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

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Pz-peptidase was purified from chicken liver as a protein of Mr 80 000 and pl 5.2. The purified enzyme hydrolysed phenylazobenzyloxy carbonyl-Pro-Leu-Gly-Pro-d-Arg, 2,4-dinitrophenyl-Pro-Leu-Gly-Pro-Trp-d-Lys, 7-methoxycoumarin-3-carboxylyl-Pro-Leu-Gly-Pro-d-(2,4-dinitrophenyl)Lys, benzoyl-Gly-Ala-Ala-Phe-p-aminobenzoate, Ac-AlaN (at the Ala-d-Ala-2 bond) and bradykinin (at the Phe-d-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 KI 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 metalloendopeptidase 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-peptidase 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
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 (NH₄)₂SO₄ 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(NH₄)₂SO₄ (800 ml) during 30 min, and centrifuged as before. The supernatant was made 3.0 M with respect to (NH₄)₂SO₄, 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 bed exchanger was packed into a chromatographic column (50 mm diam. × 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 (NH₄)₂SO₄ 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 hydroxypatite (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 (NH₄)₂SO₄ 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 × 850 mm, 171 ml) of Sephacyrl 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 CaCl₂ 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 (Tisljar 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 Kₜ 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 (Kₜ) were determined with values of [I] > [E], so that non-tight-binding assump-

\[ Kₜ = Kₜ'//(1 + [S]/Kₐ) \]  

(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 Mₘ 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). Prollyl endopeptidase [assayed with Z-Gly-Pro-7-(4-methyl)-coumarylamide (Tisljar et al., 1990)] was undetectable after the DEAE-cellulose step.

Physicochemical properties

The apparent Mₘ of the protein was estimated to be 80000; it ran fractionally more slowly than transferrin (Mₘ 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**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Purification factor (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue extract</td>
<td>118300</td>
<td>789</td>
<td>(1)</td>
<td>(100)</td>
</tr>
<tr>
<td>Supernatant at pH 5</td>
<td>20200</td>
<td>620</td>
<td>4.6</td>
<td>79</td>
</tr>
<tr>
<td>Supernatant from</td>
<td>6000</td>
<td>387</td>
<td>9.8</td>
<td>49</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>2880</td>
<td>377</td>
<td>19.8</td>
<td>48</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>241</td>
<td>165</td>
<td>103</td>
<td>21</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>20.7</td>
<td>51</td>
<td>370</td>
<td>6.4</td>
</tr>
<tr>
<td>Hydroxypatite</td>
<td>4.2</td>
<td>15.1</td>
<td>542</td>
<td>1.9</td>
</tr>
<tr>
<td>Sephacryl S-200 HR</td>
<td>0.6</td>
<td>6.5</td>
<td>1631</td>
<td>0.8</td>
</tr>
</tbody>
</table>

1990
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 $A_{280}^{\text{ww}}$ 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 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 metalloendopeptidase') and with bradykinin (for 'endo-oligopeptidase 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 units 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-Gly-Phe)Ser-Pro-Phe-Arg, as reported for endo-oligopeptidase 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-[J(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.

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (units/mg)</th>
<th>Present work</th>
<th>Literature value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pz-Pro-Leu-Gly-Pro-d-Arg</td>
<td>983</td>
<td>1060*</td>
<td></td>
</tr>
<tr>
<td>Bz-Gly-Ala-Ala-Phe-pAB</td>
<td>273</td>
<td>1220†</td>
<td></td>
</tr>
<tr>
<td>Bradykinin</td>
<td>6.5</td>
<td>1.37‡</td>
<td>12.61§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.8†</td>
<td></td>
</tr>
</tbody>
</table>

* Reported by Morales & Woessner (1977) for chicken-embryo Pz-peptidase.
† Reported by Orlowski et al. (1989) for rat testis soluble metalloendopeptidase.
‡ Reported by Camargo et al. (1987) for bovine brain endo-oligopeptidase A.
§ Reported by Kikuchi et al. (1988) for rat epidermis enzyme.
Hydrolysis of Ac-Ala₄ and Ac-Ala₃ (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 hydrolysat by h.p.l.c. revealed Ala₃, but no free alanine or Ala₄, showing that the bond cleaved was: Ac-Ala₃|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 μg 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 mM-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 mM-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 Orłowski 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; Tisijar & 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 (Tisijar & 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 μM residual EDTA). Various metal ions were tested for their ability to restore activity at 50 and 100 μM concentration, in standard assay mixtures containing 5 mM-2-mercaptoethanol. Results were essentially the same at both 50 and 100 μM 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. Orłowski 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 Orłowski 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.

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

<table>
<thead>
<tr>
<th>Metal salt</th>
<th>Rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>93</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>71</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>35</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>31</td>
</tr>
<tr>
<td>CdSO₄</td>
<td>22</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>3</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>2</td>
</tr>
<tr>
<td>NiSO₄</td>
<td>3</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>2</td>
</tr>
</tbody>
</table>

Metallo-endopeptidase inhibitors

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

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)–NH₂ structure, which contains a carboxy group located to co-ordinate with an active-site metal atom (Orłowski et al., 1988). Some of these compounds (provided by Dr. M. Orłowski, 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 mM-N-{[(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-pAB. In continuous assays with Dnp-Pro-Leu-Gly-Pro-Trp-Lys, the N-(carboxyalkyl)-peptides produced apparently instantaneous inhibition, and the new rates of product formation were linear. Kᵢ 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 $\mu$m substrate on the basis of $K_m$ values of 10.3 $\mu$m and 12.8 $\mu$m for the chicken and rat enzymes respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chicken enzyme</th>
<th>Rat enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I) $N{[(RS)\text{-Carboxy-3-phenylpropyl}]\text{-Ala-Ala-Tyr-pAB}$</td>
<td>4.6</td>
<td>12.3</td>
</tr>
<tr>
<td>(II) $N{[(RS)\text{-Carboxy-3-phenylpropyl}]\text{-Ala-Ala-Phe-pAB}$</td>
<td>7.5</td>
<td>16.7</td>
</tr>
<tr>
<td>(III) $N{[(RS)\text{-Carboxy-3-phenylpropyl}]\text{-Gly-Ala-Phe-pAB}$</td>
<td>252</td>
<td>627</td>
</tr>
<tr>
<td>(IV) $N{[(RS)\text{-Carboxybutyl}]\text{-Ala-Ala-Phe-pAB}$</td>
<td>142</td>
<td>315</td>
</tr>
<tr>
<td>(V) $N{[(RS)\text{-Carboxy-2-phenylethyl}]\text{-Ala-Ala-Phe-pAB}$</td>
<td>461</td>
<td>662</td>
</tr>
</tbody>
</table>

Liver Pz-peptidase, and also with the enzyme from rat testis purified as described by Tisijar & 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 $S_3^\prime$ and alanine in $S_4^\prime$ 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 $\mu$m-Zn$^{2+}$ or -Mn$^{2+}$ in 10 $\mu$m-EDTA for the effect of dithiothreitol. At 1 mm-dithiothreitol percentage inhibitions relative to the control without dithiothreitol were 63% (native enzyme), 60% (Zn$^{2+}$) and 6% (Mn$^{2+}$). 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-CH$_2$Cl, however, which Morales & Woessner (1977) also found to be a significant
Table 5. Effect of thiol-blocking reagents on chicken Pz-peptidase

<table>
<thead>
<tr>
<th>Compound (concentration)</th>
<th>Inhibition (%)</th>
<th>Inactivation (m⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chloromercuribenzoate (0.1 mM)</td>
<td>100*</td>
<td>—</td>
</tr>
<tr>
<td>Iodacetate (1 mM)</td>
<td>22*</td>
<td>—</td>
</tr>
<tr>
<td>N-Ethylmaleimide (1 mM)</td>
<td>82*</td>
<td>—</td>
</tr>
<tr>
<td>5-Cyano-2-nitrothiobenzoate (0.1 mM)</td>
<td>62*</td>
<td>—</td>
</tr>
<tr>
<td>Tos-Phe-CH₂Cl (0.1 mM)</td>
<td>—</td>
<td>15†</td>
</tr>
<tr>
<td>Chloroacetone (1 mM)</td>
<td>—</td>
<td>0†</td>
</tr>
</tbody>
</table>

* 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 mM-SCN, was made 1 mM with respect to diethyl pyrocarbonate. Samples withdrawn periodically for dilution and assay showed activity falling off with t₁/₂ = 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 Kᵢ 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-endopeptidase. This conclusion is in clear agreement with the literature on rat soluble metallo-endopeptidase; 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-endopeptidase, or perhaps has a less direct role.

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 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-endopeptidase has been the recommended name.

We thank Dr. C. G. Knight for providing the quenched-fluorescence substrates, and Dr. M. Orłowski 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.

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