

Chemical modification of porcine kidney aminopeptidase P indicates the involvement of two critical histidine residues

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Received 10 January 1996

Abstract Aminopeptidase P (AP-P), purified to homogeneity from porcine kidney membranes, was completely inactivated by treatment with 0.2 mM diethylpyrocarbonate (DEP) at pH 7.0. Treatment of the modified enzyme with 20 mM hydroxylamine resulted in recovery of AP-P activity. The differential absorption of native and modified AP-P at 240 nm showed that DEP modified two histidyl residues per mol of AP-P. The substrates, bradykinin(1–5) and Gly-Pro-Hyp, and also the inhibitor, apstatin, could protect against DEP inactivation. These results suggest that histidine residues are critical for AP-P activity.

Key words: Aminopeptidase P; Diethylpyrocarbonate; Metallopeptidase; Proline-peptidase; Histidine modification; Porcine kidney

1. Introduction

Aminopeptidase P (AP-P; EC 3.4.11.9) is a zinc-metallo-peptidase [1] that can catalyse the release of N-terminal amino acid residues from peptides where a proline residue is present in the penultimate position [2]. AP-P was first suggested to have a role in the degradation of collagen [2] but has subsequently been reported to be involved in pulmonary bradykinin metabolism [3,4]. The enzyme may also play a role in the metabolism of certain other bioactive peptides such as substance P, peptide YY and neuropeptide Y [3,5,6]. AP-P is located in the microvillar membranes of pig and human kidney [1], and has also been found in rat intestine [7] and lung [8], bovine lung [3], and guinea pig serum and kidney [9]. A soluble (cytosolic) form of AP-P has been identified in rat brain [10] but this may represent a distinct gene product from membrane AP-P.

We have previously identified AP-P as a cell-surface ectoenzyme anchored to the membrane by a glycosylphosphatidyl inositol (GPI) moiety [11] and exploited the selective release of AP-P by bacterial phosphatidylinositol-specific phospholipase C in the purification of the enzyme from porcine kidney membranes [1]. During the initial purification of AP-P from pig kidney, it was observed that Mn^{2+} ions were required for optimal activity of the enzyme [2]. Further work has shown that both Mn^{2+} and Co^{2+} ions stimulate the activity of pig AP-P, whereas some other bivalent cations were inhibitory. These results, together with the inhibition of AP-P activity by chelating agents such as EDTA and 1,10-phenanthroline

[1], have confirmed the earlier observations by several groups that AP-P is a metalloenzyme [3,7,12] containing 1 mol of zinc per mol of protein [13].

Unlike other brush border aminopeptidases (e.g. AP-N, AP-A, AP-W), AP-P is not inhibited by actinonin, amastatin, puromycin or bestatin [1] but a specific inhibitor of AP-P, apstatin, has recently been developed [8,14]. Rather than resembling aminopeptidases N, A and W, AP-P appears to be related to the recently described proline peptidase family of hydrolytic enzymes which includes *E. coli* aminopeptidase P, prolidase, methionine aminopeptidase and creatinase [15,16]. The amino acid sequence of pig kidney AP-P has recently been obtained by Edman degradation and mass spectrometry of the purified protein and reveals some limited sequence similarities in the C-terminal half of the protein with other members of this family [17]. The amino acid sequence of AP-P does not contain the typical HExxH motif common to many zinc peptidases nor any other recognised zinc-binding motif and no residues in the protein important for substrate or inhibitor binding, or catalysis, have yet been identified. In the present study, therefore, we show that diethylpyrocarbonate (DEP) inactivates AP-P through the modification of two critical histidine residues and substrates and inhibitor can protect against such modification.

2. Materials and methods

2.1. Materials

Bradykinin, bradykinin(1–5), bradykinin(2–9), Gly-Pro-Hyp, Pro-Hyp and diethylpyrocarbonate (DEP) were purchased from Sigma Chemical Co. Ltd., Dorset, UK. The AP-P inhibitor, apstatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-prolyl-L-prolyl-L-alaninamide), was a generous gift from Professor W.H. Simmons (Loyola University, Chicago, IL). *Bacillus cereus* phospholipase C was obtained from Fluka Chemie AG, Switzerland. Microcentrifuge filters (molecular cut-off 30 kDa) were from Sigma. All other chemicals were obtained from either BDH Chemicals Ltd. or Sigma Chemical Co. Ltd. and were of the appropriate grade.

2.2. Purification of AP-P from pig kidney

AP-P was purified by a modification of the method of Hooper et al. [1]. Pig kidney cortex (200 g) was homogenised in 0.33 M sucrose, 50 mM Tris-HCl buffer, pH 7.4 to a total volume of 1500 ml and centrifuged at 8000 × g for 15 min. The supernatant was centrifuged at 26,000 × g for 2 h and then the pellet was resuspended in 10 mM Tris-HCl, 0.1 M NaCl, pH 7.4, to 250 ml. After centrifugation at 31,000 × g for 90 min, the membrane pellet was resuspended in 10 mM Tris-HCl, 0.1 M NaCl, pH 7.4, to give a protein concentration of approx. 7.5 mg/ml and incubated with *Bacillus cereus* PLC (0.35 units/mg of protein) for 2 h at 37°C. After centrifugation at 31,000 × g for 90 min, the supernatant was dialysed extensively against 50 mM Tris-HCl, pH 8.0. The dialysed supernatant was then subjected to sequential chromatography on cilastatin-Sepharose, DEAE-cellulose, Mono-Q and alkyl-Superose as in [1] except that elution from Mono-Q was performed first isocratically with 10 mM Tris-HCl, 0.1 M KCl, pH 8.0, and then followed by a linear gradient to 0.5 M KCl in order

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Abbreviations: AP, aminopeptidase; DEP, diethylpyrocarbonate; GPI, glycosylphosphatidylinositol; Hyp, hydroxyproline; PLC, phospholipase C.

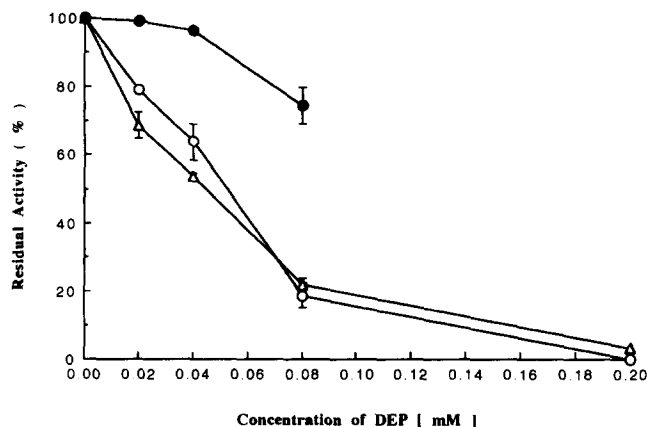


Fig. 1. The effect of DEP on AP-P activity. AP-P was reacted with various concentrations of DEP (0, 0.02, 0.04, 0.08, 0.2 mM) in 0.1 M phosphate buffer, pH 7.0, at room temperature for 30 min. Then, the activity of AP-P was assayed with Gly-Pro-Hyp or bradykinin as substrate. The reactivation experiment was performed with 20 mM hydroxylamine at 4°C for 4 h. Gly-Pro-Hyp (○), bradykinin (△), reversal with hydroxylamine (●).

to remove impurities more effectively. The final product was apparently homogeneous (M_r 91,000) as assessed by SDS-PAGE.

2.3. Assay of AP-P

The hydrolysis of Gly-Pro-Hyp (1 mM) by AP-P was assayed by an HPLC method, as described previously [1]. Preincubation of enzyme sample in 0.1 M Tris-HCl, pH 8.0 containing 4 mM $MnCl_2$ for 5 min was followed by a 30 min incubation with substrate (100 μ l total volume), all at 37°C. The reaction was terminated by boiling for 4 min. Under these conditions the formation of product (Pro-Hyp) was linear with time. Hydrolysis of Gly-Pro-Hyp was monitored using reverse-phase HPLC [1]. The product, Pro-Hyp, was quantified by calibration from a standard curve.

Hydrolysis of bradykinin by AP-P was also monitored by reverse-phase HPLC using a 20 min linear gradient of 4–45% (v/v) acetonitrile in 0.08% H_3PO_4 , followed by 5 min elution at final conditions. The product, bradykinin(2–9), was quantified by calibration from standard curves.

2.4. Chemical modification of AP-P with DEP

AP-P was incubated in 0.1 M phosphate buffer, pH 7.0, at room temperature with various concentrations of DEP for 30 min before enzyme activity was measured. To confirm whether the modified reaction could be reversed, excess DEP was removed using a microcentrifuge filter. Then, the samples were reacted with 20 mM hydroxylamine at 4°C for 4 h after which AP-P activity was assayed.

2.5. Spectroscopic studies

The differential spectra of carbethoxylated against untreated enzyme were obtained on a Uvikon 930 spectrophotometer. Spectroscopic studies in the range 220–300 nm were performed at room temperature in 0.1 M phosphate, pH 7.0, with DEP at 0.2 mM and AP-P at 10 μ M. The number of modified histidine residues was calculated by using $3200\text{ M}^{-1}\text{cm}^{-1}$ at 240 nm as the molar extinction coefficient for *N*-carbethoxyhistidine residues [18].

2.6. Protection of AP-P against DEP inactivation

AP-P was preincubated with various substrates or inhibitors, and then incubated with 0.08 mM DEP at room temperature for 30 min. After removal of excess reagents and products, the residual enzyme activity of each sample was assayed.

3. Results

3.1. Inactivation of AP-P by modification with DEP

Purified AP-P (4 μ M) from pig kidney was reacted with various concentrations of DEP (0, 0.02, 0.04, 0.08, 0.2 mM)

in 0.1 M phosphate buffer, pH 7.0, for 30 min. After treatment, AP-P activity was assayed with both Gly-Pro-Hyp or bradykinin as substrate. At 0.2 mM DEP, the activity of AP-P was completely abolished when measured with either substrate (Fig. 1). Treatment of DEP-inactivated AP-P with 20 mM NH_2OH restored a major part of the original activity (Fig. 1).

3.2. Stoichiometry of DEP modification of AP-P

AP-P (10 μ M) was treated with 0.2 mM and 5 mM DEP for 30 min and the UV absorption difference spectrum of DEP-treated against untreated-enzyme, in the range of 220–300 nm, was obtained for both concentrations. An absorption maximum in the difference spectrum was seen in the region of 240 nm and there was no decrease in absorbance around 280 nm (Fig. 2). The increase in absorption at 240 nm in 0.2 mM DEP corresponded to the modification of 2.3 ± 0.2 histidine residues per mol of the enzyme (mean $\pm$ range of two observations). Under these conditions approx 80% of the AP-P activity was inhibited. To establish whether DEP could modify additional histidine or tyrosine residues at higher concentrations, AP-P was reacted with 5 mM DEP under the same conditions as before. The absorbance increase between 220 nm and 230 nm showed excess DEP could react further with histidine residues forming di-*N*-carbethoxyhistidine derivatives (Fig. 2). The decrease in absorption at 280 nm also indicated *O*-carbethoxytyrosyl residues could be formed under conditions of excess DEP (Fig. 2). At 5 mM DEP the estimated number of modified histidyl residues per mol of AP-P was 8.

The effect of *N*-acetyl imidazole on AP-P activity was also investigated, which is known as a specific modifying reagent for tyrosyl residues [20]. At 0.5 mM *N*-acetyl imidazole, the activity of AP-P was not affected (relative activity = $101.2 \pm 11.1\%$).

3.3. Protection of AP-P against DEP inactivation

AP-P (4 μ M) was preincubated with 0.5 mM bradykinin(1–5), 1 mM Gly-Pro-Hyp or 0.05 mM apstatin in 0.1 M phosphate buffer, pH 7.0, at 37°C for 5 min and then reacted with 0.08 mM DEP at room temperature for 30 min. After excess reagents and products were removed by microcentrifuge filter, the activity of the enzyme was assayed. Both substrates, bradykinin(1–5) and Gly-Pro-Hyp, and the inhibitor, apstatin, were able to protect against DEP inactivation (Table 1).

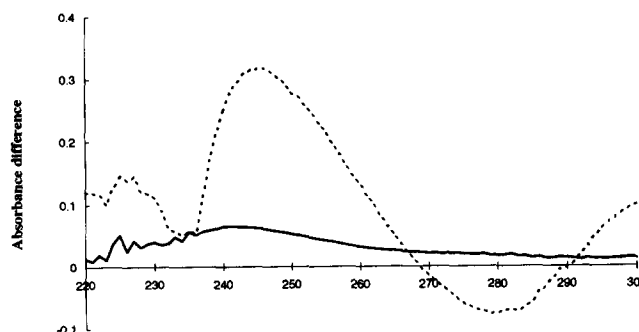


Fig. 2. Ultraviolet difference spectra of AP-P with DEP. This spectroscopic study was performed at room temperature in 0.1 M phosphate buffer, pH 7.0, with DEP at 0.2 mM (solid line) and 5 mM (broken line). The absorbance shown has been computer-corrected for the blank (enzyme in buffer with same volume of ethanol) in the range of 220–300 nm.

Table 1
Protection of AP-P against inactivation by DEP

Protecting agent	Relative activity (%)
None	33.8 ± 3.6 (3)
Bradykinin(1–5) (0.5 mM)	84.8 ± 1.6 (3)
Gly-Pro-Hyp (1 mM)	92.2 ± 2.7 (3)
Apstatin (0.05 mM)	79.8 ± 5.5 (3)

AP-P was preincubated with substrates or inhibitor, and then incubated with 0.08 mM DEP at room temperature for 30 min. After removal of excess reagents and products, the residual enzyme activity of each sample was determined with Gly-Pro-Hyp as a substrate. Activity is quoted relative to the activity of an untreated sample of enzyme (100% activity = 75.3 nmol/min/mg).

4. Discussion

The primary effect of low concentrations of DEP on proteins is the modification of histidyl residues. However, under certain conditions, DEP can also react with tyrosyl and lysyl residues. These various reactions can be distinguished spectrophotometrically. *O*-Carbethoxylation of a tyrosyl residue is readily detected by a decrease in absorbance at 280 nm whereas mono-*N*-carbethoxylation of a histidyl residue shows an absorption maximum between 230 and 250 nm. Modification of lysyl residues with DEP cannot be reversed by hydroxylamine. Also, hydroxylamine at 20 mM can decarbethoxylate selectively histidyl residues under conditions that do not decarbethoxylate 2-*O*-carbethoxy tyrosyl residues [19]. In the present work, AP-P was completely inactivated at 0.2 mM DEP and could be reactivated by treatment with 20 mM NH_2OH , implicating specific modification of histidyl residues. Under these conditions, the number of histidine residues modified, estimated by measuring absorbance difference at 240 nm, was 2.3.

Excess DEP was observed to react with a further six histidyl residues and also to form di-*N*-carbethoxyhistidine derivatives, which absorb maximally between 220 and 230 nm. Formation of *O*-carbethoxytyrosyl residues was also detected. The use of excess DEP may lead to inaccurate quantification and irreversible modification of enzyme action. It is therefore important to establish the lowest concentrations of DEP required for modification, in this case 0.2 mM. At this concentration, spectroscopic studies revealed no decrease in absorbance at 280 nm. The lack of effect of *N*-acetyl imidazole also confirmed that tyrosine is not involved in the activity of AP-P.

Protection of enzymic activity from an external inactivating reagent by substrates, products, or competitive inhibitors is generally regarded as evidence that the target residues for that reagent reside within the active site. The presence of substrates, bradykinin(1–5) and Gly-Pro-Hyp, and the inhibi-

tor, apstatin, protected against DEP inactivation implying that at least 2 of the 13 histidine residues in AP-P are important for substrate and inhibitor binding, or catalytic activity.

Sequence similarities with other members of the proline peptidase family [17] are restricted to the C-terminal half of AP-P (residues 350–623), suggesting that the active site of the enzyme resides in this part of the protein. In this region there are seven histidines but only four of these (residues 403, 493, 497 and 506) align with other members of the proline peptidase family. cDNA cloning of AP-P coupled with site-directed mutagenesis will be required to identify the critical histidine residues unequivocally and provide insight into the chemical mechanisms underlying this new class of proteolytic enzymes.

Acknowledgements: We thank the British Heart Foundation and the Wellcome Trust for support of our work on AP-P.

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