

Chemical modification of aminopeptidase isolated from Pronase

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Chemical modification of aminopeptidase from pronase has revealed two important histidines in enzyme catalysis. In the absence of metal ions, modification of the readily-modified histidine (pK_a 6.9 ± 0.5) results in a drastic loss of activity, indicating that this residue is indispensable for enzyme activity. In the presence of $CaCl_2$, the modified enzyme still retains approx. 60% of the activity, whereas modification of another histidine (pK_a 7.7 ± 0.2) leads to a dramatic loss of activity. In fact, the enzyme with the first histidine being modified is active only in the presence of metal ions. Moreover, modification of the second histidine is prevented by the presence of $Ca(II)$. These

results indicate that the second histidine is serving as a ligand for $Ca(II)$ and the bound $Ca(II)$ is directly involved in enzyme catalysis. The c.d. spectra of the modified and unmodified enzymes in the absence or presence of $CaCl_2$ are all very similar, indicating that no gross conformational changes in protein occur upon modification or by the presence of $Ca(II)$. Modification of both histidines is prevented by the presence of a competitive inhibitor, suggesting that they are located in the active centre. Modification of 11 amino groups, two tyrosines, or four arginines causes no appreciable inactivation of the enzyme, indicating that these residues are not directly involved in enzyme catalysis.

INTRODUCTION

Pronase E (*Streptomyces griseus* neutral protease from Merck) contains several exo- and endo-peptidases, and hydrolyses practically all peptide linkages in proteins and peptides. We have purified from Pronase a stable, monomeric aminopeptidase (AP) with a molecular mass of 32 kDa. The enzyme does not necessarily require a metal ion for activity. However, different metal ions can modify the enzyme activity to various extents, with the calcium ion exhibiting the most prominent effect. The study of the pH-dependence of kinetic parameters for the enzyme revealed the need for a free-base form of a group with $pK_a \sim 7$ and an undissociated residue ionized at $pH \sim 10$. In order to delineate the mechanism of action for these enzymes, it is necessary to characterize the $Ca(II)$ -binding site and to identify the amino acid residues responsible for these catalytically important deprotonation processes.

Chemical modification has been widely used to determine these catalytically important amino acid residues. In this paper, we have carried out the chemical modification of histidyl, lysyl, tyrosyl and arginyl residues of the enzyme. The results indicate that a readily modified histidine with pK_a 6.9 is essential for enzyme catalysis, another histidine, with pK_a 7.7, is serving as a ligand for $Ca(II)$ and the enzyme-bound $Ca(II)$ is catalytically essential. Modification of eleven amino groups, two tyrosines, or four arginines does not cause any appreciable inactivation of the enzyme, indicating that these residues are not directly involved in enzyme catalysis.

MATERIALS AND METHODS

Materials

Pronase E, trifluoroacetic acid (TFA), acrylamide, 2-mercaptoethanol, all metal salts, NaOH and HCl were purchased from E. Merck (Darmstadt, Germany). Diethyl pyrocarbonate (DEPC), maleic anhydride (MA), tetranitromethane (TNM), leucine hydroxamate, 9,10-phenanthrenequinone (PQ), 1,2-

cyclohexanedione (CHD), boric acid, ethyl acetate, all buffers, EDTA, Coomassie Brilliant Blue R, glycine, dialysis tubing, standard protein marker, L-leucine *p*-nitroanilide (LPNA), and KH_2PO_4 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CM-52 was from Whatman Biosystems (U.K.). G-75 and 6-aminohexanoyl-Sepharose 4B were from Pharmacia Fine Chemicals (Sweden). Acetonitrile, methanol, acetone and acetic acid were supplied by Alps Chemical Co. (Taiwan).

Metal-free procedures

The activity and kinetic measurements are markedly affected by the presence of adventitious metal ions and precautions against their contamination were taken as described [1]. Buffers and substrates were prepared in water purified to a specific resistance of $18\text{ M}\Omega/\text{cm}$ by a Milli-Q system (Millipore) and further rendered free of metals with Chelex-100 resin (Bio-Rad) and dithizone extraction. Dialysis tubing was treated as recommended [2]. The metal-free AP was prepared simply by dialysing the enzyme against 10 mM Hepes buffer (pH 8.0) at 4°C to remove the calcium ion. The purified enzyme was also analysed for its metal contents by inductively coupled plasma-mass spectrometry and the results indicate the absence of $Zn(II)$ and $Ca(II)$. Treatment with EDTA in the purification process may remove other enzyme-bound metal ions.

Enzyme purification

A 0.2 g sample of Pronase E was dissolved in 1 ml of 5 mM Tris/HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ and applied to a CM-cellulose column ($1.5\text{ cm} \times 14\text{ cm}$) which was equilibrated with the same buffer. The column was first eluted with the starting buffer and then with the same buffer containing 0.3 M NaCl.

The fractions containing the proteins in the first elution band were combined and concentrated by pressure ultrafiltration through a YM-10 membrane (Amicon). Sufficient 0.25 M EDTA (pH 8.0) was added to the retentate to give a final concentration

Abbreviations used: AP, aminopeptidase; TFA, trifluoroacetic acid; LPNA, L-leucine *p*-nitroanilide; Bz-Arg *p*-nitroanilide; $N\alpha$ -benzoyl-DL-arginine *p*-nitroanilide; DEPC, diethyl pyrocarbonate; TNM, tetranitromethane; MA, maleic anhydride; CHD, 1,2-cyclohexanedione; PQ, 9,10-phenanthrenequinone; Ches, 2-[*N*-cyclohexylamino]ethanesulphonic acid; Epps, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulphonic acid.

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of 25 mM and allowed to stand at 4 °C for 1 h. Prior treatment with EDTA is necessary for selective retention of the aminopeptidases on the affinity column and the successful separation of the enzymes by elution with CaCl_2 is probably due to the gradual removal of EDTA through complexation with Ca(II) . The EDTA-treated protein was loaded on an EAH-Sepharose column (1.5 cm \times 10 cm) and eluted with 5 mM Tris/HCl buffer (pH 8.0). After the first peak came out, the protein was washed sequentially by the following solutions each containing the starting buffer: 30 mM NaCl; 5 mM CaCl_2 ; 20 mM CaCl_2 ; and finally 1 M NaCl. All chromatographic procedures were carried out at 4 °C on a liquid chromatographic system equipped with a peristaltic pump (Minipuls 3, Gilson), a u.v./visible detector (Model 112, Gilson), a fraction collector (Model 203, Gilson) and a chart recorder.

Spectral measurements and other methods

The absorption spectra were performed on an Hitachi U-3210 spectrophotometer. The c.d. spectra were conducted on a JASCO-720 spectropolarimeter (Japan Spectroscopic Co.) using a 0.2 mm-pathlength quartz cell. PAGE in the presence of 0.1 % SDS was performed according to the procedure of Laemmli [3]. The gel was run at a constant current of 40 mA and stained with Coomassie Blue. All samples were incubated at 100 °C for 1 min before electrophoresis. Reverse-phase chromatography of Pronase and APs was performed on an Hitachi h.p.l.c. system (containing L-6200/6000 dual-gradient pumps and an L-4250 u.v./visible detector) using a column (25 cm \times 0.46 cm) packed with Nucleosil C_{18} (5 μm). Elution conditions are: mobile phase, A = 0.07 % TFA/18 % CH_3CN ; B = 0.07 % TFA/80 % CH_3CN ; linear gradient, 100 % A to 100 % B over 2–30 min; flow rate, 1.0 ml/min; detection, 280 nm.

pH-dependence of the kinetic parameters

The enzyme activity against LPNA was determined by monitoring the absorbance change at 405 nm using a molar absorptivity of 9900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [4]. For investigating the pH-dependence of the kinetic parameters, a combination of the following buffers (5 mM each), which covered the desired pH range of 6.5 to 10.5, was employed: Mes, Pipes, Hepes, Epps and Ches. These buffers were found to exhibit no adverse effects on enzyme activity. The kinetic parameters at various pH values were also determined by the initial rate method.

Modification of histidines with DEPC

Modification of histidine residues with DEPC was performed as described previously [5,6]. The stock solution in ethanol was added sequentially to 9.2 μM AP over a period of 2 h in Tris buffer (pH 8.0) at 25 °C. The enzyme activity against LPNA was measured every 15 min and the extent of modification was estimated from the absorbance change at 240 nm using a molar absorptivity of 3200 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [7,8]. Decarboxylation was performed by treating the modified enzyme with 1 M hydroxylamine in 50 mM phosphate buffer, pH 7.0, and stirred at 25 °C for 4 h.

Modification of amino groups with MA

Modification of amino groups with MA was carried out in a similar way to that described previously [9]. The stock solution of the reagent is usually prepared in dioxane. However, the enzyme activity was found to be decreased by 50 % with the addition of 1 % (v/v) dioxane, whereas no adverse effect was observed for ethyl acetate. Therefore the stock solution of the reagent was

prepared in ethyl acetate. MA in ethyl acetate was added sequentially to 2.9 μM AP in 0.1 M phosphate buffer at pH 8.5 and 25 °C. The solution was stirred for 15 min after each addition of the reagent and an aliquot of the solution was taken out for enzyme assay. The extent of modification was determined from the absorbance change at 250 nm using ϵ 3360 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [10].

Modification of tyrosines with TNM

The tyrosine residues were modified with TNM as described previously [11]. The stock solution of TNM in ethanol was introduced in two additions (at 10 and 100 molar excess over the enzyme) to 4.2 μM AP in 50 mM Tris buffer (pH 8.0) at 25 °C. An aliquot of enzyme solution was taken out for assay every 10 min. The extent of modification was estimated from the absorbance change at 428 nm using ϵ 4100 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [11].

Modification of arginines with CHD

Modification of arginine residues with CHD was performed similarly to that described elsewhere [12]. An aliquot of the stock solution of CHD in 10 mM borate buffer (pH 8.5) was added to 3.1 μM AP in the same buffer at 25 °C (the final molar ratio of CHD to AP was 2000). The enzyme activity against LPNA was measured every 10 min. The extent of modification was calculated from the number of unmodified arginine residues determined by a fluorimetric method using PQ as the fluorophore [13]. An aliquot of the enzyme solution was diluted 1000-fold. To 1 ml of the diluted solution was added 3 ml of 1 μM PQ in ethanol and 0.5 ml of 2.0 M NaOH. The mixture was stirred at 60 °C for 6 h and an equal volume 1.2 M HCl was added. The fluorescence intensity ($\lambda_{\text{ex.}} = 312 \text{ nm}$, $\lambda_{\text{em.}} = 395 \text{ nm}$) of the resulting solution was then measured to determine the number of free arginine residues using a calibration curve obtained from the reactions of PQ and the standard arginine solutions. Unless otherwise stated, all modifications were performed in the absence of metal ions.

RESULTS

Enzyme purification

The chromatography of crude Pronase through CM-cellulose displays three separate bands. The proteins in each band were assayed for enzyme activities against LPNA and the results indicate that, for a given A_{280} , the AP activities of the two latter bands are much lower (< 0.2 %) than that of the first band.

The fractions associated with the first band were collected, concentrated, equilibrated with EDTA, and then loaded on the EAH-Sepharose column. The chromatogram exhibits six separate peaks as demonstrated in Figure 1. Most of the AP activity against LPNA was found in the two bands designated as I and II. The more abundant AP (peak I) was used for the rest of the study. No activities against *N* α -benzoyl-DL-arginine *p*-nitroanilide (Bz-Arg *p*-nitroanilide) are found for proteins in these bands.

The SDS/PAGE of AP in peak I shows a single protein band with a molecular mass of 32 kDa; estimated from the calibration curve of the standard proteins (results not shown). G-75 column (1.5 cm \times 100 cm; eluted with 5 mM CaCl_2 , 5 mM Tris, 0.1 M NaCl, pH 8.0, at a flow rate of 8.8 ml/h) and reverse-phase h.p.l.c. chromatograms also exhibit a single protein band for AP in peak I, suggesting that this enzyme is monomeric and pure.

pH-dependence of the kinetic parameters

The initial rates for the hydrolysis of LPNA by AP were measured at various pH values and the kinetic parameters, k_{cat} and K_{m} ,

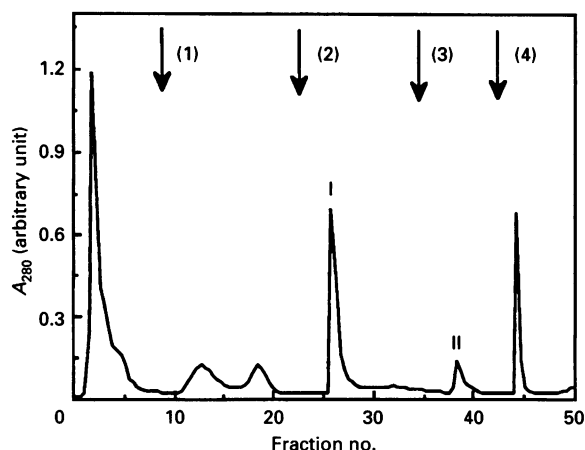


Figure 1 Chromatography of proteins through EAH-Sepharose

The proteins were first eluted with 5 mM Tris buffer (pH 8.0) followed by the same buffer which also contained: (1), 30 mM NaCl; (2), 5 mM CaCl_2 ; (3), 20 mM CaCl_2 ; (4), 1 M NaCl. The changes of washing solutions were indicated by the vertical arrows.

were determined from the non-linear least-squares fit of the Michaelis-Menten equation. The results are demonstrated in Figure 2. Small variations in K_m are observed in the range pH 7–9, indicating that the substrate affinities are nearly unchanged in this pH range. The pH-dependencies of k_{cat} and k_{cat}/K_m are bell-shaped, suggesting that at least two deprotonation processes are involved in enzyme catalysis. The lines that were drawn through the data points of Figures 2(b) and 2(c) were obtained from the non-linear least-squares fits of the data based on two-ionization equilibria. The two $\text{p}K_a$ values were estimated to be 6.9 ± 0.2 and 9.9 ± 0.2 for the enzyme-substrate complex (Figure 2b) and 7.4 ± 0.2 and 9.8 ± 0.2 for the free enzyme (Figure 2c). All the S.D. values described in the text and in Table 1 for $\text{p}K_a$, k_{cat} , and K_m were obtained from triplicate measurements of each individual experiment.

Modification of histidines by DEPC

AP ($9.2 \mu\text{M}$) was modified by successive additions of DEPC in the absence of metal ions. The enzyme activity (measured in the absence or presence of 5 mM CaCl_2) and the number of modified histidines (estimated from the absorbance change at 240 nm) were determined for each addition of DEPC and the results are illustrated in Figure 3. The enzyme was inactivated as the extent of modification increased. Modification of one histidine residue causes a drastic inactivation of the metal-free AP (Figure 3a), whereas modification of two histidines results in a dramatic loss of activity for the Ca(II) -activated enzyme (Figure 3b). The modification rates for these two histidines are different. For $[\text{DEPC}]/[\text{aminopeptidase}] = 50$ and in 50 mM phosphate buffer (pH 6.0) at 25°C , the apparent pseudo-first-order rate constants for the two histidines are $(3.2 \pm 0.2) \times 10^{-3} \text{ min}^{-1}$ and $(6.0 \pm 0.5) \times 10^{-4} \text{ min}^{-1}$ respectively. For convenience, the readily modified histidine is designated as His(1) and the less readily modified one as His(2).

In order to obtain further insight into the roles of the two histidines, the modification of AP with DEPC was also performed in the presence of CaCl_2 or L-leucine hydroxamate, a competitive inhibitor of AP. Three separate solutions each containing $5.3 \mu\text{M}$ AP in 50 mM phosphate buffer (pH 8.0), were modified with 250 molar excess of DEPC at 25°C under the following conditions:

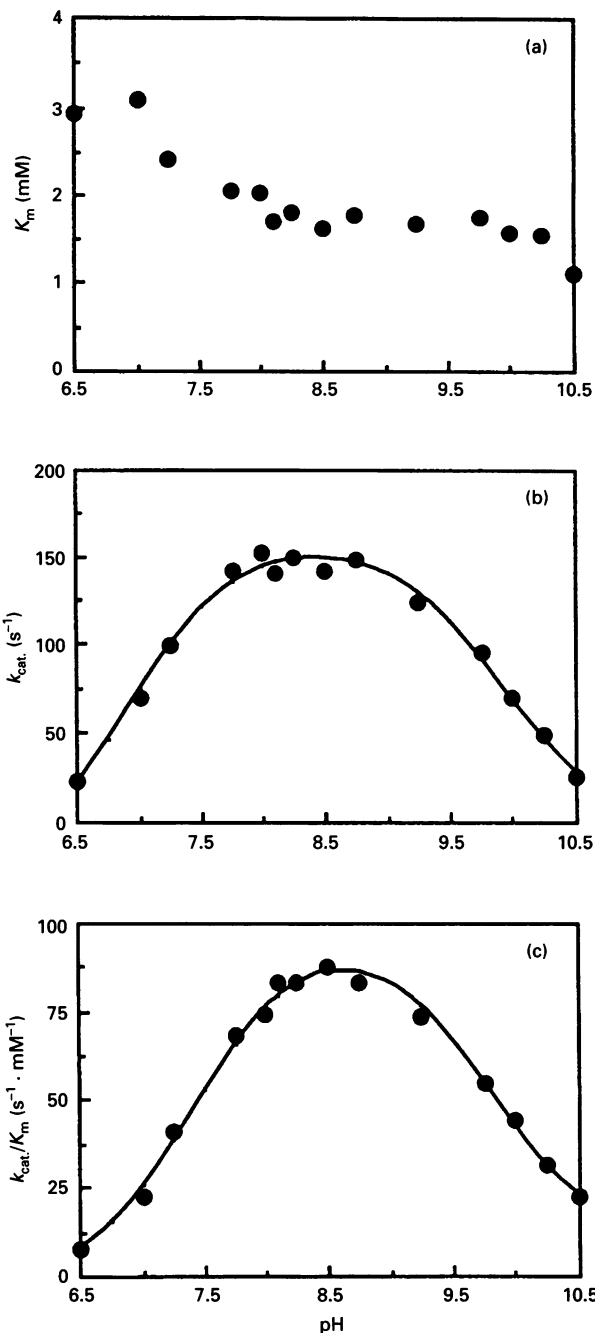


Figure 2 pH-dependence of the kinetic parameters for the hydrolysis of LPNA by AP in the absence of metal ions

(a) pH-dependence of K_m , (b) pH-dependence of k_{cat} , (c) pH-dependence of k_{cat}/K_m . The solid lines drawn through the data points are the theoretical curves calculated by the obtained $\text{p}K_a$ values as described in the text.

(1) in the absence of metal ion and inhibitor; (2) in the presence of 5 mM CaCl_2 ; and (3) in the presence of 5 mM L-leucine hydroxamate. The extent of modification was monitored over a period of 1 h and the results are shown in Figure 4. In the absence of the metal ion and inhibitor, His(1) is very reactive and is modified almost instantly, while His(2) is modified within 1 h as demonstrated in Figure 4(a). In the presence of 5 mM CaCl_2 , His(1) is still very reactive and is modified easily, but His(2) is unable to be modified as shown in Figure 4(b). In the presence of

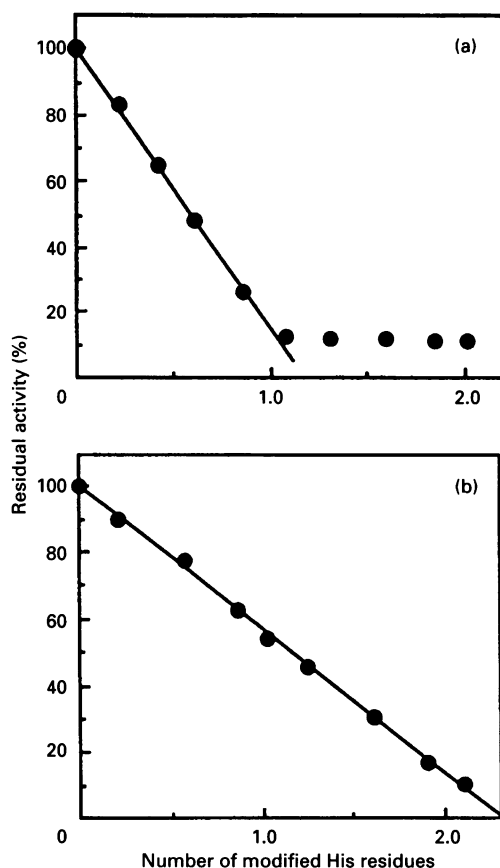


Figure 3 Correlation of the inactivation of AP with the extent of histidine modification

AP ($3.3 \mu\text{M}$) was modified with successive additions of DEPC ($[\text{DEPC}]/[\text{AP}] = 0\text{--}200$) in 50 mM phosphate buffer (pH 6.0) at 25°C . The enzyme activity was measured (a) in the absence and (b) in the presence of 5 mM CaCl_2 .

5 mM L-leucine hydroxamate, $K_i = 0.030 \text{ mM}$ at pH 8.0, both histidines are modified extremely slowly (Figure 4c), suggesting that the two histidines are located in the active centre.

The c.d. spectra of the modified and unmodified enzymes in the absence or presence of Ca(II) were also measured to investigate the possible conformational changes. The obtained c.d. spectra are all very similar, indicating that no gross conformational changes in protein occur upon modification, or due to the presence of Ca(II) .

The kinetic parameters for the hydrolysis of LPNA by the

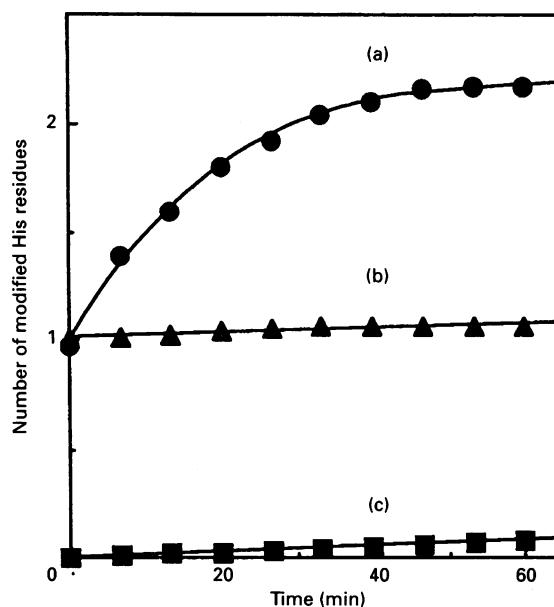


Figure 4 Effects of Ca(II) and a competitive inhibitor on the time courses for the extent of histidine modification

AP ($5.3 \mu\text{M}$) was modified with 250 molar excess of DEPC in 50 mM phosphate buffer (pH 8.0) at 25°C under the following conditions: (a), in the absence of metal ion and inhibitor; (b) in the presence of 5 mM CaCl_2 ; or (c), in the presence of 5 mM L-leucine hydroxamate.

modified and unmodified AP were also determined to study how these two histidines affect the binding or turnover of the substrate during catalysis. The results obtained from non-linear least-squares fits of the Michaelis–Menten equation were given in Table 1. Modification of histidines causes no appreciable changes in K_m , suggesting that the substrate affinity is hardly perturbed. In other words, the modified histidines do not block the active centre to prevent access of the substrate. The loss of activity upon modification is primarily due to the decreases in k_{cat} . For His(1)-modified AP, the enzyme lost most of its activity in the absence of Ca(II) , whereas only an approx. 40% decrease in activity was observed in the presence of 5 mM CaCl_2 . Modification of both histidines caused a dramatic loss of enzyme activity, even in the presence of Ca(II) .

The addition of hydroxylamine decreases the absorption peak at around 240 nm very slowly and approx. one modified histidine is decarboxylated after 4 h of incubation. The resulting enzyme shows a restoration of approx. 60% of the activity in the presence of Ca(II) , whereas essentially no recovery of the activity

Table 1 Kinetic parameters for the hydrolysis of LPNA by unmodified and modified AP

All assays were conducted at 25°C , in 10 mM HEPES/0.1 M NaCl, pH 8.0. S.D. values were obtained from triplicate measurements of each experiment.

| Enzyme (AP) | Ca(II) absent | | | Ca(II) present | | |
|-------------------|---|---------------|--|---|-----------------|--|
| | k_{cat} (s^{-1}) | K_m (mM) | k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$) | k_{cat} (s^{-1}) | K_m (mM) | k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$) |
| Unmodified | 160 ± 11 | 1.7 ± 0.1 | 94 ± 9 | 360 ± 12 | 0.31 ± 0.02 | 1160 ± 80 |
| His(1) modified | 2.7 ± 0.2 | 1.9 ± 0.2 | 1.4 ± 0.2 | 210 ± 10 | 0.31 ± 0.02 | 680 ± 50 |
| His(1,2) modified | 2.5 ± 0.2 | 2.4 ± 0.2 | 1.0 ± 0.1 | 14 ± 1 | 0.40 ± 0.03 | 35 ± 4 |

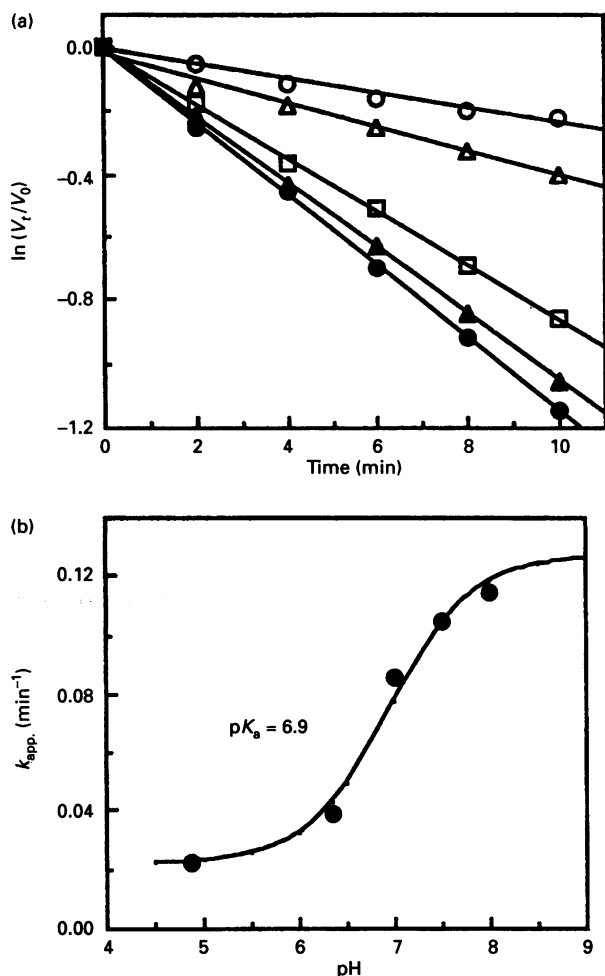


Figure 5 Time-dependence of $\ln(V_t/V_0)$ (a) and pH-dependence of k_{app} (b) for the modification of His(1) with DEPC

AP (5.0 μ M) was modified at a desired pH with 50 molar excess of DEPC in the presence of 5 mM CaCl_2 in 50 mM phosphate buffer at 25 °C. The enzyme activities were monitored over a period of 10 min. Key to pH values in (a): \circ , 4.9; \triangle , 6.4; \square , 7.0; \blacktriangle , 7.4; \bullet , 8.0.

is seen in the absence of Ca(II) . These results indicate that treatment of hydroxylamine causes the decarboxylation of His(2) but not His(1).

Metal ions other than Ca(II) were also examined to investigate their effect on enzyme activity. For the unmodified enzyme, the relative activities (against 1 mM LPNA in 10 mM Hepes, pH 8.0, at 25 °C) for AP incubated with various bivalent metal ions (Mg, Sr, Ba, Mn, Co, Ni, Zn) fall in the range of 10–54 % as compared with that of Ca(II) -AP (the relative activity of the metal-free AP is 22 %). Thus the impact of the metal ions on activity is not so pronounced for the unmodified AP. For His(1)-modified AP, the metal-incubated APs are 10–67 % as active as that of Ca(II) -AP, whereas the relative activity of the metal-free enzyme is only 0.5 %. These results indicate that His(1)-modified AP is very similar to a metalloenzyme with the metal ions being crucial to enzyme catalysis. In addition, the large difference in activity of His(1)-modified AP in the absence and presence of metal ions also indicate that the activity observed for the unmodified AP under metal-free conditions is not due to contaminations of the residual metal ions.

To determine the pK_a of His(1), the modification was carried

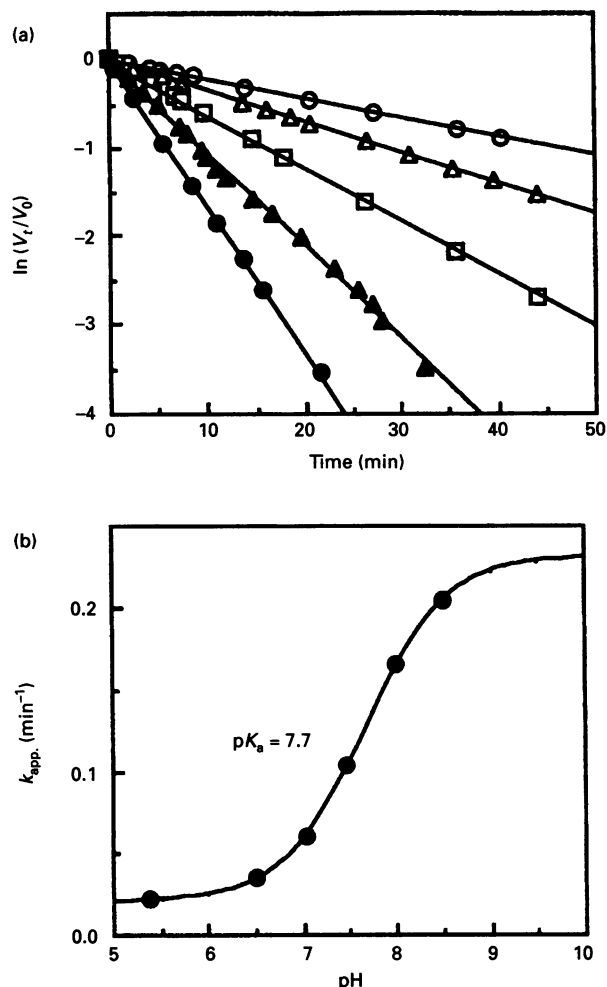


Figure 6 Time-dependence of $\ln(V_t/V_0)$ (a) and pH-dependence of k_{app} (b) for the modification of His(2) with DEPC

His(1)-modified AP (5.0 μ M) at a desired pH was subjected to further modification with 450 molar excess of DEPC in 50 mM phosphate buffer at 25 °C. The enzyme activities were monitored over a period of 45 min. Key to pH values in (a): \circ , 5.4; \triangle , 6.5; \square , 7.0; \blacktriangle , 7.5; \bullet , 8.0.

out at various pH values and in the presence of 5 mM CaCl_2 to protect His(2) from being modified. The enzyme activity for a given molar ratio of DEPC:AP and at a given pH was measured over a period of time. The reaction is assumed to be pseudo-first-order and its rate is given by: $V_t = V_0 e^{-k_{app} \cdot t}$, where V_0 and V_t are the enzyme activities at time 0 and t respectively and k_{app} is the apparent rate constant. Figure 5(a) shows the plots of $\ln(V_t/V_0)$ versus t at various pH values. The plots are all linear, consistent with the pseudo-first-order reaction, and their slopes yield values for k_{app} at various pH values. The k_{app} values are then replotted against pH as demonstrated in Figure 5(b) and a pK_a of 6.9 ± 0.5 was obtained for His(1) from the sigmoidal curve fitting.

To determine the pK_a of His(2), the enzyme was first reacted with DEPC in the presence of 5 mM CaCl_2 . After the modification of His(1) was complete, the enzyme was dialysed to remove Ca(II) and excess DEPC. The resulting enzyme was then subjected to further modification at various pH values with DEPC in the absence of CaCl_2 . The enzyme activity was again monitored over a period of time and the results are shown in Figure 6(a). The slopes of the linear plots of $\ln(V_t/V_0)$ versus t give k_{app} .

readings at various pH values and the k_{app} values are again replotted against pH as demonstrated in Figure 6(b); the pK_a was determined to be 7.7 ± 0.2 .

Modification of amino groups, tyrosines and arginines

The enzyme activities and the number of modified amino groups at various molar ratios of MA to AP were determined. As the molar ratio of MA to AP-I was raised to approx. 200, 11 amino groups were modified; however, the enzyme activity was unchanged during the modification.

The tyrosine was modified to 0.43 and 2.14 residues as 10 and 100 molar excess of TNM were added to AP. No further modification was observed even when the molar ratio of TNM to AP was raised to 1000, indicating that only approx. 2 tyrosines are accessible to TNM. The activity of the enzyme was found to be unchanged during the modification. In addition, the enzyme was not inactivated by the modification of four arginines (results not shown).

DISCUSSION

Modification of AP from Pronase with DEPC revealed two important histidine residues for enzyme catalysis. In the absence of metal ions, modification of His(1) leads to a dramatic loss of activity, which results mainly from the corresponding decrease in k_{cat} (Table 1), suggesting that this residue plays an important role in substrate turnover. When the modification was carried out in the presence of 5 mM $CaCl_2$, only one histidine was modified (Figure 4b). The modified enzyme exhibits very similar kinetic parameters to those listed in Table 1 for His(1)-modified AP, suggesting that the presence of $Ca(II)$ prevents His(2) from being modified. Modification of His(2) leads to a dramatic loss of activity even in the presence of $CaCl_2$, and the presence of $Ca(II)$ causes decreases in K_m and increases in k_{cat} for the modified and unmodified AP (Table 1). In addition, His(1)-modified AP is active only in the presence of metal ions. All these results indicate that His(2) and $Ca(II)$ must co-exist to give an active His(1)-modified AP. Consequently, it is proposed that His(2) is acting as a ligand for $Ca(II)$ and the enzyme-bound $Ca(II)$ is directly involved in enzyme catalysis.

The pK_a of His(1) is determined to be 6.9 and that of His(2) is 7.7. These values are reasonable for histidines in proteins. These two pK_a values are too close to be resolved in the study of pH-dependence of kinetic parameters, which leads to a single pK_a of

7.4 (Figure 2c). For the modification of protein with DEPC, only the free-base form of histidine is reactive as a nucleophile. High pK_a values thus result in corresponding lower reaction rates, which is consistent with the experimental observation. At the optimum pH of AP (pH 8–8.5), most of the histidines exist in the free-base form, which makes His(1) a good nucleophile (or general base) for enzyme catalysis and His(2) a good ligand for $Ca(II)$. Protonation of His(1) and His(2) at low pH thus results in a dramatic loss of activity consistent with the pH profiles of kinetic parameters (Figure 2).

The involvement of the histidines and metal ions in peptidase catalysis has already been recognized [14–18]. The role of the histidines is usually to stabilize the transition state, to participate in some proton-relay processes, or to serve as ligands for metal ions; whereas the metal ions are known to enhance the nucleophilicity of water, to polarize the peptide bond to be cleaved, or to stabilize the transition state. These are also the possible roles His(1), His(2) and $Ca(II)$ will play in the catalytic mechanism of Pronase AP, but exactly how they are participating in enzyme catalysis still awaits further structural and catalytic information.

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