Metal ion modulation of cystinyl aminopeptidase

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Cystinyl aminopeptidase has one Zn\(^{2+}\)-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-\(N,N,N',N''-\)-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 Zn\(^{2+}\) 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 Zn\(^{2+}\). The inhibitory effect of EDTA plus 1,10-phenanthroline could be completely reversed by Zn\(^{2+}\). Ca\(^{2+}\) and Mg\(^{2+}\) increased the potency of Zn\(^{2+}\) 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 \([^{125}\text{I}]\)angiotensin IV binding to the membranes and this was potentiated by the acetic acid-containing chelators. These findings support the concept that high-affinity \([^{125}\text{I}]\)angiotensin IV binding, previously referred to as 'AT\(_{4}\) 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 metalloprotease 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 \([^{125}\text{I}]\)Ang IV, which were designated previously as the AT\(_{2}\) 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 Zn\(^{2+}\)-binding motif HEXXH(X)\(_{18}\)E 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 A\(_{4}\) 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-\(N,N,N',N''-\)-penta-acetic acid), 1,10-phenanthroline and 2,2'-bipyridine

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*Abbreviations used: Ang, angiotensin; CHO-K1 cells, Chinese-hamster ovary K1 cells; DTPA, diethylenetriamine-\(N,N,N',N''-\)-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 125I 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 KH2PO4 (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% CO2 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 (20–22°C). After resuspending in PBS, the cells were washed, counted and then homogenized in 50 mM Tris/HCl (pH 7.4) using a Polytron and Potter homogenizer. The resulting homogenate was then centrifuged for 30 min (30000 g at 4°C). The pellet was resuspended in 50 mM Tris/HCl and centrifuged again for 30 min (30000 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 x 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 x 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-nitroanilide 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 Zn2+ 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 x 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 [125I]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 binding). After incubation at 37°C for 60 min, the mixture was vacuum-filtered through GF/B glass-fibre filters (Whatman) pre-soaked 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. IC50 and EC50 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. $Y = Y_{\text{control}} + (Y_{\text{max}} - Y_{\text{control}})/(1 + 10^{X-Y_{\text{IC50}}})$, where $nH$ is the Hill slope, $Y_{\text{control}}$ is the enzyme activity in the absence of chelators, $Y_{\text{max}}$ 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 ($nH = 3.32 ± 1.78$ and $2.10 ± 0.26$) with pIC50 ($-\log IC50$) 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 (≥15 µM), the 1,10-phenanthroline inhibition curves had Hill slopes close to unity (with an average value of 0.92 ± 0.08) and similar pIC50 values (with an average value of 4.98 ± 0.06). EDTA and EGTA caused a similar potentiation of
Ion modulation of cystinyl aminopeptidase

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

pIC₅₀ 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 IC₅₀ values are calculated by nonlinear regression analysis of the inhibition curves and are the means ± S.E.M. for at least three independent experiments performed in duplicate.

Enzyme activity was measured after the addition of increasing concentrations of bivalent ions to the incubation mixture. The basal concentration of free Zn²⁺ in the buffer with cell membranes was 1.3 μM. 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 Zn²⁺ (Figure 5). This effect was biphasic since a further increase of the Zn²⁺ concentration produced a nearly complete inhibition of the cystinyl aminopeptidase activity. Interestingly, the re-activation potency of Zn²⁺ was increased in the presence of Ca²⁺ at concentrations between 0.03 and 0.1 mM (Figure 5A). A similar effect was also produced by Mg²⁺, 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 [¹²⁵I]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 (□), 0.03 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 (▲) and 1 mM (▽) Mg\(^{2+}\). Values are the means ± 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 \([^{125}\text{I}]\)Ang IV binding in CHO-K1 cell membranes. Half-maximal effects occurred at 204 ± 34 µM 1,10-phenanthroline and at 1900 ± 250 µM 2,2′-bipyridine.

The acetic acid-containing chelators only produced a modest increase in \([^{125}\text{I}]\)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 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 [35S]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.

<table>
<thead>
<tr>
<th>Chelator (mM)</th>
<th>1,10-Phenanthroline</th>
<th>2,2’-Bipyridine</th>
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<tbody>
<tr>
<td>Control</td>
<td>204 ± 34</td>
<td>1900 ± 250</td>
</tr>
<tr>
<td>EGTA 0.05</td>
<td>135 ± 27</td>
<td>1640 ± 440</td>
</tr>
<tr>
<td>0.15</td>
<td>19 ± 10</td>
<td>85 ± 24</td>
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<tr>
<td>0.5</td>
<td>2.2 ± 0.5</td>
<td>26 ± 4</td>
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<tr>
<td>1.5</td>
<td>1.4 ± 0.8</td>
<td>21 ± 5.1</td>
</tr>
<tr>
<td>EDTA 0.005</td>
<td>293 ± 58</td>
<td>−0.15</td>
</tr>
<tr>
<td>0.015</td>
<td>214 ± 61</td>
<td>−0.05</td>
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<tr>
<td>0.05</td>
<td>6.7 ± 2.3</td>
<td>92 ± 55</td>
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<tr>
<td>0.15</td>
<td>6.4 ± 2.2</td>
<td>70 ± 44</td>
</tr>
<tr>
<td>0.5</td>
<td>7.4 ± 2.5</td>
<td>1.43</td>
</tr>
<tr>
<td>DTPA 0.0005</td>
<td>246 ± 4.1</td>
<td>−0.10</td>
</tr>
<tr>
<td>0.0015</td>
<td>97 ± 8.2</td>
<td>0.32</td>
</tr>
<tr>
<td>0.005</td>
<td>14 ± 2.9</td>
<td>1.15</td>
</tr>
<tr>
<td>0.015</td>
<td>8.3 ± 1.1</td>
<td>1.40</td>
</tr>
<tr>
<td>0.05</td>
<td>6.6 ± 1.7</td>
<td>1.49</td>
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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.

formation are likely to produce sigmoidal inhibition curves with Hill coefficients (equivalent to $n$ 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 Zn$^{2+}$ 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 Zn$^{2+}$. This therefore favours the explanation that 1,10-phenanthroline decreases the cystinyl aminopeptidase activity by scavenging free Zn$^{2+}$ 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 dissociation constant $K_I$) [15]. This allows the ternary enzyme–Zn$^{2+}$–chelator complex to play a predominant role in the inactivation process. The ability of 1,10-phenanthroline to interact with the catalytic Zn$^{2+}$ 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 chela-
tors and that the latter conformation is favoured in the presence
of acetic acid-containing chelators. On the other hand, the
$V_{\text{max}}$ and $K_{\text{m}}$ values for synthetic substrates like L-Leu-pNA and
the affinity for competitive inhibitors like Ang IV ([12] and re-
results not shown) appear to be quite similar for both confor-
mational 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 A4 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 metallopeptidase 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 aminope-
tidase A, a histidine residue located 60 amino acids N-ter-
minal from the HEXXH motif has been identified to play an
important role in this Ca$^{2+}$ modulation [17]. Since this histidine
residues is conserved among most of the M1 metallopeptidases, 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 bi-
nuclear 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 metallopeptidases, 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 [125I]Ang IV binding sites have initially been referred
to as ‘AT$_{4}$ 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
[125I]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, hu-
mans cystinyl aminopeptidase transiently expressed in HEK-293T
cells displayed typical ‘AT$_{4}$ receptor’-like binding characteristics
[9]. It is of interest that nearly all of the past binding studies
with [125I]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 ‘AT$_{4}$
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
[125I]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-contain-
ing 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 ‘AT$_{4}$ 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
[125I]Ang IV. These binding sites were previously referred to as
‘AT$_{4}$ receptors’ and are most likely to represent the cystinyl
aminopeptidase apoenzyme.

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