. It is expected that functional characteristics of mammalian α -amylases must be reflected

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in the roles of particular amino acid residues, since X-ray crystallographical studies show that the active site structures of PPA and TAA resemble each other.

We targeted histidine residues in α -amylases because the activity and specificity of PPA are controlled sensitively by the chemical modification of histidine residues (14,15). There are three histidine residues (101, 201, 299) in the active site of mammalian α -amylases, according to the information on the three-dimensional structure of PPA and the sequence homology among mammalian α -amylases (4,5). Human pancreatic α -amylase (HPA) has close sequence homology (83%) with PPA (4,16) and its structural gene has been obtained (17). Three mutant human pancreatic α -amylases of which each histidine residue (101, 201, 299) is converted to asparagine residue were synthesized and the functional roles of the histidine residues were examined.

EXPERIMENTAL PROCEDURES

The plasmid (YEp-HPASIG) containing the GAL10 promoter, the HPA structural gene, and the signal sequence was constructed to secrete HPA by yeast (17 and unpublished data). With site-directed mutagenesis (18), the three mutant HPA's of which each histidine residue (101, 201, 299) is converted to asparagine residue (H101N, H201N, and H299N) were prepared. The yeast transformed with the plasmids were cultured in SD media (19) containing 4% galactose as a carbon source at 30°C for 120 h. About 1 mg of purified mutant HPA's were obtained from 1L culture medium. Mutant HPA's were stable in cold neutral buffer solution for at least a few months.

Amylase activity was measured with reductometry (20) using maltohexaitol as a substrate (21). Hydrolytic activity for p-nitrophenyl α -D-maltoside (G_2-PNP) was measured with a spectrophotometrical method at 400 nm (14,15).

RESULTS AND DISCUSSION

Hydrolytic activity of mutant HPA's. HPA and other α -amylases have the activity to hydrolyze a synthesized substrate, p-nitrophenyl α -D-maltoside (G_2-PNP), to maltose and PNP. We call "maltosidase activity" for this activity in distinction from "amylase activity" which means an activity for the hydrolysis of α -1,4-glucoside bond.

The optimum pH of the wild-type HPA is 6.9 for amylase activity and 5.2 for maltosidase activity (21,22). Fig. 1 shows the pH profiles of amylase and maltosidase activities for wild-type and mutant HPA's. Amylase activities of H101N, H201N, and H299N are 1, 26, and 3% of the wild-type, respectively. The optimum pH for the amylase activity of H201N was changed to 5.2

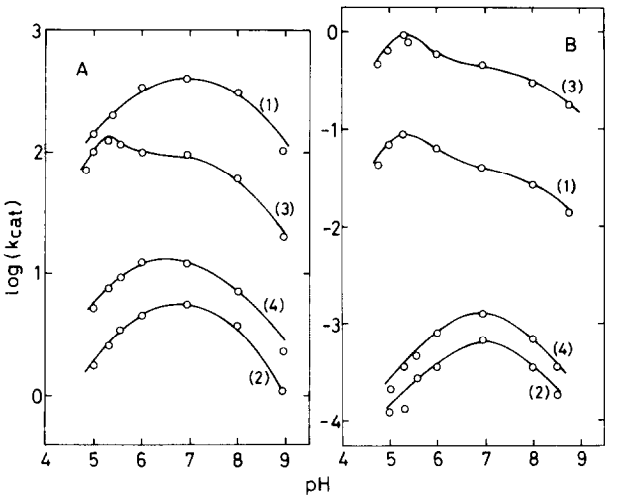

Fig. 1. pH Profiles of amylase (A) and maltosidase (B) activities for wild-type HPA (1) and HPA mutants (H101N (2), H201N (3), H299N (4)). Amylase activity was measured in the buffers (20 mM sodium acetate buffer (pH 4-5.7), 20 mM sodium phosphate buffer (pH 6-8), and 20 mM sodium borate buffer (pH 8.5-9)) containing 25 mM NaCl at 30°C with reductometry using maltohexaitol (G-G-G-G-G-GOH) as a substrate. The reaction for amylase activity is G-G-G-G-G-GOH → G-G-G + G-G-GOH, where G is a glucose and -GOH is a sorbitol bound to the reducing end of maltooligosaccharides. Maltosidase activity was measured under the same conditions as that of amylase activity with a spectrophotometrical method using p-nitrophenyl α -D-maltoside (G-G-PNP) as a substrate. The reaction is G-G-PNP→ G-G + PNP where -PNP is a p-nitrophenol bound to the reducing end of maltose.

from 6.9, whereas those of the others stayed around neutral pH. As for maltosidase activity, H101N, H201N, and H299N showed 1, 1120, and 3% of the wild-type activity, respectively. The optimum pH of maltosidase activity of H201N stayed 5.2 but those of H101N and H299N were shifted to 6.9. The substrate dependent shift of optimum pH observed in the wild-type HPA (unpublished data) and PPA (21) is lost for the H101N and H299N HPA mutants.

The drastic shift of optimum pH of H201N from 6.9 to 5.2 for amylase activity indicates that His201 plays an important role for controlling optimum pH of HPA. So far, slight optimum pH shifts have been observed mainly when the pK values of the catalytic residues are affected by neighbor charged residues (23-25). His201 of HPA contributes to both substrates specificity and the control of the optimum pH. Since both His101 and His299 are located near the catalytic center of PPA, they may be related to the catalytic mechanism directly or indirectly in spite of weak residual activities (26).

Table I. Functional change of HPA's

	wild-type HPA	H201N
Amylase act.		
k_{cat} (s^{-1}) (%)	410 (100%)	107 (26%)
K_m (mM)	0.87± 0.15	2.75± 0.32
Maltosidase act.		
k_{cat} (s^{-1}) (%)	0.040 (100%)	0.448 (1120%)
K_m (mM)	4.2± 1.18	15.1± 2.4
Residual activity of E-I complex[a]		
Amylase act.		
k_{cat} (s^{-1}) (%)	0 (0%)	4.28 (4%)
Maltosidase act.		
k_{cat} (s^{-1}) (%)	0 (0%)	0.067 (15%)
Optimum pH[b]		
Amylase act.	6.9	5.2
Maltosidase act.	5.2	5.2
Activation by Cl^-[c]		
Amylase act.	44 -fold	23 -fold
Maltosidase act.	3.2-fold	6.8-fold

Amylase activity was measured at pH 6.9 (20 mM sodium phosphate buffer containing 25 mM NaCl) with reductometry using maltohexaitol as a substrate. Maltosidase activity was measured in the same sodium phosphate buffer with a spectrophotometrical method using p-nitrophenyl α -D-maltoside as a substrate.

[a] Enzyme-inhibitor (E-I) complexes were formed at pH 6.5 and 25°C for 5 h. Complete formations of E-I complexes were confirmed changing excess inhibitor concentration.

[b] Determined from pH profiles (Fig. 1).

[c] Each ratio was determined at pH 6.9 comparing k_{cat} values in 20 mM sodium phosphate buffer in the absence or presence (25 mM) of NaCl.

Inhibition by the proteinaceous inhibitor. Further, detailed characters of the H201N mutant were investigated, since pronounced increase of maltosidase activity was observed in this mutant only. A proteinaceous α -amylase inhibitor, phaseolamin from white kidney bean (Phaseolus vulgaris) inhibits mammalian α -amylases specifically (12). Both amylase and maltosidase activities of the wild-type HPA are completely inhibited by the inhibitor (Table I). In the case of H201N, however, the enzyme-inhibitor complex still has both residual amylase and maltosidase activities (4% and 15% of H201N, respectively) as shown in Table I. These results show that His201 of HPA is essential for complete inhibition by the proteinaceous inhibitor.

Effect by the activator. Mammalian α -amylases are activated by chloride ion (Cl^-). The binding site is near the active site

cleft (5,6), and the dissociation constant is below 1mM (10). Amylase and maltosidase activities of the wild-type HPA were activated 44- and 3.2-fold by Cl^-, respectively. This activation ratio was changed in H201N; 23-fold for amylase and 6.8-fold for maltosidase activities (Table I). The amplitude of activation by Cl^- decreased for amylase activity (52% of the wild-type HPA) and increase for maltosidase activity (210% of the wild-type HPA). These results show that His201 residue is related to the activation by Cl^- which is unique to mammalian α -amylases, although it locates far from the binding site of Cl^- (5,11). Since Cl^- binds Arg195 (one of the Cl^- binding residues), the loop containing Arg195, Asp197 (one of probable catalytic residues), and His201 must be locally stabilized by Cl^- for the formation of the active conformation (6). It is speculated that configuration of Asn201 in H201N is arranged with the binding of Cl^- at Arg195.

Chemical modification of histidine residue. Increase of maltosidase activity and decrease of amylase activity has been observed by chemical modification of histidine residues of PPA and TAA with diethylpyrocarbonate (DEP) (13-15). We confirmed that the maltosidase activity of the wild-type HPA was increased by the chemical modification of histidine residues with DEP. However, maltosidase activity of the mutant enzyme, H201N, was rather decreased to 50% with the chemical modification by DEP. This fact shows that His201 in all the modifiable histidine residues solely contributes to the increase in maltosidase activity of the wild-type HPA. In the case of histidine modified HPA (mod-HPA), only amylase activity is inhibited by the inhibitor selectively, as in the case of PPA (14,15).

The main results mentioned above are summarized in Table I.

His201 of HPA plays important multi-functional roles in the catalytic activity and regulation of HPA; control of optimum pH, conversion of the substrate specificities, change in the inhibition mode of a proteinaceous inhibitor, and modulation of activator effect by Cl^-. The above roles of His201 should be peculiar not only to HPA but also to whole mammalian α -amylases due to close sequence homology among them.

In general, histidine residue may play important roles multi-functionally when it is located at the suitable position in the active site of an enzyme owing to the unique characters of the imidazol group in its structure. In the case of α -amylase,

histidine residues (101, 201, and 299) are conserved from microorganism to mammal. Therefore, it is reasonable to speculate that in the course of evolution, His201 has acquired unique multi-functional roles which are significant physiologically for the control of starch digestion in small intestine of mammal.

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