Thiol-dependent metallo-endopeptidase characteristics of Pz-peptidase in rat and rabbit

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Pz-peptidase was purified from rat testis and rabbit muscle. Zinc was detectable in the rat enzyme. The activity of the enzyme from both species was slowly but completely abolished by EDTA and restored by Zn^{2+} . Free thiol groups were also important for the catalytic activity of rat Pz-peptidase, as previously reported for the rabbit enzyme. We conclude that in both species Pz-peptidase has the characteristics of a thiol-dependent metallo-endopeptidase.

INTRODUCTION

Pz-peptidase (EC 3.4.99.31) is an endopeptidase widely distributed in mammalian tissues (Aswanikumar & Radhakrishnan, 1973). Recent work with rat Pz-peptidase showed it to be identical with 'endopeptidase 24.15' (EC 3.4.24.15) (Barrett & Tisljar, 1989), an enzyme regarded as a metallo-peptidase (Orlowski *et al.*, 1989). In contrast, our study of rabbit muscle Pz-peptidase (Tisljar & Barrett, 1989) highlighted its cysteine-endopeptidase-like characteristics, and indeed we showed rabbit Pz-peptidase to be identical with 'endopeptidase A' (EC 3.4.22.19) (Tisljar *et al.*, 1989), an enzyme considered to be a cysteine peptidase (Cicilini *et al.*, 1988). The emphasis that has been placed on the metal-ion-dependence of rat Pz-peptidase as opposed to the thiol-dependence of the rabbit enzyme could appear to raise the question of whether these are separate enzymes rather than forms of a single enzyme. The present study further addresses this question.

EXPERIMENTAL

Enzyme assay

The quenched fluorescence substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) was used as described by Tisljar *et al.* (1990). In brief, enzyme and substrate (10 μ M) were incubated in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.05 % Brij 35 at 30 °C. The dithiothreitol concentration in the assay was 0.1 mM for rat Pz-peptidase and 0.2 mM for the rabbit enzyme. When di-isopropyl phosphorofluoridate-sensitive activity was present, samples were pretreated with 1 mM-di-isopropyl phosphorofluoridate for at least 5 min at 0 °C before assay (Tisljar *et al.*, 1990). One unit of activity was defined as that hydrolysing 1 μ mol of substrate/min.

Apparent inhibition constants (K_i') were determined with values of $[I] \gg [E]$, so non-tight-binding assumptions were valid, and the values were corrected for the effect of substrate by use of the equation:

$$K_i = K_i' / (1 + [S] / K_m)$$

Protein determination

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

Enzyme purification

Pz-peptidase was purified from rabbit muscle as previously described (Tisljar & Barrett, 1989). For the purification of the enzyme from rat testis, all solutions contained 0.1 mM-dithiothreitol. Acid precipitation at pH 5.2 was followed by

DEAE-cellulose chromatography and gel chromatography as described for the rabbit enzyme. The preparation was then run on a Mono Q f.p.l.c. column eluted with a linear gradient of 0–300 mM-NaCl in 20 mM-Tris/HCl buffer, pH 7.9, containing 0.1 mM-dithiothreitol and 0.05 % Brij 35. The active fractions (peak at 150 mM-NaCl) were collected and applied to a Mono P f.p.l.c. chromatofocusing column, eluted with Polybuffer 74 in the pH range 7.1–4.0. The active fractions were collected, and 2 M-Tris was added to adjust the pH from 4.8 to about 7.2. The enzyme was stored at 4 °C. All experiments with purified Pz-peptidase were done within 5 days of completion of the purification.

SDS/PAGE

Purified rat testis Pz-peptidase was precipitated from 50 % (w/v) trichloroacetic acid before SDS/PAGE (total acrylamide concentration 7 %, w/v) with reduction as described by Bury (1981) with the same standards. PAGE without denaturation was done with the same buffer system, but with no SDS or 2-mercaptoethanol. Following electrophoresis without denaturation, enzymic activity was assayed in small pieces cut out of the gel before staining.

Metal analysis

Purified rat Pz-peptidase was dialysed against distilled water and subjected to analysis in a Perkin–Elmer 4000 atomic absorption spectrophotometer equipped with a graphite furnace. Samples of the diffusate were analysed as controls.

Inhibition by *N*-ethylmaleimide

To avoid interference by the thiol activator, the following procedure was used for the study of inhibition by *N*-ethylmaleimide. Pz-peptidase was activated with 0.1 mM-dithiothreitol (0.2 mM for the rabbit enzyme) in a total volume of 25 μ l at 30 °C for 2 min. *N*-Ethylmaleimide in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.05 % Brij 35 was added to a volume of 250 μ l and a final *N*-ethylmaleimide concentration of 0.5 mM. After 1 min, the volume was further increased with the same Brij 35/Tris/HCl buffer to 2.5 ml including 2-mercaptoethanol (5 mM) and the quenched fluorescence substrate. The increase in fluorescence was monitored as in the standard assay.

RESULTS

The results of the purification procedure for rat testis Pz-peptidase are summarized in Table 1. The final product ran as a

Table 1. Purification of Pz-peptidase from rat testis

A 124 g portion of tissue was used in a procedure as described in the text. One unit of activity corresponds to the hydrolysis of 1 μ mol of substrate/min at 30 °C, and protein was determined by the Bio-Rad assay.

Purification step	Protein (mg)	Activity (units)	Purification factor	Yield (%)
Crude homogenate	24 700	98	(1)	(100)
Acid-precipitation supernatant	3980	79	5	81
DE-52 DEAE-cellulose chromatography	383	43	28	44
Sephacryl S-200 HR chromatography	118	26	56	26
Mono Q f.p.l.c.	12.4	12	244	12
Mono P f.p.l.c.	1.26	2.5	501	2.8

Fig. 1. SDS/PAGE of purified rat Pz-peptidase

Approx. 10 μ g of purified rat Pz-peptidase was precipitated from 50% (w/v) trichloroacetic acid and subjected to SDS/PAGE alongside M_r standards.

single major band in SDS/PAGE (Fig. 1). A single major band was also obtained after PAGE without denaturation. Enzymic activity was associated with this band.

The rabbit enzyme was as described by Tisljar & Barrett (1989). Specific activities were 1.98 units/mg of protein for the rat enzyme and 0.0065 unit/mg for that from rabbit muscle. The rat enzyme contained between 1.7 and 2.0 g-atoms of Zn/mol of enzyme, on the basis of M_r 74000 for Pz-peptidase.

The activity of the enzyme from both species was completely abolished by incubation in 10 mM-EDTA at 4 °C for 24 h. Respectively 86% and 85% of the original activities of the enzyme from rat and rabbit were restored by adding 50 μ M-ZnCl₂ to a reaction mixture also containing 10 μ M-EDTA. Both forms of the enzyme were inhibited by *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoic acid, but with very different potencies; thus the K_i for the rat enzyme was 17 nM, whereas the rabbit enzyme retained 70% of activity at 2 μ M of this inhibitor. The low K_i value of the rat enzyme agrees well with that reported by Orlowski *et al.* (1988).

To investigate the thiol-dependence of Pz-peptidase from rat testis, 10 μ l of enzyme solution in 0.1 mM-dithiothreitol was diluted into 2.5 ml of reaction mixture without any thiol compound. During the first 3 min the hydrolysis rate was comparable with that in an assay with 0.1 mM-dithiothreitol. The activity then declined rapidly, but was restored by addition of dithiothreitol to a final concentration of 0.1 mM (Fig. 2). However,

Fig. 2. Effect of dithiothreitol on activity of Pz-peptidase from rat testis in a continuous rate assay

The enzyme was diluted from a stock solution in 0.1 mM-dithiothreitol into the incubation mixture with a final concentration of 1 μ M-dithiothreitol. After 35 min (arrow), dithiothreitol was added to 0.1 mM.

higher concentrations of dithiothreitol were inhibitory, the relative activities in assays with 0.1 mM-, 0.2 mM- and 0.4 mM-dithiothreitol being 100, 95 and 74 respectively.

After 1 min incubation with 0.5 mM-*N*-ethylmaleimide the residual activity was 46% and 42% for the rat and rabbit enzyme respectively.

DISCUSSION

Influence of metal ions

Our finding of inhibition of Pz-peptidase by EDTA and re-activation by Zn²⁺ ions is in good agreement with the reports by Lukac & Koren (1977) and Orlowski *et al.* (1989) for the rat testis enzyme. The similar metal-ion-dependence of freshly prepared rabbit Pz-peptidase contrasts with previous reports for the rabbit brain enzyme (Camargo *et al.*, 1973) and our own study of rabbit muscle Pz-peptidase (Tisljar & Barrett, 1989). The results obtained by Camargo and co-workers can be attributed to the use of rather mild treatment with EDTA. Thus, with the heart enzyme, Cicilini *et al.* (1988) used only 2 mM-EDTA for 15 min, obtaining up to 35% inactivation. Our own work with the rabbit muscle enzyme involved aged preparations and an assay procedure that was less specific for Pz-peptidase (lacking the diisopropyl phosphorofluoridate treatment of the samples).

The zinc content of the rat protein further emphasizes the metallo-peptidase characteristics. Our failure to detect metals

in rabbit Pz-peptidase (Tisljar & Barrett, 1989) is probably attributable to the very low specific activity of the enzyme preparation from that species.

Influence of thiol groups

The activity of Pz-peptidase is markedly increased and stabilized by low concentrations of thiols, as is shown for the rat enzyme with 0.1 mM-dithiothreitol in Fig. 2. However, concentrations of thiols above 0.2 mM are inhibitory. These findings are consistent with the reports by Orlowski *et al.* (1983), describing activation of rat brain soluble metallo-endopeptidase by low dithiothreitol concentration, and by Orlowski *et al.* (1989), who found the more highly purified enzyme from rat testis to be less active in 0.4 mM-dithiothreitol than in the 0.2 mM concentration used in their standard assay. The thiol activation of the rabbit enzyme has been clearly demonstrated (Cicilini *et al.*, 1988; Tisljar & Barrett, 1989).

We conclude that the Pz-peptidase found in soluble fractions from both rat and rabbit tissues shows both thiol- and metal-ion-dependence. The enzyme is identical with endo-oligopeptidase A and soluble metallo-endopeptidase, although these enzymes have been thought to be distinct. The metal-ion-dependence of the enzyme as endo-oligopeptidase A was probably missed because the metal ion is tightly bound, so that inactivation is a slow process. Conversely, the thiol-dependence of the enzyme as soluble metallo-endopeptidase has been obscured by the fact that, although low thiol concentrations activate, higher concentrations inhibit. Despite the important common characteristics of Pz-peptidases from rat and rabbit, the two forms of the enzyme show distinct differences, for example in sensitivity to inhibition by *N*-[1(*RS*)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoic acid.

All three of the names by which this enzyme has been known in the past are unsatisfactory in one way or another. We therefore

suggest the new name 'thimet peptidase', based on the distinctive thiol-dependent metallo-endopeptidase character of the enzyme. The number EC 3.4.24.15 seems appropriate.

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