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PIG KIDNEY PARTICULATE AMINOPEPTIDASE A ZINC METALLOENZYME*

PAVEL LEHKY, JOSEF LISOWSKI**, DON P. WOLF***, HANS WACKER AND ERIC A. STEIN
Department of Biochemistry, University of Geneva (Switzerland)

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SUMMARY

1. Particulate aminopeptidase (EC 3.4.1.2) from pig kidney, which contains 2 gatoms of zinc per mole protein as the only metal component (Wacker *et al.* (1971) *Helv. Chim. Acta* 54, s473–485) is converted to an inactive metal-free apoaminopeptidase by electrodialyis, gel filtration, or treatment with chelating agents.

2. Metal depletion does not significantly affect the molecular weight and electrophoretic mobility of the protein, but reduces the resistance of the enzyme towards denaturation under extreme conditions.

3. Zn^{2+}, Cu^{2+}, Co^{2+} or Ni^{2+}, but no other cations, restore activity to the apoaminopeptidase.

4. Restoration of enzyme activity is directly proportional to the amount of divalent metal ions added up to 2 gatoms per mole of apoaminopeptidase; there is no increase of activity upon further addition.

5. The data indicate that particulate aminopeptidase from pig kidney is a zinc metalloenzyme, and suggest that both zinc atoms are essential for activity.

INTRODUCTION

Particulate aminopeptidase (EC 3.4.1.2) from pig kidney has been shown to be a metallo-glycoprotein, containing 2 atoms of zinc and approx. 400 carbohydrate residues (glucosamine, galactose, mannose, fucose and sialic acid), amounting to 20% of the molecular weight of 280 000 (ref. 2). During purification, the concentrations of all metals other than zinc decrease below detection limits, whereas the zinc:protein ratio increases 2.5-fold, suggesting that zinc is an intrinsic component of the enzyme

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** Present address: Ludwik Hirszfeld's Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

*** Present address: University of Pennsylvania, School of Medicine, Division of Reproductive Biology, Philadelphia, 19104.

molecule[2]. Conflicting results have been published as to whether metal ions were implicated in the catalytic activity of this aminopeptidase. Pfleiderer[3] reported that the enzyme was not inhibited by EDTA, and that metal ions such as Co^{2+} had little or no effect on the enzymatic activity, whereas Hanson *et al.*[4] observed a marked activation with Co^{2+} for the same enzyme. More recently, Femfert and Pfleiderer[5] have reported that, in contrast to their earlier results, Co^{2+} and certain other divalent cations were inhibitory.

In the present work, various procedures allowing the reversible removal of divalent cations from this particulate aminopeptidase are described, as are some properties of the metal-free apoenzyme. The role of metal ions in the enzymatic activity is reappraised, and evidence is presented that particulate aminopeptidase from pig kidney is a zinc metalloenzyme.

MATERIALS AND METHODS

All reagents were of analytical grade; Dowex resins were purchased from Fluka (Buchs, Switzerland), Chelex-100 from Calbiochem (Lucern, Switzerland), and "Specpur" divalent Mg, Mn, Ca, Co, Ni, Cu, Zn, Cd, Hg salts from Johnson Matthey Chemicals, London. Metal-free water was obtained by double distillation of deionized water in a quartz still (Westdeutsche Glasschmelze, Geesthacht). Metal analyses were performed on a Perkin-Elmer Model 303 atomic absorption spectrophotometer fitted with a recorder; as a control, zinc was also assayed by the dithizone method[6].

The enzyme was prepared as described previously[2], yielding preparations which are homogeneous on polyacrylamide gel electrophoresis and in the ultracentrifuge; the zinc-free apoenzyme was routinely prepared by treatment with Chelex resin (100–200 mesh), as described under Results.

To minimize contamination of solutions by heavy metals, polyethylene vessels and columns, as well as glassware, were cleaned according to Thiers[7]; whenever possible, glass was avoided. Metal-free buffers were prepared by extraction with dithizone[7] or by passage through a column of chelating resin[8]. Sephadex was freed of heavy metal contaminants by rinsing with 0.1 M EDTA at pH 8.0 followed by double-distilled water, 0.1 M HCl (Merck suprapure) and double-distilled water again.

The determinations of protein concentration and enzyme activity were carried out as described previously[2], except that the substrate was made 0.1 mM in EDTA in order to neutralize the effects of extraneous metals during assay. EDTA present in the substrate prevents the adventitious reactivation of metal-depleted preparations, but does not affect the intrinsic activity of zinc-aminopeptidase under the conditions of the assay (*i.e.* presence of substrate, short exposure to EDTA at concentrations below 10 mM). For reactivation experiments, zinc, instead of EDTA, was added to the substrate (final concentration 2 μM). Unless otherwise stated, solutions and assay system were buffered at pH 7.0 with 0.05 M TES (*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid) which has little affinity for multivalent metals[9]. Under these conditions, the specific activity of the pure enzyme was 27 to 33 units per mg. When dipeptides were used as substrates (1–2 mM), the hydrolysis was followed by measuring the decrease in absorbance at 228 nm (ref. 10). The dipeptide substrates were prepared from 0.1 M stock solution previously extracted with 0.01% dithizone in CCl_4.

RESULTS

Reversible removal of zinc

Several procedures for the removal of zinc from the enzyme were investigated in order to ascertain that the properties of apoaminopeptidase (and particularly the requirement of the protein for zinc) were not affected by the technique used for metal depletion: (a) Upon electrodialysis, conducted as described by Stein *et al.*[11], the pH of the enzyme solution dropped to the isoionic point (approx. pH 4) within 30 min, followed by rapid release of metal. (b) Gel filtration through a 30 cm × 2.5 cm column of non buffered Sephadex G-25, equilibrated with 0.1 M NaCl, also brought the enzyme (2–3 mg in 0.5 ml) to its isoelectric point with concomitant loss of metal. (c) Exposure to 1,10-phenanthroline. 1 mg enzyme in 0.25 ml buffer was applied to a 1.5 cm × 14 cm column of metal-free Sephadex G-25 equilibrated with 1 mM 1,10-phenanthroline and buffer (flow rate: 70 ml/h; retention time: 8 min); 1,10-phenanthroline was then removed from the enzyme solution by passage through a buffer-equilibrated column of Sephadex G-25. (d) Incubation with EDTA. The enzyme (10 mg/ml) in buffer was incubated for 24 h in the presence of 0.1 mM EDTA at pH 7.0, then passed through a 2.5 cm × 25 cm column of Sephadex G-25 equilibrated with buffer only. (e) Treatment with a chelating resin. 1 ml of wet Chelex was added to 15 mg enzyme in 2.5 ml of buffer. After 18 h of stirring at 22 °C in the presence of toluene (20 μl), the resin was removed by centrifugation and the treatment was repeated with a second portion of 0.5 ml of wet Chelex.

All five procedures yielded preparations that were 90 to 100% inactive and which could be reactivated by the addition of zinc. The least effective methods were electrodialysis, which yielded preparations with 10% of the initial zinc content and activity, and treatment with 1,10-phenanthroline (10% zinc and 2% activity); gel filtration on Sephadex gave preparations with 5% of the initial zinc content and 1.5–2% activity. The most effective procedures for metal depletion were treatment with EDTA or Chelex, which removed 99.5% of the zinc with concomitant loss of more than 99% of initial activity. For the routine preparation of metal-free apoaminopeptidase, the Chelex method was chosen because it can easily be adapted to large scale preparations and has the further advantage of bringing about very little dilution of the sample.

Properties of apoaminopeptidase

Zinc-free apoaminopeptidase was found to be inactive, not only towards the substrate routinely used in this work (leucine-*p*-nitroanilide), but also towards the *p*-nitroanilides of glycine, alanine and phenylalanine, and towards dipeptides such as Leu–Gly, Gly–Gly, Gly–Leu, Gly–Ala, Ala–Gly. Upon the addition of Zn^{2+}, the apoenzyme reverts to the active form virtually instantaneously, irrespective of the substrate used.

On the basis of the elution volumes from columns of Sephadex G-200, the molecular weight of the apoenzyme is identical to that of the native enzyme. The electrophoretic migration in EDTA-containing polyacrylamide gels is likewise not altered. Most of the heat- and pH-stability of the native enzyme is retained by the apoenzyme: no loss of potential activity could be detected after a 40-h preincubation at pH values ranging from 6.0 to 8.5 at 22 °C; at neutrality, the apoenzyme could be

TABLE I

STABILITIES OF AMINOPEPTIDASE AND APOAMINOPEPTIDASE

Enzyme (or apoenzyme), 35 μM, in buffer in Expts (1) and (2); in 0.1 M sodium acetate in (3), or in 0.1 M sodium phosphate in (4) was treated as indicated above. Samples were removed at selected times and assayed in zinc substrate.

Treatment	*Half life (min)*	
	Native or reconstituted[a] *aminopeptidase*	*Apoaminopeptidase*[b]
1 68 °C	70	4
2 Urea, 6.4 M	8	1
3 pH 3.8	300	8
4 pH 11.6	3000	5

[a] A solution of apoenzyme, 5 μM in EDTA, was reactivated by addition of $ZnSO_4$ to a final concentration of 7.5 μM prior to treatment.

[b] 5 μM in EDTA during treatment.

kept at 37 °C for 40 h while retaining 100% of its potential activity. However, under extreme conditions (Table I), a difference in stability between the apoenzyme and the native aminopeptidase becomes apparent; in contrast, the reconstituted enzyme is indistinguishable from native aminopeptidase in its resistance to denaturation, even under extreme conditions.

Correlation between zinc content and aminopeptidase activity

The influence of Zn^{2+} on the catalytic activity of particulate aminopeptidase was studied as the metal was progressively removed from the native enzyme, as well as when the metal-free apoenzyme was replenished with Zn^{2+}. Fig. 1 shows that there is a direct correlation between loss of enzyme activity and removal of zinc. These results were confirmed by titration of the apoenzyme with Zn^{2+}. Fig. 2 (triangles) shows that the activity is directly proportional to zinc content from 0 to 2.0 gatoms per mole, and is not affected by further additions of Zn^{2+} (large excesses of metal, however, inhibit the enzyme).

Reactivation of apoaminopeptidase by metal ions other than zinc

The apoenzyme is instantaneously converted to active aminopeptidase not only by the addition of Zn^{2+} but also by the addition of Cu^{2+}, Co^{2+} or Ni^{2+}. Other metal ions were investigated too, namely Be^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}, Sc^{3+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Sr^{2+}, Pd^{2+}, Ag^{+}, Cd^{2+}, Sn^{2+}, Ba^{2+}, La^{3+}, Hg^{2+} and Pb^{2+}, but failed to bring about any restoration of activity.

Fig. 2 illustrates the titration of the metal-free enzyme with Zn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+}, which restore 100, 240, 190 and 50%, respectively, of the activity of native aminopeptidase. In each case, the activation is a linear function of metal ion concentration up to a metal content very close to 2 gatoms per mole of enzyme.

The percentage specific activities plotted in Fig. 2 were obtained by measurements of initial reaction rates. Additional information can be gained by studying the effect of enzyme–metal interaction on enzyme activity as a function of time. Reaction rates measured with native aminopeptidase or with Zn^{2+}-reactivated apo-

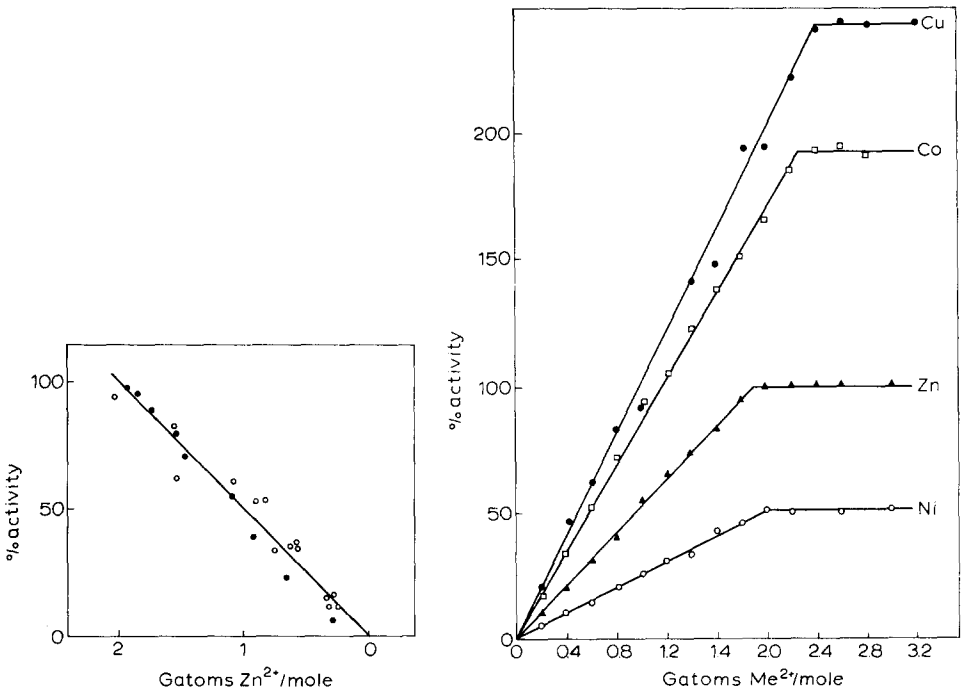

Fig. 1. Correlation between Zn content and enzyme activity. Enzyme (2.1 μM) was incubated in 50 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.0 at 25 °C with Chelex (○), or 0.1 mM EDTA (●). In the latter case, aliquots were filtered through Sephadex G-25 prior to analysis.

Fig. 2. Reactivation of the metal-free aminopeptidase with various cations. 2 mg of apoaminopeptidase in 4 ml buffer were titrated with various metals. Enzyme activity was determined using EDTA-containing substrate 5 min after each successive addition of metal (0.2 gatom per mole of enzyme).

enzyme were identical whether the substrate contained EDTA or zinc, and remained constant. Apoenzyme reactivated with metal ions other than Zn^{2+} gave initial reaction rates which were the same in either EDTA- or zinc-containing substrates; however time-dependant changes in reaction rates were observed. When EDTA-containing substrate was used there was always a slow levelling off (Fig. 3a); the addition of excess zinc restored the initial reaction rate, showing that substrate depletion was not involved. With zinc-containing substrate the reaction rate changed to that of the native enzyme (Fig. 3b), suggesting replacement by Zn^{2+} of the Ni^{2+}, Co^{2+} or Cu^{2+} bound to the enzyme.

The apoenzyme activated by Zn^{2+}, Cu^{2+}, Co^{2+} or Ni^{2+} was also capable of catalyzing the cleavage of a number of different substrates such as the *p*-nitroanilides of glycine, alanine and phenylalanine, as well as the dipeptides Leu–Gly, Gly–Gly, Gly–Leu, Gly–Ala and Ala–Gly. Dipeptide cleavage was monitored at 228 nm (ref. 10); the Cu^{2+} enzyme could not be tested since it forms a colored complex with dipeptide substrates. In all cases, the lowest hydrolysis rates were obtained with the Ni^{2+}- and the highest rates with the Cu^{2+}- (or Co^{2+}-) activated apoenzyme.

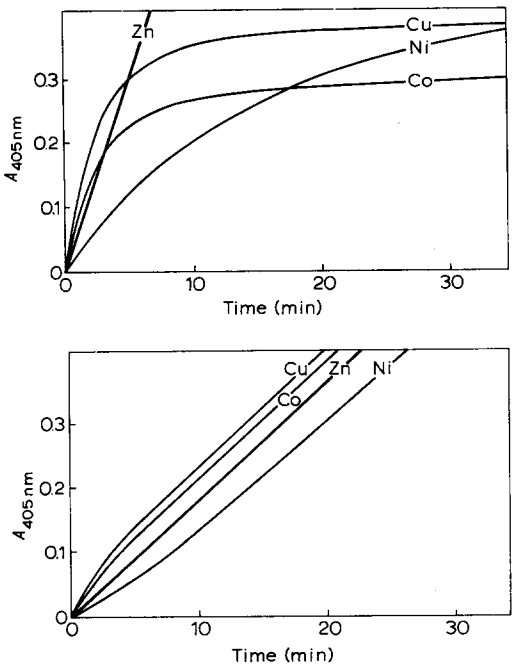

Fig. 3. Hydrolysis of leucine-*p*-nitroanilide by Zn-, Co-, Ni- and Cu-aminopeptidase in the presence of EDTA or zinc. The peptidases were prepared by reactivating the apoenzyme with a two-fold excess of the various metals. The reaction was started by the addition of the enzyme to the EDTA- or zinc-containing substrate (3a and 3b, respectively). The final enzyme concentration was 0.75 μM in the assay system containing EDTA, and 0.25 μM in the one containing zinc.

DISCUSSION

Aminopeptidase can be characterized as a zinc-metalloenzyme[12,13] since it complies with all the generally accepted criteria for this class of enzymes: (1) it is a homogeneous protein which can be isolated with its full metal complement and with maximum activity; (2) the intrinsic metal to protein ratio increases during enzyme purification and becomes constant with complete purification, whereas the extrinsic metal to protein ratios decreases to zero; (3) the ratio of gatoms of zinc to moles of protein in the pure enzyme is a small integral number[2]; (4) metal-binding agents inactivate the enzyme; (5) the inactivation can be reversed by the addition of metals; (6) removal of the intrinsic metal abolishes activity, with activity loss being directly proportional to metal loss; (7) restoration of activity to the metal-free apoenzyme is directly proportional to the amount of zinc added until the enzyme has regained its full metal complement.

Zinc can be removed from aminopeptidase either by exposing the enzyme to a metal-binding agent or by taking advantage of the greatly increased dissociation of the enzyme-metal complex at low pH values. Regardless of the method used, there is good correlation between the extent of metal removal and loss of enzyme activity, except for 1,10-phenanthroline treatment where too low an enzyme activity relative to the metal content is observed; this might be due to inactive enzyme-inhibitor complexes in which protein-bound metal is still present.

Removal of Zn^{2+} from aminopeptidase as such does not grossly change the architecture of the protein. The molecular weight and electrophoretic mobility of the metal-free inactive apoenzyme are very similar to that of the native aminopeptidase. However, Zn^{2+} may play some role in stabilizing the structure of the native enzyme, as the zinc-free apoenzyme is less resistant to denaturation than the native enzyme under extreme conditions. The reconstituted, active enzyme, on the other hand, is identical to aminopeptidase both in regard to its catalytic properties with all substrates tested and its resistance to denaturation.

For catalysis, the importance of zinc is decisive, irrespective of the nature of the substrate. The linear correlation between catalytic activity and zinc content from 0 to 2.0 gatoms of zinc per mole of enzyme seems to exclude the possibility that the two atoms of zinc have different functions. Aminopeptidase, like certain other zinc-metalloenzymes[14,15], is active with a number of divalent cations other than zinc. However, the effects of extrinsic metals on the activity of the zinc enzyme are not straightforward, and have led to contradictory interpretations[3–5]. In contrast, a study of the apoenzyme yields a clear picture: Zn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} are the only cations capable of conferring significant aminopeptidase activity. Titration of apoaminopeptidase with "activating" cations shows that the metal content required to confer full activity is very close to 2 gatoms per mole (mol. wt 280 000) in all cases, suggesting that the enzyme molecule might consist of two identical catalytic units, or that the active center possesses two indistinguishable metal-binding sites. As can be expected, replacement of zinc by another "activating" metal affects the specific activity of the native enzyme; the Cu^{2+}- and Co^{2+}-substituted aminopeptidases are more active than the zinc enzyme; however, the affinity of the protein for Zn^{2+} is probably higher than that for Cu^{2+}, Co^{2+} or Ni^{2+}. The progressive decrease in reaction rate observed with the Ni-, Co- or Cu-enzyme in Fig. 3a, in contrast to the constant rate of hydrolysis displayed by zinc aminopeptidase, suggests that Ni^{2+}, Co^{2+} and Cu^{2+} are released from the enzyme whereas Zn^{2+} is retained by the protein under the same conditions.

The conclusions reached here concerning aminopeptidase contrast with the vague and sometimes conflicting interpretations found in past reports[3–5]. The present findings suggest that previous difficulties encountered in work with particulate aminopeptidase were mainly due to extraneous metals. If significant results are to be obtained in work with this enzyme, it is essential to recognize and counteract the effect of such contaminants. In the present work, this was accomplished by the use of substrate-EDTA combination and of substrate-zinc combination. The methods described here may also be useful with other metalloenzymes, especially those which are as exquisitely sensitive to the effects of extraneous metal as aminopeptidase.

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REFERENCES

1 Wacker, H., Lehky, P. and Stein, E. A. (1970) *Experientia* 26, 688
2 Wacker, H., Lehky, P., Fischer, E. H. and Stein, E. A. (1971) *Helv. Chim. Acta* 54, 473–485
3 Pfleiderer, G., Celliers, P. G., Stanulovic, M., Wachsmuth, E. D., Determann, H. and Braunitzer, G. (1964) *Biochem. Z.* 340, 552–564
4 Hanson, H., Hütter, H. J., Mannsfeldt, H. G., Kretschmer, K. and Sohr, Ch. (1967) *Z. Physiol. Chem.* 348, 680–688
5 Femfert, U. and Pfleiderer, G. (1970) *FEBS Lett.* 14, 89–91
6 Malmström, B. G. (1956) in *Methods of Biochemical Analysis* (Glick, D., ed.), Vol. 3, 327–352, Interscience Publishers, New York
7 Thiers, R. E. (1957) in *Methods of Biochemical Analysis* (Glick, D., ed.), Vol. 5, pp. 273–335, Interscience Publishers, New York
8 Himmelhoch, S. R., Sober, H. A., Vallee, B. L. and Fuwa, K. (1966) *Biochemistry* 5, 2523–2530
9 Good, N. E., Winget, D., Winter, W., Connolly, T. N., Iyawa, S. and Singh, R. M. M. (1966) *Biochemistry* 5, 467–477
10 Vescia, A. and Traniello, S. (1964) *Arch. Biochem. Biophys.* 105, 465–469
11 Stein, E. A., Hsiu, J. and Fischer, E. H. (1964) *Biochemistry* 3, 56–66
12 Vallee, B. L. (1955) *Adv. Prot. Chem.* 10, 317–384
13 Vallee, B. L. and Wacker, W. E. C. (1970) in *The Proteins*, 2nd edn (Neurath, H. ed.), Vol. 5, pp. 129–143, Academic Press, New York and London
14 Coleman, J. E. and Vallee, B. L. (1960) *J. Biol. Chem.* 235, 390–395
15 Lazdunski, C., Petitclerc, C. and Lazdunski, M. (1969) *Eur. J. Biochem.* 8, 510–517