Chicken liver Pz-peptidase, a thiol-dependent metallo-endopeptidase

Alan J. BARRETT* and Molly A. BROWN

Department of Biochemistry, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

Pz-peptidase was purified from chicken liver as a protein of M_r 80000 and pI 5.2. The purified enzyme hydrolysed phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, 2,4-dinitrophenyl-Pro-Leu-Gly-Pro-Trp-D-Lys, 7-methoxycoumarin-3-carboxyl-yl-Pro-Leu-Gly-Pro-D-(2,4-dinitrophenyl)Lys, benzoyl-Gly-Ala-Ala-Phe-*p*-aminobenzoate, Ac-Ala₄ (at the Ala-1-Ala-2 bond) and bradykinin (at the Phe-5-Ser-6 bond). No hydrolysis of proteins was detected. Loss of activity in the presence of EDTA or 1,10-phenanthroline was time-dependent. Metal ions found to restore activity after treatment with EDTA were Zn²⁺, Mn²⁺, Ca²⁺, Co²⁺ and Cd²⁺, in decreasing order of effectiveness. Ni²⁺, Fe²⁺ and higher concentrations of Zn²⁺ were inhibitory. Inhibition by *N*-[1-(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate and related compounds showed K_i values (down to 5 nM) somewhat lower than those for the rat enzyme. Pz-peptidase was activated by low concentrations of 2-mercaptoethanol and dithiothreitol, but inhibited by higher concentrations. *p*-Chloromercuribenzoate and some other thiol-blocking reagents were inhibitory. Inactivation by diethyl pyrocarbonate that was reversible by hydroxylamine showed the presence of essential histidine residue(s). We conclude that chicken Pz-peptidase is a metallo-endopeptidase with thiol-dependence. Moreover, the properties of chicken Pz-peptidase agree well with those described for mammalian soluble metallo-endopeptidase and endo-oligopeptidase A, consistent with the view that these three types of activity are all attributable to the single enzyme for which the name thimet peptidase has been proposed.

INTRODUCTION

Pz-peptidase (or tissue-endopeptidase degrading collagenase-synthetic-substrate, EC 3.4.99.31) is a widely distributed enzyme that hydrolyses Pz-Pro-Leu-Gly-Pro-D-Arg at the Leu-Gly bond, and is also active on the related quenched fluorescence substrates Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys (Barrett *et al.*, 1989) and Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) (Tisljar *et al.*, 1990). The results of recent work with the enzymes of rat and rabbit indicate that the activities of mammalian Pz-peptidase, soluble metallo-endopeptidase or endopeptidase 24.15 (EC 3.4.24.15) and endo-oligopeptidase A (endo-oligopeptidase, EC 3.4.22.19) are due to a single enzyme with species variants (Barrett & Tisljar, 1989; Tisljar *et al.*, 1989; Barrett, 1990; Tisljar & Barrett, 1990).

Naturally, there is a distinct literature associated with each of the three names under which Pz-peptidase has been known. As Pz-peptidase, the enzyme has been most thoroughly characterized from chicken embryo by Morales & Woessner (1977). Because the Pz-peptide substrate is also a substrate of clostridial collagenase, Pz-peptidase has been suspected of being involved in the degradation of collagen (Morales & Woessner, 1977; Rajabi & Woessner, 1984), but this now seems unlikely (Barrett & Tisljar, 1990). As soluble metallo-endopeptidase, the enzyme is best known from the rat (Orlowski *et al.*, 1983, 1988, 1989), and it was in this species that the identity of Pz-peptidase with soluble metallo-endopeptidase was shown (Barrett & Tisljar, 1989). Under the name of endo-oligopeptidase A, Pz-peptidase has been characterized from tissues of rabbit and other species (Camargo *et al.*, 1985), and it was with the rabbit enzyme that Pz-peptidase and endo-oligopeptidase were shown to be identical (Tisljar *et al.*, 1989).

A major question regarding Pz-peptidase has been the nature

of the catalytic site. The effects of inhibitors readily exclude it from the serine and aspartic types of endopeptidase (e.g. Morales & Woessner, 1977), but it has characteristics of both cysteine and metallo-proteinases, hence the 3.4.22 and 3.4.24 Enzyme Commission numbers associated with the names endo-oligopeptidase and soluble metallo-endopeptidase respectively. The purpose of the present paper is to extend the characterization of chicken Pz-peptidase, to compare the properties of the enzyme with those reported for mammalian soluble metallo-endopeptidase and endo-oligopeptidase A, and to consider the nature of the catalytic site.

EXPERIMENTAL

Materials

Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys, Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) and Mcc-Pro-Leu (Barrett *et al.*, 1989; Tisljar *et al.*, 1990) were prepared by Dr. C. G. Knight of this Laboratory.

Purification of chicken Pz-peptidase

Chicken liver was obtained from a local poultry packing station, minced and stored frozen until required. The tissue (800 g) was allowed to thaw partially at 20 °C, but subsequent steps were at 4 °C. The tissue was dispersed in 1.6 litres of 10 mM-sodium acetate/acetic acid buffer, pH 4.9, with a Silverson homogenizer. The pH was adjusted to 4.9 with 1.0 M-acetic acid (about 48 ml) from a measured volume. Then 1.0 M-sodium acetate/acetic acid buffer, pH 4.9, was added to bring the total concentration of acetate/acetic acid to 50 mM. The homogenate was stirred for 30 min, and centrifuged at 10000 *g* for 30 min. The supernatant was stirred with 20 g of CM-cellulose (Whatman

Abbreviations used: Ac, *N*-acetyl; *p*AB, *p*-aminobenzoate; Dnp, 2,4-dinitrophenyl; Bz, benzoyl; Mcc, 7-methoxycoumarin-3-carboxyl-yl; Pz, phenylazobenzoyloxycarbonyl; Tos, toluene-*p*-sulphonyl; Z, benzyloxycarbonyl.

* To whom correspondence should be addressed.

CM-52, previously equilibrated with 50 mM-sodium acetate/acetic acid buffer, pH 4.9)/100 ml of supernatant for 15 min at 4 °C. The CM-cellulose was removed by filtration and discarded.

The pH of the filtrate was adjusted to 7.8 with solid Tris (8–12 g), and $(\text{NH}_4)_2\text{SO}_4$ was added to 3.0 M. After 30 min, the preparation was centrifuged at 10000 g for 15 min and the supernatant was discarded. The pellet was extracted with 25 mM-Tris/HCl buffer, pH 7.8, containing 1.8 M- $(\text{NH}_4)_2\text{SO}_4$ (800 ml) during 30 min, and centrifuged as before. The supernatant was made 3.0 M with respect to $(\text{NH}_4)_2\text{SO}_4$, stirred for 30 min, and further centrifuged to give a pellet, which was redissolved in 130 ml of 25 mM-Tris/HCl buffer, pH 7.8, and dialysed against several changes of the same.

Whatman DE-52 DEAE-cellulose was equilibrated with 25 mM-Tris/HCl buffer, pH 7.8, and 320 g of the damp exchanger was packed into a chromatographic column (50 mm diam. $\times$ 300 mm long). A further 80 g of the exchanger was stirred with the dialysed preparation for 30 min, removed by filtration and packed on top of the remainder of the DEAE-cellulose in the column. The column was eluted with a gradient (1 litre plus 1 litre) of 0–300 mM-NaCl in the Tris buffer, at a flow rate of 200 ml/h. The fractions (20 ml) were assayed, activity being found at about 150 mM-NaCl. Active fractions were combined, made 3.0 M with respect to $(\text{NH}_4)_2\text{SO}_4$ and centrifuged. The pellet was taken up in 10 ml of 10 mM-potassium phosphate buffer, pH 7.0, and dialysed against the same. The sample was run on a 40 ml (16 mm diam.) column of Bio-Gel HT hydroxyapatite (Bio-Rad Laboratories), eluted with a gradient (200 ml plus 200 ml) of 10–200 mM-potassium phosphate buffer, pH 7.0. Active fractions were again concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$, and the pellet was taken up in 3 ml of 50 mM-Tris/HCl buffer, pH 7.8, containing 100 mM-NaCl and 0.1 % Brij 35.

The sample was next run on a column (16 mm $\times$ 850 mm, 171 ml) of Sephacryl S-200 HR in the above Tris/HCl buffer at a flow rate of 50 ml/h, 2 ml fractions being collected. Fractions containing activity were combined, and then transferred into 25 mM-Bistris/HCl buffer, pH 6.3, and concentrated to 5 ml by ultrafiltration. The sample was run on the Pharmacia f.p.l.c. Mono-P column at a flow rate of 0.5 ml/min. The column had been pre-equilibrated with the Bistris buffer, and was developed with Polybuffer 74 diluted 1:10 (pH 4.0). The purified enzyme was stored at pH 7.0, in 50 mM-Tris/HCl buffer.

Enzyme assays

The normal assay in this study was the quenched-fluorescence assay with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys as substrate [as described by Barrett *et al.* (1989), except that CaCl_2 was omitted from the buffer], 1 unit being defined as that activity hydrolysing 1 μmol of substrate/min. Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was used in a similar procedure, with excitation at 345 nm and emission at 405 nm (Tislar *et al.*, 1990). For both quenched-fluorescence substrates it was necessary to correct the data for the effects of absorptive quenching by use of standard(s) containing substrate at the relevant concentration in addition to the 1 μM product. Assays with other substrates were made by published methods, as described in the text.

Determination of kinetic constants

This was by standard methods. For determination of K_m values, rates of hydrolysis of substrate at a range of substrate concentrations were fitted to the Michaelis–Menten equation by non-linear-regression analysis (Enzfitter, Elsevier–Biosoft, Cambridge, U.K.). Apparent inhibition constants (K_i') were determined with values of $[\text{I}] \gg [\text{E}]$, so that non-tight-binding assump-

tions were valid, and the values were corrected for the effect of substrate by use of eqn. (1):

$$K_i = K_i'/(1 + [\text{S}]/K_m) \quad (1)$$

Determination of protein

The Bio-Rad Laboratories protein assay (Bradford, 1976) was used, with BSA as standard.

Gel electrophoresis and isoelectric focusing

SDS/PAGE was as described by Bury (1981), with the same M_r standards. PAGE without denaturation was done with the same buffer system, but with gels containing 5 % (w/v) total acrylamide, and no SDS or 2-mercaptoethanol. In some experiments a broad sample lane was cut longitudinally after the run, and one half was stained for protein and the other was cut transversely into narrow strips for enzyme assay.

Analytical isoelectric focusing in polyacrylamide gel was as described by Barrett (1973), with gels containing 7.5 % (w/v) polyacrylamide and 4 % (w/v) of LKB Ampholine, range pH 4–6.

RESULTS AND DISCUSSION

Purification of chicken Pz-peptidase

The results of a typical purification are summarized in Table 1, and the progress of the purification to a single major band in SDS/PAGE is shown in Fig. 1. In non-denaturing PAGE a single band of protein was again obtained, and this was associated with enzymic activity (Fig. 1c). The same result was obtained in analytical isoelectric focusing in polyacrylamide gel (not shown). Prolyl endopeptidase [assayed with Z-Gly-Pro-7-(4-methyl)-coumarylamide (Tislar *et al.*, 1990)] was undetectable after the DEAE-cellulose step.

Physicochemical properties

The apparent M_r of the protein was estimated to be 80000; it ran fractionally more slowly than transferrin (M_r 78000) in SDS/PAGE (Fig. 1b). This value is in close agreement with that of 77000 reported by Morales & Woessner (1977) for the enzyme from chicken embryo.

The peak of enzymic activity from the chromatofocusing column appeared at pH 5.0, and in analytical isoelectric focusing (see above) the pH of the gel segments containing the peak of

Table 1. Purification of Pz-peptidase from chicken liver

The results are those for a typical preparation from 800 g of tissue. Activity was assayed with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys, and protein by the Bio-Rad Laboratories method. In the early stages (before DEAE-cellulose chromatography) up to 10 % of the activity recorded was inhibitable by di-isopropyl phosphorofluoridate and therefore attributable to some enzyme other than Pz-peptidase (perhaps prolyl endopeptidase).

Purification step	Protein (mg)	Activity (units)	Purification factor (fold)	Yield (%)
Tissue extract	118 300	789	(1)	(100)
Supernatant at pH 5	20 200	620	4.6	79
Supernatant from CM-cellulose	6000	387	9.8	49
$(\text{NH}_4)_2\text{SO}_4$ fraction	2880	377	19.8	48
DEAE-cellulose	241	165	103	21
Hydroxyapatite	20.7	51	370	6.4
Sephacryl S-200 HR	4.2	15.1	542	1.9
Chromatofocusing	0.6	6.5	1631	0.8

Fig. 1. PAGE of chicken liver Pz-peptidase
(a) SDS/PAGE with reduction, in 10% (w/v) polyacrylamide, samples being (i) tissue extract, (ii) CM-cellulose supernatant, (iii) (NH₄)₂SO₄ fraction, (iv) DEAE-cellulose fraction, (v) hydroxyapatite fraction, (vi) Sephacryl S-200 HR fraction and (vii) final sample from chromatofocusing. (b) SDS/PAGE with reduction, in 7% polyacrylamide gel, samples being (i) *M_r* standards and (ii) purified Pz-peptidase as in part (a), lane (vii) (2.2 μg). (c) Purified Pz-peptidase run without denaturation in 5% polyacrylamide, and stained for protein (below) or assayed for activity (above).

activity was 5.2. We conclude that 5.2 is the best estimate of the pI. A pI value of 5.0 was reported for chicken embryo Pz-peptidase by Morales & Woessner (1977).
On the basis of protein assays by the Bio-Rad Laboratories method, the *A*₂₈₀^{1%} of the purified enzyme was 10.0.

Catalytic activity

Preparations of chicken liver Pz-peptidase that were essentially homogeneous as protein ranged from 7 to 21 units/mg in specific activity on Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys. For comparison

Table 2. Action of chicken Pz-peptidase on various substrates, compared with published values

A single preparation of Pz-peptidase (20.3 units/mg on Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys) was assayed exactly as described in the publications cited, with the same arbitrary units of activity.

Substrate	Specific activity (units/mg)	
	Present work	Literature value
Pz-Pro-Leu-Gly-Pro-D-Arg	983	1060*
Bz-Gly-Ala-Ala-Phe-pAB	273	1220†
Bradykinin	6.5	1.37‡ 12.61§ 18.8†

* Reported by Morales & Woessner (1977) for chicken-embryo Pz-peptidase.
† Reported by Orlowski *et al.* (1989) for rat testis soluble metallo-endopeptidase.
‡ Reported by Camargo *et al.* (1987) for bovine brain endo-oligo-peptidase A.
§ Reported by Kikuchi *et al.* (1988) for rat epidermis enzyme.

with the enzyme under other names from other sources, assays were also made with Pz-Pro-Leu-Gly-Pro-D-Arg (for 'Pz-peptidase'), Bz-Gly-Ala-Ala-Phe-pAB (for 'soluble metallo-endopeptidase') and with bradykinin (for 'endo-oligo-peptidase A') (Table 2). It can be seen that the activity of the chicken enzyme on all of these substrates was of the same order as that of the preparations of the enzyme from other sources by other methods.
In order to establish the point of cleavage of bradykinin by Pz-peptidase, the peptide (0.1 mM) in 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-2-mercaptoethanol was treated with 4.2 munits of chicken Pz-peptidase/ml during 4 h at 40 °C, and 50 μl samples were run in h.p.l.c. on a C₁₈ reverse-phase column in a gradient of 5–70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, with monitoring at 220 nm. The peptide was found to be converted into two new components, which were eluted earlier. These were collected and hydrolysed for amino acid analysis (Barrett *et al.*, 1989), with results that showed them to be Ser-Pro-Phe-Arg and Arg-Pro-Pro-Gly-Phe, in order of elution. Clearly, there had been a single peptide-bond cleavage, at Phe-5-Ser-6 in the sequence of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), as reported for endo-oligo-peptidase A by Camargo *et al.* (1973) and soluble metallo-endopeptidase by Orlowski *et al.* (1983). Morales & Woessner (1977) failed to detect cleavage of bradykinin by chicken Pz-peptidase.
The action of Pz-peptidase on bradykinin was almost completely (> 90%) blocked by 1 μM-*N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB, a specific inhibitor of soluble metallo-endopeptidase (see below). The activity was unaffected by pretreatment of the enzyme for 5 min with 1 mM-di-isopropyl phosphorofluoridate, a potent inhibitor of prolyl endopeptidase, which also cleaves bradykinin, although at a different bond.
The *K_m* of chicken Pz-peptidase for Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys was 13.7 μM, and that for Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was 12.3 μM.

Hydrolysis of Ac-Ala₃ and Ac-Ala₄ (both 5 mM in the Tris/HCl buffer, pH 7.8) was monitored by the fluorescamine reaction for the released α -amino groups (Pace & Barrett, 1984). Ac-Ala₄ proved to be rapidly cleaved, whereas there was no action on Ac-Ala₃. Analysis of the hydrolysate by h.p.l.c. revealed Ala₃, but no free alanine or Ala₂, showing that the bond cleaved was: Ac-Ala-Ala-Ala-Ala. Ac-Ala₄ is the smallest and simplest substrate molecule yet discovered for Pz-peptidase.

To test for possible proteolytic activity of Pz-peptidase, BSA, rabbit muscle phosphorylase *b* and gelatin produced by heat denaturation of collagen, each at 1 mg/ml, were incubated with 100 μ g of Pz-peptidase/ml for 1 h at 37 °C in 50 mM-Tris/HCl buffer, pH 7.8, containing 5 mM-2-mercaptoethanol. Samples subjected to SDS/PAGE showed no sign of degradation of the substrate proteins. This is consistent with a previous report that chicken Pz-peptidase does not degrade proteins (Morales & Woessner, 1977).

pH-dependence of activity

In 50 mM-Tris/HCl buffer, the enzyme showed a rather flat pH optimum at pH 8.0.

Removal of essential metal ion

Solutions of Pz-peptidase were made up in 50 mM-Tris/HCl buffer, pH 7.0, containing 2 mM-2-mercaptoethanol and 10 mM-EDTA or 1 mM-1,10-phenanthroline and stored at 4 °C. Samples were removed periodically for assay in the standard system, but with 10 μ M-EDTA. For the EDTA experiment activities expressed as percentages of the zero-time value were 78% (1 h) and 1% (16 h), and for 1,10-phenanthroline the values were 22% (10 min), 9% (20 min) and 3% (60 min). A control without EDTA or phenanthroline showed no loss of activity over the same period. The loss of activity in 10 mM-EDTA at pH 7.8 was at least 10-fold slower than at pH 7.0. The enzyme was also incubated with 1,10-phenanthroline in parallel with the non-chelating analogue 4,7-phenanthroline for 16 h at 4 °C in assay buffer. Residual activities (measured in the presence of 10 μ M-EDTA and expressed relative to a control without phenanthroline) were 0% for 1,10-phenanthroline and 93% for 4,7-phenanthroline.

The progressive loss of enzymic activity in the presence of EDTA or 1,10-phenanthroline is best explained by a mechanism of inactivation in which a transient enzyme-metal ion-chelator ternary complex is formed, and metal ion is lost from this more rapidly than from the binary enzyme-metal ion complex (Stöcker *et al.*, 1988). The time-dependence of inactivation, taken together with its almost complete reversal by Zn²⁺ (see below), precludes the possibility that EDTA is a simple inhibitor of the enzyme.

Inactivation of the enzyme by chelating agents has previously been reported by Lukac & Koren (1977), Morales & Woessner (1977) and Orlowski *et al.* (1983). In some studies inactivation by EDTA has not been seen, and the effect of 1,10-phenanthroline has been reversible by dialysis without the addition of metal ions (Camargo *et al.*, 1973; Tislar & Barrett, 1989). These results may have been due to species differences in tightness of binding of the metal ion, incubations with the chelators that were too brief or at too high a pH, or the presence of traces of metal ions in the buffers (Tislar & Barrett, 1990).

Re-activation of Pz-peptidase by metal ions

Pz-peptidase was stored in 10 mM-EDTA, pH 7.0, as above for 24 h, after which time it showed negligible activity (in reaction mixtures containing 10 μ M residual EDTA). Various metal ions were tested for their ability to restore activity at 50 and 100 μ M concentration, in standard assay mixtures containing 5 mM-2-mercaptoethanol. Results were essentially the same at both 50

Table 3. Re-activation of Pz-peptidase by metal ions after it has been inactivated by exposure to EDTA

The enzyme was inactivated by exposure to 10 mM-EDTA for 24 h at pH 7.0, and was then diluted to 10 μ M-EDTA for assay. All metal salts were used at 50 μ M, and activities are expressed relative to that of a control enzyme sample not treated with EDTA.

Metal salt	Rate (% of control)
None	2
ZnCl ₂	93
MnCl ₂	71
CaCl ₂	35
CoCl ₂	31
CdSO ₄	22
MgCl ₂	3
CuSO ₄	2
NiSO ₄	3
FeCl ₂	2

and 100 μ M for all ions except Zn²⁺, for which activity was 15% lower at the higher concentration. Zn²⁺ was the most effective ion in re-activating the enzyme, followed by Mn²⁺, Ca²⁺, Co²⁺ and Cd²⁺ (Table 3). Activation appeared instantaneous except with Ca²⁺, for which the rate of reaction increased progressively over 2 min. Although Mg²⁺ and Cu²⁺ did not activate, they did not prevent subsequent activation by Zn²⁺; in contrast, after the partial activation by Ca²⁺, or non-activation by Ni²⁺ or Fe²⁺, there was no further activation by Zn²⁺.

Our finding that the most effective metal ions were Zn²⁺ and Mn²⁺ is consistent with the report by Lukac & Koren (1977) of re-activation of rat testis Pz-peptidase preferentially by these ions after treatment with chelators. Morales & Woessner (1977) failed to detect such re-activation, but this is attributable to their use of the metal ions at 3 mM concentration, at which most are strongly inhibitory. Orlowski *et al.* (1983) reported re-activation of rat brain soluble metallo-endopeptidase by Zn²⁺, Mn²⁺, Ca²⁺ and Co²⁺, qualitatively in agreement with the results for the chicken enzyme, although there were quantitative differences. The rather surprising partial re-activation of a metallo-endopeptidase by Ca²⁺ has previously been reported for 'endopeptidase 24.11' (Kerr & Kenny, 1974). We did not see the super-activation by Co²⁺ reported by Orlowski *et al.* (1989) for the rat testis enzyme under rather different conditions. All metallo-endopeptidases that have been fully characterized to date have proved to be zinc enzymes, and this is very probably true of Pz-peptidase also.

Metallo-endopeptidase inhibitors

Chicken Pz-peptidase was not significantly inhibited (< 10%) by phosphoramidon or thiorphan (10 μ M).

Inhibitors of rat soluble metallo-endopeptidase have been designed as substrate analogues in which the structure in the vicinity of the scissile peptide bond is replaced by the unhydrolysable -CH(CO₂H)-NH- structure, which contains a carboxy group located to co-ordinate with an active-site metal atom (Orlowski *et al.*, 1988). Some of these compounds (provided by Dr. M. Orlowski, Mount Sinai School of Medicine, New York NY, U.S.A.) have now been used with chicken Pz-peptidase. The cleavage of Pz-Pro-Leu-Gly-Pro-D-Arg by the enzyme was completely inhibited by 100 nM-*N*-[1(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*AB. In continuous assays with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys, the *N*-(carboxyalkyl)-peptides produced apparently instantaneous inhibition, and the new rates of product formation were linear. *K*_i values were determined for chicken

Table 4. Active-site-directed reversible inhibitors of Pz-peptidase

Inhibition of chicken liver Pz-peptidase was compared with that of the enzyme from rat testis, assays being made with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys at pH 7.8. The K_i values have been corrected for the effect of the 10 μM substrate on the basis of K_m values of 10.3 μM and 12.8 μM for the chicken and rat enzymes respectively.

Compound	K_i (nM)	
	Chicken enzyme	Rat enzyme
(I) <i>N</i> -[1(<i>RS</i>)-Carboxy-3-phenylpropyl]-Ala-Ala-Tyr- <i>p</i> AB	4.6	12.3
(II) <i>N</i> -[1(<i>RS</i>)-Carboxy-3-phenylpropyl]-Ala-Ala-Phe- <i>p</i> AB	7.5	16.7
(III) <i>N</i> -[1(<i>RS</i>)-Carboxy-3-phenylpropyl]-Gly-Ala-Phe- <i>p</i> AB	252	627
(IV) <i>N</i> -[1(<i>RS</i>)-Carboxybutyl]-Ala-Ala-Phe- <i>p</i> AB	142	315
(V) <i>N</i> -[1(<i>RS</i>)-Carboxy-2-phenylethyl]-Ala-Ala-Phe- <i>p</i> AB	461	662

liver Pz-peptidase, and also with the enzyme from rat testis purified as described by Tislar & Barrett (1990) for comparison (Table 4). The ranking of the inhibitors was the same for the enzyme from both species, but the absolute K_i values were about 2-fold lower for the chicken enzyme. Compound (I) was the most effective, and the higher values of K_i for compounds (II) and (III) confirm the preference of the enzyme for tyrosine in subsite S_3' and alanine in S_1' previously reported by Orlowski *et al.* (1988) for the rat brain enzyme in a different assay system. The importance of the 3-phenyl substituent is demonstrated by the results for compound (IV), in which it is replaced by a methyl group, and compound (V), in which there is a 2-phenyl substituent. Inhibition by compound (I) was confirmed to be competitive with substrate.

Effects of thiol compounds

Chicken Pz-peptidase was assayed alone and in the presence of 2-mercaptoethanol (0.2–25 mM) or dithiothreitol (0.025–0.5 mM). Linear rates of activity were obtained in the period 1–5 min. It

was found that both thiol compounds produced maximal activation at low concentrations (1 mM and 0.05 mM respectively) and that activity fell off as the concentrations were increased (Fig. 2). The shapes of the two curves were similar, but the effects of dithiothreitol occurred at about 30-fold lower concentrations than those of 2-mercaptoethanol.

The inhibition at 5 mM-dithiothreitol was fully reversible on dilution of the dithiothreitol to 0.1 mM, which would be consistent with inhibition being due to weak interactions between the thiol compounds and the essential metal atom. The tightness of such an interaction would depend upon the thiophilicity of the metal atom. We therefore compared native enzyme and EDTA-inactivated enzyme re-activated with 50 μM -Zn²⁺ or -Mn²⁺ in 10 μM -EDTA for the effect of dithiothreitol. At 1 mM-dithiothreitol percentage inhibitions relative to the control without dithiothreitol were 63 % (native enzyme), 60 % (Zn²⁺) and 6 % (Mn²⁺). Since manganese is of lower thiophilicity than zinc (Murphy & Pratt, 1989), these results are consistent with the proposed mechanism of inhibition by dithiothreitol. Also, they would be consistent with the probability that the metal atom in native Pz-peptidase is zinc.

Previously Morales & Woessner (1977) reported activation of chicken-embryo Pz-peptidase by 0.1 mM-dithiothreitol, with inhibition at higher concentrations, and comparable results have been obtained for rat soluble metallo-endopeptidase (Orlowski *et al.*, 1983) and bovine endo-oligopeptidase. Orlowski *et al.* (1989) did not detect activation of the rat enzyme by dithiothreitol (0.4 mM), after purifying it in the presence of 0.5 mM-2-mercaptoethanol; probably the enzyme was already fully activated, or 0.4 mM was too high a concentration of dithiothreitol (cf. Fig. 2).

Inactivation by thiol-blocking reagents

The effects of thiol-blocking reagents on chicken liver Pz-peptidase were determined in the light of previous reports of inhibition (Morales & Woessner, 1977; Camargo *et al.*, 1987; Orlowski *et al.*, 1989). The enzyme was treated with 5 mM-dithiothreitol (5 min) and diluted into reaction mixtures containing 0.01 mM-dithiothreitol for determination of the effects of thiol-blocking reagents (Table 5). The reagents expected to substitute thiol groups irreversibly in a time-dependent way, iodoacetate, *N*-ethylmaleimide and 5-cyano-2-nitrothiobenzoate, gave only partial inhibition, and this was not progressive. This is consistent with the evidence from activation studies that the most active form of the enzyme contains one or more free thiol groups, but is not characteristic of the behaviour of an enzyme that depends directly on a thiol group for its catalytic activity. Progressive inactivation was seen with Tos-Phe-CH₂Cl, however, which Morales & Woessner (1977) also found to be a significant

Fig. 2. Effects of 2-mercaptoethanol and dithiothreitol on the activity of chicken liver Pz-peptidase

The enzyme was assayed in the presence of various concentrations of 2-mercaptoethanol (○) or dithiothreitol (●), as described in the text. Specific activity in the absence of thiol compounds (designated 100%) varied somewhat according to the history of the enzyme sample.

Table 5. Effect of thiol-blocking reagents on chicken Pz-peptidase

Compound (concentration)	Inhibition (%)	Inactivation ($M^{-1} \cdot s^{-1}$)
<i>p</i> -Chloromercuribenzoate (0.1 mM)	100*	—
Iodoacetate (1 mM)	22*	—
<i>N</i> -Ethylmaleimide (1 mM)	82*	—
5-Cyano-2-nitrothiobenzoate (0.1 mM)	62*	—
Tos-Phe-CH ₂ Cl (0.1 mM)	—	15†
Chloroacetone (1 mM)	—	0†

* Inhibition was instantaneous (< 60 s), and no further progressive inactivation was seen.

† There was progressive inactivation, for which the apparent second-order rate constant is given.

inhibitor; the inactivity of the control compound, 1-chloroacetone, suggested that the covalent reaction of Tos-Phe-CH₂Cl is facilitated by reversible binding of the Tos-Phe- moiety. Our data do not permit us to decide whether Tos-Phe-CH₂Cl inhibits by reacting with thiol or other groups, however.

Detection of essential histidine residue(s)

A solution of Pz-peptidase in 50 mM-Mes/NaOH buffer, pH 6.5, containing 0.2 M-NaCl, was made 1 mM with respect to diethyl pyrocarbonate. Samples withdrawn periodically for dilution and assay showed activity falling off with $t_{0.5} = 42$ s. After 10 min no activity was detectable, and the solution was made 50 mM with respect to hydroxylamine hydrochloride and stood for 4 h at 23 °C. Activity returned to 59% of the original value. These results are consistent with the presence of one or more essential histidine residues in Pz-peptidase (Bateman & Hersh, 1987).

Inhibition by dynorphin-(1–13)

Dynorphin-(1–13) proved to be a potent inhibitor of Pz-peptidase, with an apparent K_i value of 26 nM.

CONCLUSIONS

On the basis of inhibition by EDTA and 1,10-phenanthroline, re-activation by Zn²⁺ and other metal ions after EDTA, and potent competitive inhibition by substrate analogues designed to co-ordinate a metal atom close to the scissile bond, we conclude that chicken Pz-peptidase is a metallo-endoropeptidase. This conclusion is in clear agreement with the literature on rat soluble metallo-endoropeptidase; data on Pz-peptidase have been ambiguous, and endo-oligopeptidase A has been considered not to be a metallo-peptidase.

Pz-peptidase has thiol-dependence shown both by the activation with low concentrations of thiol compounds and by inhibition by some thiol-blocking reagents. It is not yet clear whether the thiol group involved in the activity of the enzyme

contributes directly to the catalytic mechanism in a way that has not previously been reported for any metallo-endoropeptidase, or perhaps has a less direct role.

The properties of chicken Pz-peptidase agree well with those described for mammalian soluble metallo-endoropeptidase and endo-oligopeptidase A, consistent with the view that these three types of activity are all attributable to a single enzyme. The name thimet peptidase, proposed for the mammalian enzyme by Tisljar & Barrett (1990), is equally applicable to the chicken enzyme, in view of its thiol- and metal-dependence. Thimet peptidase probably merits the EC number 3.4.24.15, for which soluble metallo-endoropeptidase has been the recommended name.

We thank Dr. C. G. Knight for providing the quenched-fluorescence substrates, and Dr. M. Orlowski for the gift of inhibitors as listed in the text. Not least, Ursula Tisljar and our other colleagues gave much help and advice, and Wendy Gilbey provided skilled technical assistance.

REFERENCES

- Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
- Barrett, A. J. (1990) *Biol. Chem. Hoppe-Seyler* **371**, 311–320
- Barrett, A. J. & Tisljar, U. (1989) *Biochem. J.* **261**, 1047–1050
- Barrett, A. J. & Tisljar, U. (1990) in *Matrix Metalloproteinases* (Birkedal-Hansen, H., ed.), Gustav Fischer, Stuttgart, in the press
- Barrett, A. J., Knight, C. G., Brown, M. A. & Tisljar, U. (1989) *Biochem. J.* **260**, 259–263
- Bateman, R. C. & Hersh, L. B. (1987) *Biochemistry* **26**, 4237–4241
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bury, A. F. (1981) *J. Chromatogr.* **213**, 491–500
- Camargo, A. C. M., Shapanka, R. & Greene, L. J. (1973) *Biochemistry* **12**, 1838–1844
- Camargo, A. C. M., Ribeiro, M. J. V. F. & Schwartz, W. N. (1985) *Biochem. Biophys. Res. Commun.* **130**, 932–938
- Camargo, A. C. M., Oliveira, E. B., Toffoletto, O., Metters, K. M. & Rossier, J. (1987) *J. Neurochem.* **48**, 1258–1263
- Kerr, M. A. & Kenny, A. J. (1974) *Biochem. J.* **137**, 489–495
- Kikuchi, M., Fukuyama, K. & Epstein, W. L. (1988) *Biochim. Biophys. Acta* **956**, 176–184
- Lukac, J. & Koren, E. (1977) *J. Reprod. Fertil.* **49**, 95–99
- Morales, T. I. & Woessner, J. F., Jr. (1977) *J. Biol. Chem.* **252**, 4855–4860
- Murphy, B. P. & Pratt, R. F. (1989) *Biochem. J.* **258**, 765–768
- Orlowski, M., Michaud, C. & Chu, T. G. (1983) *Eur. J. Biochem.* **135**, 81–88
- Orlowski, M., Michaud, C. & Molineaux, C. J. (1988) *Biochemistry* **27**, 597–602
- Orlowski, M., Reznik, S., Ayala, J. & Pierotti, A. R. (1989) *Biochem. J.* **261**, 951–958
- Pace, C. N. & Barrett, A. J. (1984) *Biochem. J.* **219**, 411–417
- Rajabi, M. & Woessner, J. F. (1984) *Am. J. Obstet. Gynecol.* **150**, 821–826
- Stöcker, W., Wolz, R. L., Zwilling, R., Strydom, D. J. & Auld, D. S. (1988) *Biochemistry* **27**, 5026–5032
- Tisljar, U. & Barrett, A. J. (1989) *Arch. Biochem. Biophys.* **274**, 138–144
- Tisljar, U. & Barrett, A. J. (1990) *Biochem. J.* **267**, 531–533
- Tisljar, U., Camargo, A. C. M., da Costa, C. & Barrett, A. J. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1460–1464
- Tisljar, U., Knight, C. G. & Barrett, A. J. (1990) *Anal. Biochem.* **186**, 112–115

Received 15 March 1990/13 July 1990; accepted 17 July 1990