Identification of the thyrotropin-releasing-hormone-degrading ectoenzyme as a metallopeptidase

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A time-dependent inhibition of the thyrotropin-releasing-hormone (TRH)-degrading ectoenzyme (EC 3.4.19.–) from rat brain by the metal-complexing agents imidazole, NaCN, EDTA and 1,10-phenanthroline could be demonstrated. In contrast, the inhibition by the non-chelating analogues 4,7- and 1,7-phenanthroline was not time-dependent. At a concentration of 100 μ M EDTA the enzymic activity decreased by 50% only after pretreatment for 6 h. It could be restored by addition of Zn^{2+} , Ni^{2+} and Co^{2+} but not by other transition metal ions and also not by Ca^{2+} and Mg^{2+} . Without pretreatment, the enzyme was activated by Co^{2+} and inhibited by Cu^{2+} , Cd^{2+} and Hg^{2+} in a time-dependent manner, but remained unaffected by Ni^{2+} and

Mn^{2+} , as well as by Ca^{2+} and Mg^{2+} . Compatible with the His-Glu-Xaa-Xaa-His consensus sequence of most zinc-containing metallopeptidases, chemical modification studies with carbodiimide revealed the presence of an essential acidic amino acid residue, probably located at the active site of the enzyme. The catalytically active metal ion could be exchanged for ^{65}Zn and the enzyme could be effectively inhibited by L-pyroglutamyl hydroxamic acid, the chelating derivative of the TRH cleavage product pyroglutamic acid. The TRH-degrading ectoenzyme thus classifies as a member of the zinc-dependent metallopeptidase family.

INTRODUCTION

Thyrotropin-releasing hormone (TRH; pyroGlu-His-Pro-NH₂), the first hypothalamic hypophysiotrophic neuropeptide hormone to be structurally elucidated, stimulates thyrotropin, prolactin and, in certain cases, growth hormone secretion [1,2]. In addition to these endocrine functions, TRH elicits a variety of behavioural changes and neuropharmacological effects [3,4], suggesting that TRH may be involved in neurotransmission or other forms of cellular communication. This hypothesis is supported by the observation that TRH is widely distributed in extra-hypothalamic brain areas [5–7] and that high-affinity receptors for TRH are found throughout the central nervous system [8,9]. In addition, biochemical studies suggest that TRH signals might be terminated by a particulate enzyme hydrolysing TRH at the pyroGlu-His bond.

The particulate TRH-degrading enzyme is located in synaptosomal [10] and adenohipophyseal [11] membranes with its active site oriented towards the extracellular space [12–14] and thus represents a true ectoenzyme. In addition, the observations that relatively high enzymic activities are associated with neuronal cells while only low activities are found in pituitary aggregates and glial cells are almost devoid of it indicated that this enzyme is not a general constituent of plasma membranes, thereby suggesting that it may serve very specialized functions for the inactivation of TRH after its release. This interpretation is supported by the observation that the activity of the adenohipophyseal ectoenzyme is stringently controlled by oestradiol and thyroid hormones [15–18], whereas the activity of the brain enzyme is not [15,19]. Furthermore, and most importantly, the TRH-degrading ectoenzyme exhibits a very high degree of substrate specificity [20] like the TRH-degrading serum enzyme studied before [21]. The biochemical data presently available strongly indicate that the TRH-degrading ectoenzyme and the serum enzyme may be derived from the same gene since they

share many similarities with respect to pH optimum, sensitivity to inhibitors, etc. [22]. Previous studies by various investigators clearly demonstrated that these enzymes are insensitive towards serine- and cysteine-specific reagents or peptidic inhibitors of aspartic proteases, but are sensitive towards the chelating reagents EDTA and 1,10-phenanthroline [10,23–26]. Therefore it is widely assumed that these enzymes are metallopeptidases. However, significant inhibition of the TRH-degrading ectoenzyme and the TRH-degrading enzyme in serum was only observed when EDTA was added in millimolar concentrations (and even at these high concentrations the reported values differ considerably), while extensively characterized metalloproteases such as neutral endopeptidase or angiotensin-converting enzyme are effectively inhibited by micromolar concentrations of EDTA [27–29]. Strong inhibition of the TRH-degrading ectoenzyme by 1,10-phenanthroline was observed by all investigators, but appropriate controls with non-chelating isomers of this compound had not been performed. In addition, it had not been demonstrated that inhibition of the enzymic activity could be reversed by addition of metal ions. Furthermore, while the active site of most metalloproteases contains glutamic acid, the recently reported study on the chemical modification of the TRH-degrading ectoenzyme failed to identify glutamic acid as a catalytically essential residue [30].

We decided to re-investigate the catalytic mechanism of the TRH-degrading ectoenzyme and report here that it is indeed a metalloenzyme, albeit one which exhibits some unusual properties.

MATERIALS AND METHODS

Materials

Lentil lectin–Sepharose 4B, Q-Sepharose FF, phenyl-Sepharose CL-4B and Sephadex G-200 were obtained from Pharmacia, trypsin from Boehringer–Mannheim and 1-ethyl-3-(3-dimethyl-

Abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; LHRH, luteinizing-hormone-releasing hormone; pyroGlu, pyroglutamic acid; TRH, thyrotropin-releasing hormone.

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aminopropyl)carbodiimide (EDC) from Aldrich. Di-isopropyl fluorophosphate, methyl- α -D-glucopyranoside and 1,10-, 1,7- and 4,7-phenanthroline were purchased from Sigma. All other chemicals were analytical-grade reagents from Merck.

Enzyme purification

Rat brains (100 g) were homogenized by use of an Ultra Turrax homogenizer (Jahnke and Kunkel) in 0.02 M sodium phosphate buffer, pH 7.3 (400 ml), containing 6 mM NaN_3 (buffer A). The homogenate was centrifuged at 100 000 *g* for 45 min. The pellet was resuspended in 400 ml of buffer A and recentrifuged. This procedure was repeated five times to yield the washed pellet. After rehomogenization trypsin (1 $\mu\text{g}/\text{ml}$) was added and the suspension was incubated for 15 min at 4 °C. The reaction was stopped by addition of di-isopropyl fluorophosphate (1 mM). The solubilized enzyme was separated from the particulate matter by centrifugation at 100 000 *g* for 90 min. The supernatant was applied to a Q-Sepharose column (2.6 cm $\times$ 16 cm) equilibrated with buffer A. After washing with 300 ml of buffer A the enzyme activity was eluted with a linear gradient of NaCl (0–0.3 M; total volume of 500 ml) in buffer A at a flow rate of 50 ml/h. The active fractions of the Q-Sepharose column were pooled and applied to a lentil lectin-Sepharose column (1 cm $\times$ 20 cm) at a flow rate of 20 ml/h. The column was washed with 10 vol. of buffer B (0.01 M diethyl malonate, pH 7.3/0.3 M NaCl/6 mM NaN_3) and the enzyme activity was eluted with 0.5 M methyl- α -D-glucopyranoside in buffer B at a flow rate of 5 ml/h. Enzyme-containing fractions were pooled and concentrated in an Amicon ultrafiltration cell to a volume of 5 ml. This material was then subjected to gel filtration on a Sephadex G-200 column followed by hydrophobic interaction chromatography on a phenyl-Sepharose column as described by O'Connor and O'Cuinn [20].

Synthesis of pyroglutamic acid (pyroGlu) derivatives

L- and D-pyroglutamyl hydroxamic acid were prepared by reacting L- and D-pyroglutamic acid ethyl ester with hydroxylamine in methanol as described [31]. Synthesis of L-pyroglutamyl-(*N*-methylhydroxamic acid) and L-pyroglutamyl-[(*N*)-*O*-methylhydroxamic acid] followed the same protocol using *N*-methylhydroxylamine and *O*-methylhydroxylamine to replace hydroxylamine. Both products were purified by column chromatography on silica gel. L-Pyroglutamyl-(*N*-methylhydroxamic acid): eluant, chloroform/methanol (2:1, v/v), R_F = 0.66, m.p. = 148 °C; and L-pyroglutamyl-[(*N*)-*O*-methylhydroxamic acid]: eluant, chloroform/methanol/water (3:1:0.08, by vol.), R_F = 0.51, m.p. = 141 °C. The identity of the compounds was confirmed by n.m.r. spectroscopy. L-Pyroglutamyl hydrazide and L-pyroglutamyl amide were synthesized from L-pyroglutamic acid ethyl ester according to Angier et al. [32].

Enzyme assays

The activity of the TRH-degrading ectoenzyme was measured by the radiochemical assay described in [14] using [pyroGlu- ^3H]-TRH, or by using L-pyroglutamyl- β -naphthylamide as fluorogenic substrate. For the fluorimetric assay, the reaction mixture contained 500 μl of the enzyme and 2.5 ml of substrate solution (0.1 mM L-pyroglutamyl- β -naphthylamide/0.02 M phosphate, pH 7.3). The time course of β -naphthylamine liberation was followed fluorimetrically [33] in a thermostatically controlled cuvette at 30 °C using a Perkin-Elmer LS-3B fluorescence spectrometer (excitation wavelength 340 nm, emission wave-

length 410 nm). For the reactivation experiments, enzyme samples (500 μl) were pre-incubated for 8 h at 20 °C in the presence of 0.6 mM EDTA before 2.5 ml of the substrate solution was added.

Labelling with ^{65}Zn

The enzyme samples were incubated overnight at 4 °C in 250 μl of 0.1 M diethyl malonate/0.02 M NaCl, pH 7.3 containing 50 μCi of $^{65}\text{ZnCl}_2$ (3.36 mCi/mg, Du Pont de Nemours). Then, they were subjected to h.p.l.c. analysis on a Pharmacia-LKB TSK-G-3000 SW gel filtration column. The material was eluted with 0.1 M diethyl malonate, pH 7.3, at a flow rate of 0.2 ml/min. Fractions of 200 μl were collected. Enzymic activity was assayed fluorimetrically after adding 2.5 ml of the fluorogenic substrate as described above, and radioactivity was measured using a Packard 500 CGD Prias γ -counter (window limits 400 and 1600 keV).

Modification by EDC

Enzyme samples (20 μl) were diluted with 400 μl of reaction buffer (0.05 M Mes, pH 6.0, or 0.1 M potassium phosphate, pH 6.0). Then, 100 μl of a freshly prepared solution of EDC (0.1 M) in the same buffer was added. In addition, some reaction mixtures contained 1 mM glycine methyl ester or 20 μM luteinizing-hormone-releasing hormone (LHRH) (Hoechst). After incubation at 20 °C for the time periods indicated in the text, 2.5 ml of the fluorogenic substrate was added and the residual catalytic activity was determined as described above. For the reactivation experiments, 0.5 ml of 1 M hydroxylamine was added after incubation with the carbodiimide for 3 h at 20 °C. The reaction mixture was then incubated at room temperature for an additional 60 min before substrate was added to determine the enzymic activity. Control reactions, without modifying reagent, were incubated in parallel for the 100 % values.

RESULTS

Inhibition by metal-complexing agents

The influence of metal-complexing agents on the enzymic activity of the TRH-degrading ectoenzyme is summarized in Table 1. Without pre-incubation, only weak inhibition by submillimolar concentrations of EDTA was observed. After pre-incubation with the chelating agent for several hours, however, the enzymic activity was strongly reduced. Pre-incubation for 24 h with as little as 30 μM EDTA inhibited the initial enzyme activity by 80 %. Enzyme activity was not significantly reduced in control samples. Like EDTA, the metal-chelating compounds 1,10-phenanthroline, NaCN and imidazole (results not shown) also inactivated the TRH-degrading ectoenzyme in a time-dependent manner. Compared with 1,10-phenanthroline, reduced but significant inhibition could be observed with the non-chelating substances 1,7-phenanthroline and 4,7-phenanthroline, but this was non-time-dependent.

To study the reactivation by metal ions, the enzyme sample was first incubated in the presence of 0.6 mM EDTA for 8 h. Then, 5 vol. of the fluorogenic substrate was added to determine the residual enzyme activity (16 %) and, 40 min later, the metal salt was added (see Figure 1). Immediate reactivation, reaching 53 % of the initial activity, was observed when an equivalent amount of ZnSO_4 was added to the EDTA-containing solution. Reactivation was not observed with 0.9 equivalent of ZnSO_4 (corresponding to an excess of 10 μM EDTA remaining in the reaction mixture) or when 1 equivalent CaSO_4 , MgSO_4 , FeSO_4 , MnSO_4 , CuSO_4 , CdSO_4 or HgSO_4 was added. In contrast,

Table 1 Inhibition of the TRH-degrading ectoenzyme by metal-complexing agents

Enzyme and inhibitor were pre-incubated at 4 °C for the time periods indicated before the reaction was initiated by addition of [pyroGlu-³H]TRH as substrate.

Inhibitor	Pre-incubation time (h) ...	Inhibition of activity (%)		
		0	6	24
EDTA				
(30 μ M)		17	41	80
(100 μ M)		21	49	87
(300 μ M)		24	59	92
1,10-Phenanthroline				
(30 μ M)		6	7	29
(100 μ M)		19	36	68
(300 μ M)		69	78	91
4,7-Phenanthroline				
(300 μ M)		29	27	26
1,7-Phenanthroline				
(1 mM)		39	32	36
NaCN				
(1 mM)		3	2	5
(3 mM)		6	17	43
(10 mM)		97	98	98

Figure 1 Reactivation of the EDTA-inactivated TRH-degrading ectoenzyme by metal salts

The enzyme was pre-incubated at 20 °C with 0.6 mM EDTA for 8 h. The reaction was started by addition of pyroglutamyl- β -naphthylamide as substrate. The liberation of β -naphthylamine was followed fluorimetrically as described in the Materials and methods section. The metal salts were added (arrow) after incubation for 40 min.

enzyme activity could be restored to 43 % and 226 % when 1 equivalent of NiSO_4 or CoSO_4 was added respectively.

Inhibition by transition metal salts

Like most metalloproteinases, the TRH-degrading ectoenzyme is also inhibited by low concentrations of transition metal ions, but not by Ca^{2+} or Mg^{2+} . Figure 2 shows the inactivation of the ectoenzyme after addition of CuSO_4 , ZnSO_4 , CdSO_4 or HgSO_4 to a final concentration of 15 μM each. Since the enzyme is not inhibited by thiol-modifying reagents, it is unlikely that this inhibition is due to interactions with essential thiol groups. In agreement with this interpretation, we observed that the TRH-degrading enzyme is not retained on a organomercurial agarose column (Bio-Rad).

It should be noted that the inhibition by copper, cadmium or mercury ions is time-dependent also. In contrast, Zn^{2+} im-

Figure 2 Effect of transition metal salts on TRH-degrading ectoenzyme activity

Enzyme activity was determined by the fluorimetric assay described in the Materials and methods section. The metal salts (15 μM final concentration) were added at the time indicated by the arrow.

mediately decreases the reaction rate. Addition of 15 μM MnSO_4 , FeSO_4 or NiSO_4 does not affect the activity of the TRH-degrading ectoenzyme, but addition of 15 μM CoSO_4 slowly increases the reaction rate to 170 % within 45 min (results not shown), as expected from the reactivation experiments described above.

The binding of Zn^{2+} ions

Metal analysis of the TRH-degrading enzyme by atomic-absorption spectrometry would require relatively large amounts of purified protein, which are difficult to obtain due to its very low abundance. Therefore we tried to detect the enzyme-bound zinc after exchanging it for its radioactive isotope ^{65}Zn . For this purpose, an enzyme sample was incubated overnight at 4 °C in the presence of a large excess of $^{65}\text{ZnCl}_2$ and then subjected to gel-filtration chromatography on a TSK-3000 SW column. Fractions were screened for ^{65}Zn content and enzyme activity. As shown in Figure 3(a), the only peak in the u.v.-absorption profile (at 72 min) contained radioactivity as well as enzymic activity, thus indicating the binding of Zn^{2+} ions by the TRH-degrading enzyme. Unbound ZnCl_2 (5×10^6 c.p.m.) was eluted from the column after 114 min (results not shown). To test the specificity of the zinc binding, the experiment was repeated using a partially purified enzyme sample. Again, a peak at 72 min contained both ^{65}Zn and the entire enzymic activity (Figure 3b). Despite the fact that large amounts of other proteins were present in the partially purified enzyme sample (note the different scales in the u.v. absorbance in Figure 3), only one small additional peak of radioactivity appeared at 49 min, whereas the major protein peaks eluting at 58 min and 64 min contained no ^{65}Zn . Thus it seems unlikely that Zn^{2+} ions are unspecifically adsorbed to the TRH-degrading enzyme under the conditions employed.

The presence of a glutamic acid residue at the catalytic site

In a previously published study, O'Connor and O'Cuinn [30] could not detect any acidic amino acid residue essential for the activity of the TRH-degrading enzyme. They attempted to modify the enzyme by incubation with the water-soluble EDC but 97 % of the enzyme activity still remained after incubation with EDC for 90 min. Under the conditions used by these authors, we also observed that the enzymic activity was reduced by only 22 % and, surprisingly, did not decrease further when incubation with

Figure 3 Binding of $^{65}\text{Zn}^{2+}$ by the TRH-degrading ectoenzyme

Samples of highly purified (a) or partially purified (b) ectoenzyme were incubated overnight with 50 μCi of $^{65}\text{ZnCl}_2$ at 4 °C. The samples were then subjected separately to analytical gel filtration (see the Materials and methods section). The fractions collected were assayed for enzyme activity (Δ) and ^{65}Zn content ($\bullet$) as described in the Materials and methods section.

Figure 4 Inactivation of the TRH-degrading ectoenzyme by EDC

The enzyme samples were incubated at 20 °C for the time periods indicated with EDC (20 mM) in phosphate buffer ($\square$), Mes buffer ($\blacksquare$), Mes buffer containing 20 μM LHRH ($\bullet$), or Mes buffer containing 10 mM glycine methyl ester ($\blacktriangle$). Enzyme activity was determined as described in the Materials and methods section and expressed as a percentage of the control values.

the modifying reagent was carried out for prolonged periods of time.

In contrast, strong inactivation of the enzyme by EDC was observed when the phosphate buffer used by O'Connor and O'Cuinn was replaced by Mes buffer, which is commonly used for this purpose. After 5 h of incubation with the carbodiimide in Mes buffer, the enzymic activity decreased by 87 % (Figure 4).

The weak effect of EDC in the presence of phosphate buffer is due to the instability of the modifying reagent in this buffer. This could be shown by pre-incubating the carbodiimide solution for

Table 2 Inhibition of the TRH-degrading ectoenzyme by pyroGlu and some of its derivatives

The activity of the TRH-degrading ectoenzyme was assayed in the presence of the sodium salt of L-pyroGlu (I), L-pyroglyutamyl hydroxamic acid (II), D-pyroglyutamyl hydroxamic acid (III), L-pyroglyutamyl-(*N*-methylhydroxamic acid) (IV), L-pyroglyutamyl-[(*N*)-*O*-methylhydroxamic acid] (V), L-pyroglyutamyl hydrazide, and L-pyroglyutamyl amide (VII) by using the fluorimetric test described in the Materials and methods section.

		Inhibition of activity (%)					
Concn (mM)...		0.01	0.1	0.3	1.0	3.0	10
I		—	—	0	7	20	53
II		25	53	62	97	100	—
III		—	—	0	4	8	21
IV		4	8	11	12	32	59
V		—	—	0	9	20	40
VI		—	—	0	5	15	34
VII		—	—	0	0	0	22

90 min in phosphate buffer which leads to complete loss of the reagent's ability to inactivate the enzyme, whereas in Mes buffer its reactivity is only slightly affected.

After modification with carbodiimide, the enzymic activity could not be restored by incubation with 0.5 M hydroxylamine for 60 min (results not shown). This indicates that the observed inactivation is not due to the modification of a tyrosine residue, but is probably caused by the modification of a carboxyl group [34]. The presence of glycine methyl ester as a nucleophile had no influence on the rate of inactivation by EDC (Figure 4), which is in agreement with the results obtained for the modification of the metalloprotease angiotensin-converting enzyme by cyclohexyl-morpholinoethylcarbodiimide [35].

When the enzyme was incubated with carbodiimide in the presence of 20 μM LHRH, a non-cleavable, competitive inhibitor of the ectoenzyme, the rate of inactivation decreased significantly (Figure 4).

Inhibition by L-pyroglyutamyl hydroxamic acid

The TRH-degrading ectoenzyme is weakly inhibited by L-pyroGlu (Table 2). Based on the assumption that this inhibition reflects an interaction of the TRH-cleavage product with the

Figure 5 A Dixon plot of the inhibition of the TRH-degrading ectoenzyme by L-pyroglutamyl hydroxamic acid

As described in the Materials and methods section, reaction rates were determined at 30 °C using radiolabelled TRH [10 nM (□) or 20 nM (△)] as substrate. The vertical error bars indicate the S.D. of the values where it exceeds the size of the symbol.

catalytic site of the enzyme, we tried to enhance the inhibitory potency by substituting the carboxyl group for a hydroxamic acid group, since hydroxamic acid derivatives of peptides or amino acids are known to inhibit various metalloproteases very effectively [36]. As expected, the resulting L-pyroglutamyl hydroxamic acid was a good inhibitor of the ectoenzyme. A Dixon plot revealed competitive inhibition with a K_i value of 160 μM (Figure 5). In contrast, the enantiomer, D-pyroglutamyl hydroxamic acid had almost no inhibitory effect (Table 2).

When L-pyroglutamyl hydroxamic acid was methylated at the hydroxamic nitrogen atom, the inhibitory potency decreased significantly and was furthermore reduced when the *N*-hydroxy group was methylated, or when the hydroxamic acid group was replaced by the non-chelating hydrazide or amide group (Table 2).

DISCUSSION

The presented results clearly demonstrate that the TRH-degrading ectoenzyme is a metallopeptidase. Interestingly, two other brain ectoenzymes that are presumably involved in the degradation of neuropeptides, neutral endopeptidase ('enkephalinase') and aminopeptidase N, have also been identified as metallopeptidases [27,37].

Typical metalloproteases such as thermolysin [38], angiotensin converting enzyme [28,29] or neutral endopeptidase [27] are strongly, and almost immediately, inhibited by low concentrations of EDTA. The TRH-degrading ectoenzyme, in contrast, is effectively inhibited by micromolar concentrations of EDTA only when incubated for prolonged periods of time. A time-dependent inhibition of metalloenzymes by chelating agents is not unprecedented, but the inhibition has required several hours of incubation in only very few cases. A most unusual example is the inhibition of the 'low-molecular-weight protease' from *Astacus fluviatilis* by EDTA which requires about 6 days of incubation to reach its half-maximal value [39]. Therefore it is understandable that the zinc-catalysed mechanism of this enzyme remained hidden for a long time.

The TRH-degrading ectoenzyme and the 'low-molecular-weight protease' are also inhibited by 1,10-phenanthroline in a time-dependent manner. In contrast, the inhibition of the 'low-

molecular-weight protease' by the non-chelating analogue 1,7-phenanthroline [39], as well as the inhibition of the TRH-degrading ectoenzyme by 1,7-phenanthroline and 4,7-phenanthroline (Table 1), are not time-dependent and are probably due to unspecific hydrophobic interactions of the enzyme with the aromatic structures of these compounds. This interpretation is supported by the observation that the TRH-degrading enzyme interacts strongly with phenyl-Sepharose, a distinctive property that has been effectively exploited for purifying the TRH-degrading ectoenzyme [20]. This feature may also contribute to the inhibition of the TRH-degrading enzyme by α -N-(1-carboxy-2-phenylethyl)-1-benzylhistidinyl- β -naphthylamide, the only substrate-related inhibitor described as yet [40].

Like the other metalloproteases such as thermolysin [38] or neutral endopeptidase [27], the TRH-degrading ectoenzyme is also inhibited by NaCN and imidazole. Cyanide ions exert no influence on the enzyme through hydrophobic interactions and do not form stable complexes with Ca^{2+} ions. Therefore NaCN can be considered a rather specific inhibitor of zinc-containing metallopeptidases.

Zinc has been identified as the catalytically active metal ion for almost all metalloproteases extensively characterized and analysed by physical methods. Thus it may be assumed that the TRH-degrading ectoenzyme also belongs to the family of zinc-metallopeptidases. This notion is supported by our exchange experiments with ^{65}Zn . Moreover, the observation that after inactivation by EDTA treatment the enzymic activity could be restored by addition of Zn^{2+} , but not by Ca^{2+} and Mn^{2+} , lends further credence to this hypothesis and indicates that Ca^{2+} ions are not important for the structural stabilization of this enzyme.

Like other metallopeptidases, the TRH-degrading ectoenzyme is also inhibited by the transition metal ions Hg^{2+} , Cd^{2+} , Cu^{2+} and Zn^{2+} . The inhibition by Hg^{2+} , Cd^{2+} and Cu^{2+} proceeds in a time-dependent manner. An excess of Zn^{2+} results in an immediate inhibition. This difference may be explained by the different inhibition mechanism of Zn^{2+} , which occupy an inhibitory metal binding site in zinc-dependent proteases [41], whereas the inhibition by other transition metal ions is also due to the exchange of the catalytic Zn^{2+} ion.

The activity of the TRH-degrading ectoenzyme is significantly increased in the presence of Co^{2+} . The same effect is also observed with other zinc-proteases [28,37,42,43].

The active sites of almost all metalloproteases contain a glutamic acid residue, which serves as proton acceptor for the hydrolysis of the substrate [44]. Modification of this residue by carbodiimides leads to inactivation of the enzymes [35,45–48]. Under appropriate conditions, the TRH-degrading ectoenzyme is also inactivated by EDC. The rate of inactivation is significantly decreased in the presence of LHRH, a competitive inhibitor of the TRH-degrading serum enzyme [21] and the TRH-degrading ectoenzyme [20]. The protective effect of LHRH therefore suggests that the modified carboxyl group is part of the catalytic centre.

Besides the glutamic acid, the active sites of metalloproteases usually contain a tyrosine residue and basic amino acids which are essential for their activity [35,46–48a]. The presence of tyrosine, histidine and arginine residues as catalytically important elements of the TRH-degrading ectoenzyme has been clearly demonstrated by O'Connor and O'Cuinn [30]. Taken together, the modification studies are in full agreement with the classification of this enzyme as a metalloenzyme.

Based on the zinc-catalysed reaction mechanism, inhibition of the TRH-degrading enzyme by L-pyroglutamyl hydroxamic acid could be predicted and demonstrated. X-ray crystallography of a thermolysin–L-leucyl hydroxamic acid complex revealed that

the essential Zn^{2+} ion is complexed in a bidentate fashion by both hydroxamic oxygen atoms of the inhibitor [49]. Hence, alkylation of the *N*-hydroxyl group reduces the metal-chelating ability of the compound and consequently also its inhibitory potency [50]. Yet, methylation at the hydroxamic nitrogen atom also decreases the inhibitory efficacy, perhaps due to sterical hindrance [50]. The same observations were made for the inhibition of the TRH-degrading ectoenzyme by L-pyroglutamyl hydroxamic acid and the methylated analogues.

There is hope that the identification of the TRH-degrading ectoenzyme as a metallopeptidase may provide the basis for the rational design and synthesis of site-directed inhibitors which may be invaluable tools with which to further our understanding on the biological function of the TRH-degrading ectoenzyme and its possible role as a terminator of TRH signals.

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