Metal ion modulation of cystinyl aminopeptidase

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Cystinyl aminopeptidase has one Zn²⁺-binding motif and is a member of the M1 aminopeptidase family. Ion modulation of its catalytic activity was studied in membranes of CHO-K1 cells (Chinese-hamster ovary K1 cells) using L-leucine-*p*-nitroanilide as substrate. The planar bidentate chelators 1,10-phenanthroline and 2,2'-bipyridine inhibited the activity in a concentration-dependent manner with Hill slopes of 3.32 ± 1.78 and 2.10 ± 0.26 respectively. The acetic acid-containing chelators EDTA, EGTA and DTPA (diethylenetriamine-*N,N,N',N',N''*-penta-acetic acid) weakly affected the activity, but they increased the potency of the planar chelators up to a limit, at which Hill slopes became close to unity. Moreover, competition between 1,10-phenanthroline and the substrate only took place in the presence of EDTA. These findings are compatible with a model in which the bidentate chelators inhibit enzyme activity by decreasing the free Zn²⁺ concentration. By removing a modulatory ion from an

allosteric site at the enzyme, the acetic acid-containing chelators facilitate the direct interaction between the bidentate chelators and the catalytic Zn²⁺. The inhibitory effect of EDTA plus 1,10-phenanthroline could be completely reversed by Zn²⁺, Ca²⁺ and Mg²⁺ increased the potency of Zn²⁺ for this process. This is expected if they interact with the modulatory site to decrease the sensitivity of the enzyme towards 1,10-phenanthroline. Conversely, the bidentate chelators increased the high-affinity [¹²⁵I]angiotensin IV binding to the membranes and this was potentiated by the acetic acid-containing chelators. These findings support the concept that high-affinity [¹²⁵I]angiotensin IV binding, previously referred to as 'AT₄ receptor binding', only occurs for the cystinyl aminopeptidase apoenzyme.

Key words: allosteric, angiotensin, chelator, cystinyl aminopeptidase, insulin-regulated aminopeptidase (IRAP), metal ion.

INTRODUCTION

Cystinyl aminopeptidase (EC 3.4.11.3) is a membrane-associated Zn²⁺-dependent metallopeptidase of the M1 aminopeptidase family. Initially, this enzyme was isolated and cloned from human retroplacental serum and denoted as placental leucine aminopeptidase [1,2]. This enzyme is reported to cleave N-terminal amino acids from bioactive peptides such as oxytocin, vasopressin and somatostatin *in vitro* [2–4]. During pregnancy, the serum level of cystinyl aminopeptidase increases with gestational age until just before the onset of labour. This may suggest that this enzyme has a role in the suppression of uterine contractions by its degradation of oxytocin [5]. The comparison of the amino acid sequence of the human form of this enzyme revealed 85 % sequence similarity to the rat IRAP (insulin-regulated aminopeptidase). IRAP has been identified in intracellular endosome-derived vesicles along with GLUT4, an insulin-regulated glucose transporter that is abundantly expressed in muscle and adipose cells [6,7]. In these cells, insulin was found to increase the translocation rate of these vesicles to the plasma membrane, resulting in an increased surface expression of GLUT4 and cystinyl aminopeptidase in healthy subjects [7]. Yet, both appear to exhibit trafficking/targeting defects in muscle and adipose cells of type II diabetic patients [7]. Interestingly, cystinyl aminopeptidase is inhibited by Ang (angiotensin) IV, the 3–8 peptide fragment of the cardiovascular peptide hormone Ang II [8]. Albiston et al. [9] have demonstrated that the high-affinity binding sites of [¹²⁵I]Ang IV, which were designated previously as the AT₄ receptors, correspond to cystinyl aminopeptidase.

Structural and mutational analysis of the cystinyl aminopeptidase cDNA indicated that it is a type II membrane-spanning protein containing the Zn²⁺-binding motif HEXXH(X)18E typical of the M1 family as well as a GXMEN motif that is proposed to be involved in the stabilization of the transition state of the enzyme–substrate complex [2]. Taken together, these results support the idea that the organization of the catalytic site of cystinyl aminopeptidase is similar to that of related aminopeptidases such as aminopeptidase A, aminopeptidase N, aminopeptidase B and leukotriene A4 hydroxylase [10]. In a recent study, the catalytic and pharmacological properties of endogenous cystinyl aminopeptidase in membranes from CHO-K1 cells (Chinese-hamster ovary K1 cells) were found to be very similar to those of human recombinant cystinyl aminopeptidase [11]. Interestingly, previous experiments revealed that, whereas EDTA did not affect enzyme activity in CHO-K1 cell membranes, it potentiated the inhibition by 1,10-phenanthroline [12]. To get further insight into the molecular mechanism(s) by which EDTA and 1,10-phenanthroline affect cystinyl aminopeptidase, we compared the effects of these and other structurally related chelators as well as the reversal of these effects by bivalent cations.

MATERIALS AND METHODS

Materials

Ang IV was obtained from NeoMPS (Strasbourg, France). The chelators EDTA, EGTA, DTPA (diethylenetriamine-*N,N,N',N',N''*-penta-acetic acid), 1,10-phenanthroline and 2,2'-bipyridine

Abbreviations used: Ang, angiotensin; CHO-K1 cells, Chinese-hamster ovary K1 cells; DTPA, diethylenetriamine-*N,N,N',N',N''*-penta-acetic acid; L-Leu-pNA, L-leucine-*p*-nitroanilide; IRAP, insulin-regulated aminopeptidase.

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and the synthetic substrate L-Leu-pNA (L-leucine-*p*-nitroanilide) were obtained from Sigma–Aldrich (Bornem, Belgium). Tyr-4 of Ang IV was iodinated using the Iodogen® iodination reagent from Perbio Science (Erembodegem, Belgium) and ^{125}I from ICN Biomedicals (Asse, Belgium). Monoiodinated Ang IV was isolated on a Hypersil BDS C18 reversed-phase HPLC column and stored at -20°C in 10 mM KH_2PO_4 (pH 6.5) containing 45 % (v/v) ethanol. Other reagents were of the highest grade commercially available.

Cell culture and membrane preparation

CHO-K1 cells (kindly provided by the Pasteur Institute, Brussels, Belgium) were cultured and membranes were prepared as described previously [11]. In short, cells were grown in 5 % CO_2 at 37°C in Dulbecco's modified essential medium, supplemented with L-glutamine, penicillin, streptomycin, non-essential amino acids, sodium pyruvate and foetal bovine serum (Invitrogen), until they were confluent. The cells were harvested after a brief treatment with 0.2 % EDTA (in PBS, pH 7.4) and centrifuged at 500 *g* for 5 min at room temperature ($20\text{--}22^\circ\text{C}$). After resuspending in PBS, the cells were washed, counted and then homogenized in 50 mM Tris/HCl (at pH 7.4) using a Polytron and Potter homogenizer. The resulting homogenate was then centrifuged for 30 min (30 000 *g* at 4°C). The pellet was resuspended in 50 mM Tris/HCl and centrifuged again for 30 min (30 000 *g* at 4°C). The resulting pellets were stored at -20°C until use.

Enzyme assay

As described previously [11], the enzyme assays were performed using L-Leu-pNA as substrate. In short, the pellets were thawed and resuspended using a Polytron homogenizer in enzyme buffer containing 50 mM Tris/HCl (pH 7.4), 140 mM NaCl, 0.1 % (w/v) BSA and 100 μM PMSF for the chelator studies. The incubation mixture was composed of 50 μl of membrane homogenate (at a concentration corresponding to 4×10^5 cells/incubation), 150 μl of L-Leu-pNA (1.5 mM unless specified otherwise), 50 μl of EDTA, EGTA or DTPA or buffer and 50 μl of the second test compound (1,10-phenanthroline or 2,2'-bipyridine or buffer). For the ion studies, the incubation mixture consisted of 50 μl of membrane homogenate (at a concentration corresponding to 4×10^5 cells/incubation), 150 μl of L-Leu-pNA (1.5 mM), 50 μl of a mixture of 0.1 mM EDTA and 0.1 mM 1,10-phenanthroline and 25 μl of the first ion and 25 μl of the second ion. The membrane homogenate was incubated at 37°C in 96-well plates (Medisch Labo Service, Menen, Belgium) and the formation of the cleavage product *p*-nitroaniline was monitored by measuring the absorbance *A* at 405 nm for 10–50 min in a BioWhittaker ELISA reader. The corresponding rate constants (further denoted as enzymatic activities) were calculated by linear regression analysis of these absorbance versus time curves. The free Zn^{2+} concentration in the buffer containing the membrane homogenate was determined by inductively coupled plasma MS in a PerkinElmer Dynamic Reaction cell (DRC-e).

Binding assay

Radioligand binding was performed as described previously [11]. Briefly, the membrane pellets were thawed and resuspended in enzyme buffer at a concentration corresponding to 4×10^5 cells/incubation. The incubations were carried out in a final volume of 300 μl /well consisting of 150 μl of membrane homogenate, 50 μl of [^{125}I]Ang IV, 50 μl of (EDTA, EGTA and DTPA) or 10 μM unlabelled Ang IV (for measuring non-specific binding) and 50 μl of 1,10-phenanthroline, 2,2'-bipyridine or buffer containing 0.1 mM EDTA and 0.1 mM 1,10-phenanthroline (for total

Figure 1 Influence of DTPA on 1,10-phenanthroline concentration-inhibition curves of enzyme activity

Enzyme activity in CHO-K1 cell membranes was measured either in the absence (●) or presence of increasing concentrations of DTPA: 0.0015 mM (○), 0.005 mM (■) and 0.05 mM (▲). Values are the means $\pm$ S.E.M. for three independent experiments performed in duplicate and are normalized according to the control activity in the absence of chelator.

binding). After incubation at 37°C for 60 min, the mixture was vacuum-filtered through GF/B glass-fibre filters (Whatman) presoaked for 30 min in 1 % BSA. After drying, the radioactivity retained in the filters was measured using a PerkinElmer γ -counter.

Data analysis

All experiments were performed at least three times, with each determination being the average of at least duplicate determinations. IC_{50} and EC_{50} values were calculated by non-linear regression analysis using GraphPad Prism 4.0. The curves plotted in Figures 1, 3 and 4 correspond to the equation of a one-site concentration–response curve having a variable Hill slope, i.e. $Y = Y_{\text{control}} + (Y_{\text{min}} - Y_{\text{control}}) / [1 + 10^{(X - \log \text{IC}_{50})nH}]$, where nH is the Hill slope, Y_{control} is the enzyme activity in the absence of chelators, Y_{min} is the enzyme activity after maximal inhibition by the chelators and X is the logarithm of the chelator concentration.

RESULTS

Enzyme activity

The catalytic activity of cystinyl aminopeptidase was determined by incubating CHO-K1 cell membranes with the synthetic substrate L-Leu-pNA as outlined previously [11]. The bivalent ion chelators 1,10-phenanthroline and 2,2'-bipyridine produced a full and concentration-dependent inhibition of the enzyme activity. The inhibition curves were steep ($nH = 3.32 \pm 1.78$ and 2.10 ± 0.26) with pIC_{50} ($-\log \text{IC}_{50}$) values of 3.71 ± 0.03 and 2.43 ± 0.05 respectively. In contrast, the chelators DTPA, EDTA and EGTA did not noticeably affect the enzyme activity during a 40 min incubation of the membranes. Interestingly, the inhibitory effect of 1,10-phenanthroline was potentiated in the presence of these acetic acid-containing chelators. As shown in Figure 1, low concentrations of DTPA (1.5 and 5 μM) caused a leftward shift of the 1,10-phenanthroline inhibition curve accompanied by a reduction of the Hill slope. At higher DTPA concentrations ($\geq 15 \mu\text{M}$), the 1,10-phenanthroline inhibition curves had Hill slopes close to unity (with an average value of 0.92 ± 0.08) and similar pIC_{50} values (with an average value of 4.98 ± 0.06). EDTA and EGTA caused a similar potentiation of

Figure 2 Potentiation of the 1,10-phenanthroline and 2,2'-bipyridine inhibition by DTPA, EDTA or EGTA

pIC_{50} values of 1,10-phenanthroline (**A**) and 2,2'-bipyridine (**B**) from concentration–inhibition curves are shown in the absence (ctrl) or presence of increasing concentrations of DTPA (■), EDTA (□) and EGTA (●). The IC_{50} values are calculated by nonlinear regression analysis of the inhibition curves and are the means $\pm$ S.E.M. for at least three independent experiments performed in duplicate.

1,10-phenanthroline until a state with pIC_{50} values of 4.99 ± 0.03 and 5.00 ± 0.03 respectively. The efficacy of the acetic acid-containing chelators to potentiate 1,10-phenanthroline inhibition decreased in the order: DTPA > EDTA > EGTA (Figure 2A).

The influence of the substrate concentration on the inhibitory effect of 1,10-phenanthroline was investigated in the absence or presence of 0.1 mM EDTA (Figure 3). In the absence of EDTA, the 1,10-phenanthroline concentration–inhibition curves were unaffected upon increasing the L-Leu-pNA concentrations ($pIC_{50} = 3.88 \pm 0.03$, 3.85 ± 0.04 and 3.71 ± 0.03 in the presence of 0.15, 0.3 and 1.5 mM L-Leu-pNA). However, in the presence of EDTA, the 1,10-phenanthroline concentration–inhibition curves were shifted to the right by increasing the L-Leu-pNA concentrations ($pIC_{50} = 5.43 \pm 0.06$, 5.30 ± 0.07 and 4.57 ± 0.04 respectively).

The acetic acid-containing chelators potentiated the inhibitory effect of 2,2'-bipyridine in the same way as for 1,10-phenanthroline (Figure 4). Their efficacy also decreased in the order: DTPA > EDTA > EGTA (Figure 2B). The pIC_{50} values for 2,2'-bipyridine were 3.44 ± 0.04 , 3.35 ± 0.06 and 3.53 ± 0.09 in the presence of maximal effective concentrations of DTPA, EDTA and EGTA respectively. In the absence of acetic acid-containing chelators, the pIC_{50} value for 2,2'-bipyridine was 2.43 ± 0.05 .

Enzyme activity was measured after the addition of increasing concentrations of bivalent ions to the incubation mixture. The basal concentration of free Zn^{2+} in the buffer with cell membranes was $1.3 \mu M$. The combined and complete inhibition of the enzyme activity by 0.1 mM EDTA and 0.1 mM 1,10-phenanthroline was completely reversed by the addition of 0.04–0.1 mM Zn^{2+} (Figure 5). This effect was biphasic since a further increase of the Zn^{2+} concentration produced a nearly complete inhibition

Figure 3 Influence of substrate concentration on 1,10-phenanthroline concentration–inhibition curves

1,10-Phenanthroline concentration–inhibition curves of enzymatic activity in CHO-K1 cell membranes were measured in the absence (**A**) or presence of 0.1 mM EDTA (**B**). Enzyme activity was measured with three substrate concentrations: 0.15 mM (■), 0.3 mM (□) and 1.5 mM (●) L-Leu-pNA. Each value is the average $\pm$ S.E.M. for at least three independent experiments performed in duplicate.

Figure 4 Influence of DTPA on 2,2'-bipyridine concentration–inhibition curves of enzyme activity

Enzyme activity was measured either in the absence (●) or presence of increasing concentrations of DTPA: 0.0015 mM (○), 0.005 mM (■), 0.015 mM (□) and 0.05 mM (▲). Values are the means $\pm$ S.E.M. for three independent experiments performed in duplicate and are normalized according to the control activity in the absence of chelator.

of the cystinyl aminopeptidase activity. Interestingly, the re-activation potency of Zn^{2+} was increased in the presence of Ca^{2+} at concentrations between 0.03 and 0.1 mM (Figure 5A). A similar effect was also produced by Mg^{2+} , except that it occurred at higher concentrations, i.e. between 0.1 and 1 mM (Figure 5B).

Radioligand binding experiments

The high-affinity binding of [^{125}I]Ang IV to cystinyl aminopeptidase has been shown to be inversely regulated by bivalent ion

Figure 5 Reversal of 1,10-phenanthroline/EDTA-mediated enzyme inhibition by Zn^{2+} .

(A) CHO-K1 cell membranes were incubated in a buffer containing 0.1 mM 1,10-phenanthroline and 0.1 mM EDTA in the presence of increasing concentrations of Zn^{2+} in the absence (■) or presence of 0.001 mM (□), 0.01 mM (●), 0.03 mM (○), 0.05 mM (▲) and 0.1 mM (△) Ca^{2+} . (B) A similar experiment but now in the absence (■) or presence of 0.001 mM (□), 0.01 mM (●), 0.03 mM (○), 0.1 mM (△), 0.3 mM (◆) and 1 mM (◇) Mg^{2+} . Values are the means $\pm$ S.E.M. for three independent experiments performed in duplicate and are normalized according to the control activity in the absence of chelator (i.e. 100 %).

chelators. Whereas no binding could be observed in the absence of chelators, 1,10-phenanthroline and 2,2'-bipyridine caused a concentration-dependent increase of the [^{125}I]Ang IV binding in CHO-K1 cell membranes. Half-maximal effects occurred at $204 \pm 34 \mu\text{M}$ 1,10-phenanthroline and at $1900 \pm 250 \mu\text{M}$ 2,2'-bipyridine.

The acetic acid-containing chelators only produced a modest increase in [^{125}I]Ang IV binding when taken alone. Instead, they produced a leftward shift of the binding versus 1,10-phenanthroline concentration curves (results not shown) and of the binding versus 2,2'-bipyridine concentration curves (Figure 6). The acetic acid-containing chelators potentiated the effects of 1,10-phenanthroline and 2,2'-bipyridine with the same order of efficacy, i.e. DTPA > EDTA > EGTA (Table 1).

DISCUSSION

Cystinyl aminopeptidase is a member of the M1 aminopeptidase family containing a typical Zn^{2+} -binding motif (HEXXHX18E) and the exopeptidase sequence GXMEN. In a previous study, it was shown that 1,10-phenanthroline inhibits the catalytic activity of cystinyl aminopeptidase and that this effect is potentiated by EDTA and EGTA, two chelators that only minimally affect the enzyme activity on their own [12]. The present findings provide better insights into the molecular mechanisms by which

Figure 6 Stimulation of [^{125}I]Ang IV binding by 2,2'-bipyridine

[^{125}I]Ang IV binding to CHO-K1 cell membranes was measured in the presence of increasing concentrations of 2,2'-bipyridine in the absence (■) or presence of: (A) 0.05 mM (○), 0.15 mM (●), 0.5 mM (□) and 1.5 mM (◆) EGTA; (B) 0.005 mM (□), 0.015 mM (●), 0.05 mM (○) and 0.15 mM (◆) EDTA; (C) 0.0005 mM (□), 0.0015 mM (●), 0.005 mM (○), 0.015 mM (◆) and 0.05 mM (◇) DTPA. Results are expressed as specific binding and are the means $\pm$ S.E.M. for at least three independent experiments performed in duplicate. Control binding (i.e. 0 %) was obtained in the absence of chelator and maximal binding (i.e. 100 %) was obtained in the presence of 0.1 mM of EDTA and 0.1 mM 1,10-phenanthroline.

these and other structurally related chelators affect the cystinyl aminopeptidase molecule.

First, there are striking structural similarities among enzyme-inactivating and -potentiating chelators. In this respect, a concentration-dependent and complete inhibition of the cystinyl aminopeptidase activity is observed with 1,10-phenanthroline as well as with 2,2'-bipyridine. Both are planar aromatic bidentate chelators, with two pyridine moieties whose nitrogen atoms are well known to interact with bivalent cations like Zn^{2+} [13]. On the other hand, EDTA, EGTA and DTPA potentiate the inhibitory effects of 1,10-phenanthroline as well as 2,2'-bipyridine. The common characteristic of these potentiating chelators is that they contain four (EDTA and EGTA) or five (DTPA) chelating acetic acid moieties. The structural dissimilarity between these two classes of chelators constitutes a plausible explanation for the observed differences in their effects.

Secondly, it is worthy to note that the concentration-inhibition curves of 1,10-phenanthroline and 2,2'-bipyridine are remarkably steep (the corresponding Hill slopes are > 1) in the absence of the acetic acid-containing chelators. This would imply that these chelators remove the metal ion from the enzyme and/or there are

Table 1 1,10-Phenanthroline and 2,2'-bipyridine induced [¹²⁵I]Ang IV binding to CHO-K1 cell membranes in the absence or presence of EGTA, EDTA or DTPA

The EC₅₀ values were calculated by non-linear regression analysis of the concentration–effect curves; n.d., not determined; log *F* = log (EC₅₀ control/EC₅₀ in the presence of chelator). The number of determinations was 3–7.

Chelator (mM)	1,10-Phenanthroline		2,2'-Bipyridine	
	EC ₅₀ value (μM)	log <i>F</i>	EC ₅₀ value (μM)	log <i>F</i>
Control	204 ± 34		1900 ± 250	
EGTA	0.05	135 ± 27	0.18	1640 ± 440
	0.15	19 ± 10	1.00	85 ± 24
	0.5	2.2 ± 0.5	1.96	26 ± 4.7
	1.5	1.4 ± 0.8	2.16	21 ± 5.1
EDTA	0.005	293 ± 58	−0.15	3010 ± 240
	0.015	214 ± 61	−0.05	2080 ± 950
	0.05	6.7 ± 2.3	1.48	92 ± 55
	0.15	6.4 ± 2.2	1.51	70 ± 44
	0.5	7.4 ± 2.5	1.43	n.d.
DTPA	0.0005	246 ± 41	−0.10	1280 ± 100
	0.0015	97 ± 8.2	0.32	287 ± 53
	0.005	14 ± 2.9	1.15	45 ± 25
	0.015	8.3 ± 1.1	1.40	48 ± 11
	0.05	6.6 ± 1.7	1.49	28 ± 5.9
				1.83

Scheme 1 Proposed mechanisms by which a chelator can affect the metalloenzyme activity

E, M and L correspond to the enzyme, metal ion and the chelator respectively. K_{Me} and K_{MeL} are the equilibrium dissociation constants for the interaction between the enzyme and the free metal and for the interaction between the enzyme and the chelator-bound metal respectively. K_i is the equilibrium constant for dissociation of L from the enzymatically inactive ternary complex EML. Note that *n* chelator molecules can bind to one metal ion. Numbers 1 and 2 represent the proposed pathways for the 1,10-phenanthroline/2,2'-bipyridine effects in the absence or presence of acetic acid-containing chelators respectively.

more than one chelator-binding sites on the enzyme [14]. The presence of only a single Zn²⁺ ion in one enzyme molecule [4] makes the latter explanation unlikely, hence the more plausible mechanisms are summarized in Scheme 1. This general model was proposed to describe the different ways in which metal–buffer ligands affect the active sites of metalloenzymes [15]. With respect to the present study, the bidentate chelators like 1,10-phenanthroline and 2,2'-bipyridine may interact with the cystinyl aminopeptidase-bound catalytic Zn²⁺ to form a ternary enzyme–Zn²⁺–1,10-phenanthroline complex. As a result, this could prevent the binding and/or hydrolysis of the substrate and, in addition, facilitate the dissociation of Zn²⁺ from the enzyme [15]. Alternatively, provided that the enzyme–Zn²⁺ complex is in equilibrium with its free constituents (apoenzyme and Zn²⁺, with an equilibrium dissociation constant K_{Me}), chelators like 1,10-phenanthroline may decrease the catalytic activity through depletion of the free Zn²⁺ and, hence, without necessarily interacting with the enzyme itself [15]. Whereas mechanisms involving ternary complex

Figure 7 Potentiation of 1,10-phenanthroline inhibition by EDTA: kinetics

CHO-K1 cell membranes and 1.5 mM L-Leu-pNA were incubated at 37°C for increasing time and the formation of *p*-nitroanilide [measured as absorbance (OD) at 405 nm] was measured during two subsequent time periods (0–30 and 40–70 min); 25 μl of buffer, EDTA or 1,10-phenanthroline was added 10 min before the first period, after which buffer, EDTA or 1,10-phenanthroline was added at 35 min (arrow), giving the following combinations and final concentrations: ■, only adding buffer; □, 0.1 mM EDTA then adding 0.5 mM 1,10-phenanthroline; ●, 0.1 mM EDTA then adding buffer; ○, 0.5 mM 1,10-phenanthroline then adding buffer; and ▲, 0.5 mM 1,10-phenanthroline then adding 0.1 mM EDTA. Lines correspond to the linear regression at the two time periods.

formation are likely to produce sigmoidal inhibition curves with Hill coefficients (equivalent to $\bar{n}$ mentioned in [15]) close to unity, steeper curves (i.e. with Hill coefficients > 1) such as the ones observed for 1,10-phenanthroline and 2,2'-bipyridine are taken as an indication that the metal has been removed from the enzyme and that it is now present in the form of a multichelator–metal complex. This complies with the ability of one Zn²⁺ ion to combine with up to three molecules of 1,10-phenanthroline or 2,2'-bipyridine in solution [14]. In agreement, the 1,10-phenanthroline inhibition curve is unaffected by the substrate concentration, indicating that both do not compete for interacting with the catalytic Zn²⁺. This therefore favours the explanation that 1,10-phenanthroline decreases the cystinyl aminopeptidase activity by scavenging free Zn²⁺ in solution.

Although the acetic acid-containing chelators only weakly affect the cystinyl aminopeptidase activity in the enzyme assays, a long preincubation of the membranes with 1 mM EDTA revealed the gradual appearance of enzyme inhibition, with a 50% inhibition after approx. 2 h of preincubation (results not shown). Instead, the EDTA-like chelators potentiate the inhibitory action of the aromatic bidentate chelators in a concentration-dependent manner until a maximal effect was obtained, and this effect occurs within less than 5 min (Figure 7). In the presence of EDTA-like potentiating chelators, the 1,10-phenanthroline and 2,2'-bipyridine concentration–inhibition curves have Hill coefficients that are close to unity. A similar phenomenon has been produced in simulation studies based on the equations given in Scheme 1 and also by increasing the affinity of the bidentate chelator for the enzyme–metal complex (i.e. upon decreasing the equilibrium dissociation constant K_i) [15]. This allows the ternary enzyme–Zn²⁺–chelator complex to play a predominant role in the inactivation process. The ability of 1,10-phenanthroline to interact with the catalytic Zn²⁺ in the presence of EDTA is also supported experimentally by the observation that the 1,10-phenanthroline inhibition curve is shifted to the right upon increasing the substrate concentration. Indeed, this finding indicates that 1,10-phenanthroline and the substrate have now become capable of competing with each other. Taken together, the present findings and analysis extend our former conclusion about the

action mechanisms of 1,10-phenanthroline and EDTA [12]. We now propose that the cystinyl aminopeptidase enzyme is able to adopt two interconvertible conformational states with low and high sensitivities respectively towards aromatic bidentate chelators and that the latter conformation is favoured in the presence of acetic acid-containing chelators. On the other hand, the $V_{\max}$ and K_m values for synthetic substrates like L-Leu-pNA and the affinity for competitive inhibitors like Ang IV ([12] and results not shown) appear to be quite similar for both conformational states. To account for these observations, it is further suggested that the acetic acid-containing chelators may affect the enzyme's conformation by an allosteric mechanism involving the removal of a modulatory cation rather than by acting on the catalytic Zn^{2+} . Positive identification of such a modulatory cation-binding site on cystinyl aminopeptidase will have to await the acquisition of its X-ray crystal structure. However, this technical approach appears to be extremely difficult in the case of M1 aminopeptidase family members and, so far, it has only been successfully achieved for the leukotriene A_4 hydrolase enzyme [16]. Yet, there is substantial functional and sometimes structural evidence for bivalent cations other than Zn^{2+} to provoke important structural changes in related Zn^{2+} metalloproteinases. In this respect, it has been reported that aminopeptidase A (EC 3.4.11.7, a multimeric M1 metalloproteinase that is closely related to cystinyl aminopeptidase) contains one Ca^{2+} ion-binding site for each monomer and that the presence of this cation increases the enzyme activity and the affinity of synthetic substrates [17]. In aminopeptidase A, a histidine residue located 60 amino acids N-terminal from the HEXXH motif has been identified to play an important role in this Ca^{2+} modulation [17]. Since this histidine residue is conserved among most of the M1 metalloproteinases, it should be of interest to find out whether a similar role is played by its homologue in cystinyl aminopeptidase. For the more distantly related protein phosphatase 1 enzyme (a Ser/Thr protein phosphatase), the activity was increased by adding a combination of Fe^{2+} and Zn^{2+} but not the individual metal ions [18]. In agreement with the catalytic activity measurements, the crystal structure of this enzyme revealed the presence of a binuclear ion centre consisting of two bivalent ions involved in the catalytic activity [19]. As another example, Ca^{2+} was found to be essential for maintaining the fibronectin and heparin binding properties of the human matrix metalloproteinase-2 [20]. Finally, Ca^{2+} was shown to modulate the affinity of *Streptomyces griseus* aminopeptidase (a bacterial Zn^{2+} metalloproteinase of the M28 family) for certain substrates as well as for inhibitors like bestatin and amastatin [21]. Here again, X-ray crystallography of the apoenzyme revealed the presence of a Ca^{2+} -binding site at a considerable distance from the double Zn^{2+} -binding amino acid residues [22].

In line with previous observations, Zn^{2+} was found to fully reverse the inhibitory effect of an EDTA/1,10-phenanthroline combination in a concentration-dependent manner. Full activity was obtained in the presence of 10^{-4} M Zn^{2+} and, as described for many other metalloproteinases, the enzyme activity decreased again upon further increase of the Zn^{2+} concentration [1,3]. Restoration of the enzyme activity by Zn^{2+} could be ascribed to its ability to reduce the concentration of free 1,10-phenanthroline and to replace potentially released catalytic Zn^{2+} ions. A new and intriguing finding in the present study is that Ca^{2+} and (to a lesser extent) Mg^{2+} ions were able to increase the potency by which Zn^{2+} reverses the inhibitory effect of the EDTA/1,10-phenanthroline combination. According to the model presented above, this phenomenon is compatible with the potential of Ca^{2+} and Mg^{2+} to bind to the modulatory cation-binding site, to decrease the sensitivity of the enzyme towards 1,10-phenanthroline and, hence,

to increase the stability and prevalence of the active enzyme- Zn^{2+} complex.

High-affinity [^{125}I]Ang IV binding sites have initially been referred to as 'AT₄ receptors' and they were shown to be present on membrane preparations from various tissues and cell types [9,23,24]. Based on the purification and partial sequencing of the [^{125}I]Ang IV binding protein from bovine brain, Albiston et al. [9] provided convincing evidence that they correspond to the cystinyl aminopeptidase/IRAP protein. In the same vein, human cystinyl aminopeptidase transiently expressed in HEK-293T cells displayed typical 'AT₄ receptor'-like binding characteristics [9]. It is of interest that nearly all of the past binding studies with [^{125}I]Ang IV and related radioligands were performed in the presence of bivalent cation chelators such as EDTA and 1,10-phenanthroline. On the basis of the much higher affinity of Ang IV in such binding experiments as compared with its ability to compete with the synthetic substrate in cystinyl aminopeptidase catalytic activity assays, Lew et al. [23] proposed that the presence of Zn^{2+} in the catalytic site decreases the apparent affinity of 'AT₄ receptor' ligands like Ang IV. In agreement with this proposal, we observed in a previous study using a limited amount of chelators as well as in the present study that the high-affinity binding of [^{125}I]Ang IV is inversely proportional to the enzymatic activity [12]. This phenomenon took place under all conditions tested so far, including experiments performed in the presence of bidentate chelators either alone or in the presence of acetic acid-containing chelators and with or without added Zn^{2+} . Since it is likely to occur to the cystinyl aminopeptidase apoenzyme, high-affinity binding of Ang IV and related 'AT₄ receptor' ligands should be differentiated from the physiological effects of the same ligands.

In summary, the present results suggest that the planar aromatic bidentate chelators 1,10-phenanthroline and 2,2'-bipyridine are able to decrease the cystinyl aminopeptidase catalytic activity by chelating Zn^{2+} in solution. On the other hand, acetic acid-containing chelators such as DTPA, EDTA and EGTA increase the affinity of the bidentate chelators for the enzyme- Zn^{2+} complex, probably by removing a bivalent cation from an allosteric modulatory site on the enzyme. Whereas the bidentate chelators can now directly interact with the catalytic Zn^{2+} , the binding affinity of ligands like Ang IV is unaffected by the presence of acetic acid-containing chelators [12]. On the other hand, the loss in enzyme activity in the presence of bidentate chelators (either in the presence or absence of acetic acid-containing chelators) is directly proportional to the gain of the high-affinity binding of [^{125}I]Ang IV. These binding sites were previously referred to as 'AT₄ receptors' and are most likely to represent the cystinyl aminopeptidase apoenzyme.

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REFERENCES

- 1 Rogi, T., Tsujimoto, M., Nakazato, H., Mizutani, S. and Tomoda, Y. (1996) Human placental leucine aminopeptidase/oxytocinase: a new member of type II membrane spanning zinc metalloproteinase family. *J. Biol. Chem.* **271**, 56–61.
- 2 Laustsen, P. G., Rasmussen, T. E., Petersen, K., Pedraza-Diaz, S., Moestrup, S. K., Gliemann, J., Sottrup-Jensen, L. and Kristensen, T. (1997) The complete amino acid sequence of human placental oxytocinase. *Biochim. Biophys. Acta* **1352**, 1–7.
- 3 Tsujimoto, M., Mizutani, S., Adachi, H., Kimura, M., Nakazato, H. and Tomoda, Y. (1992) Identification of human placental leucine aminopeptidase as oxytocinase. *Arch. Biochem. Biophys.* **292**, 388–392.

- 4 Matsumoto, H., Rogi, T., Yamashiro, K., Kodama, S., Tsuruoka, N. and Hattori, A. (2000) Characterization of a recombinant soluble form of human placental leucine aminopeptidase/oxytocinase expressed in Chinese hamster ovary cells. *Eur. J. Biochem.* **267**, 46–52
- 5 Mizutani, S. and Tomoda, Y. (1992) Placental cystine aminopeptidase or placental leucine aminopeptidase (P-LAP). *Semin. Reprod. Endocrinol.* **10**, 146–153
- 6 Keller, S. R., Scott, H. M., Mastick, C. C., Aebersold, R. and Lienhard, G. E. (1995) Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. *J. Biol. Chem.* **270**, 23612–23618
- 7 Keller, S. R. (2004) Role of the insulin-regulated aminopeptidase IRAP in insulin action and diabetes. *Biol. Pharm. Bull.* **27**, 761–764
- 8 Herbst, J. J., Ross, S. A., Scott, H. M., Bobin, S. A., Morris, N. J. and Lienhard, G. E. (1997) Insulin stimulates cell surface aminopeptidase activity toward vasopressin in adipocytes. *Am. J. Physiol.* **272**, 600–606
- 9 Albiston, A. L., McDowall, S. G., Matsacos, D., Sim, P., Clune, E. and Mustafa, T. (2001) Evidence that the angiotensin IV (AT₄) receptor is the enzyme insulin-regulated aminopeptidase. *J. Biol. Chem.* **276**, 48623–48626
- 10 Albiston, A. L., Ye, S. and Chai, S. Y. (2004) Membrane bound members of the M1 family: more than aminopeptidases. *Protein Pept. Lett.* **11**, 491–500
- 11 Demaegd, H., Vanderheyden, P. M. L., De Backer, J. P., Mosselmans, S., Laeremans, H., Le, M. T., Kersemans, V., Michotte, Y. and Vauquelin, G. (2004) Endogenous cystinyl aminopeptidase in Chinese hamster ovary cells: characterization by [¹²⁵I]Ang IV binding and catalytic activity. *Biochem. Pharmacol.* **68**, 885–892
- 12 Demaegd, H., Laeremans, H., De Backer, J. P., Mosselmans, S., Le, M. T., Kersemans, V., Michotte, Y., Vauquelin, G. and Vanderheyden, P. M. L. (2004) Synergistic modulation of cystinyl aminopeptidase by divalent cation chelators. *Biochem. Pharmacol.* **68**, 893–900
- 13 Koning, B., de Boer, J. W., Meetsma, A. and Kellogg, R. M. (2004) Synthesis and complexation characteristics of phenanthroline and bipyridine diols. *ARKIVOC* **2004**, 189–205
- 14 Auld, D. S. (1995) Removal and replacement of metal ions in metalloproteinases. *Methods Enzymol.* **248**, 228–242
- 15 Baker, J. O. (1988) Metal-buffered systems. *Methods Enzymol.* **158**, 33–55
- 16 Thunnissen, M. M., Nordlund, P. and Haeggstrom, J. Z. (2001) Crystal structure of human leukotriene A(4) hydrolase, a bifunctional enzyme in inflammation. *Nat. Struct. Biol.* **8**, 131–135
- 17 Iturrioz, X., Vazeux, G., Celerier, J., Corvol, P. and Llorens-Cortes, C. (2000) Histidine⁴⁵⁰ plays a critical role in catalysis and, with Ca²⁺, contributes to the substrate specificity of aminopeptidase A. *Biochemistry* **39**, 3061–3068
- 18 Chu, Y., Ernest, Y. C., Lee, Y. and Schlender, K. K. (1996) Activation of protein phosphatase 1. *J. Biol. Chem.* **271**, 2574–2577
- 19 Egloff, M. P., Cohen, P. T. W., Reinemer, P. and Barford, D. (1995) Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* **254**, 942–959
- 20 Margaretha-Wallon, U. and Christopher, M. (1997) The hemopexin-like domain (C domain) of human gelatinase A (matrix metalloproteinase-2) requires Ca²⁺ for fibronectin and heparin binding: binding properties of recombinant gelatinase A, C domain to extracellular matrix and basement membrane components. *J. Biol. Chem.* **272**, 7473–7481
- 21 Hasselgren, C., Park, H. I. and Ming, L. J. (2001) Metal ion binding and activation of *Streptomyces griseus* dinuclear aminopeptidase: cadmium(II) binding as a model. *J. Biol. Inorg. Chem.* **6**, 120–127
- 22 Papir, G., Spungin-Bialik, A., Ben-Meir, D., Fudim, E., Gilboa, R., Greenblatt, H. M., Shoham, G., Lessel, U., Schomburg, D., Ashkenazi, R. et al. (1998) Inhibition of *Streptomyces griseus* aminopeptidase and effects of calcium ions on catalysis and binding—comparisons with the homologous enzyme *Aeromonas proteolytica* aminopeptidase. *Eur. J. Biochem.* **258**, 313–319
- 23 Lew, R. A., Mustafa, T., Ye, S., McDowall, S. G., Chai, S. Y. and Albiston, A. L. (2003) Angiotensin AT₄ ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). *J. Neurochem.* **86**, 344–350
- 24 Lee, J., Mustafa, T., McDowall, S. G., Mendelsohn, F. A., Brennan, M. and Lew, R. A. (2003) Structure–activity study of LVV-hemorphin-7: angiotensin AT₄ receptor ligand and inhibitor of insulin-regulated aminopeptidase. *J. Pharmacol. Exp. Ther.* **305**, 205–211

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