Published in final edited form as:

J Inorg Biochem. 2012 January ; 106(1): 84–89. doi:10.1016/j.jinorgbio.2011.09.020.

Probing the Metal Ion Selectivity in Methionine Aminopeptidase via Changes in the Luminescence Properties of the Enzyme Bound Europium Ion

Nitesh Sule, Raushan K. Singh, Pinjing Zhao, and D. K. Srivastava*

Department of Chemistry and Biochemistry, North Dakota State University, Fargo, ND 58108, USA

Abstract

We report herein, for the first time, that Europium ion (Eu^{3+}) binds to the “apo” form of *E. coli* methionine aminopeptidase (*EcMetAP*), and such binding results in the activation of the enzyme as well as enhancement in the luminescence intensity of the metal ion. Due to competitive displacement of the enzyme-bound Eu^{3+} by different metal ions, we could determine the binding affinities of both “activating” and “non-activating” metal ions for the enzyme via fluorescence spectroscopy. The experimental data revealed that among all metal ions, Fe^{2+} exhibited the highest binding affinity for the enzyme, supporting the notion that it serves as the physiological metal ion for the enzyme. However, the enzyme-metal binding data did not adhere to the Irving-William series. On accounting for the binding affinity vis a vis the catalytic efficiency of the enzyme for different metal ions, it appears evident that the “coordination states” and the relative softness” of metal ions are the major determinants in facilitating the *EcMetAP* catalyzed reaction.

Keywords

Methionine aminopeptidase; europium; luminescence spectroscopy; metal ion selectivity; metal binding affinity

1. Introduction

The drug discovery endeavour involving pathogenic metalloenzymes has often been hampered (at least in part) due to difficulty in identifying their physiological metal ions. Based on their binding affinities, metalloenzymes typically fall in two categories [1]: (i) The enzymes which interact with their physiological metal ions fairly tightly ($K_d < 10 \text{ nM}$) such that the metal ions are retained during the course of protein purification, and (ii) the enzymes which possess weaker binding affinities for their metal ions ($K_d > 10 \text{ nM}$), and such enzymes usually lose their metal ions during the course of purification. Unfortunately, the assignment of physiological metal ions to the latter metalloenzymes relies on the assumption that the enzyme substrates (under steady-state condition) do not alter the intrinsic binding affinities of those metal ions. In other words, the kinetically derived activation constants (K_a 's) of metal ions are taken to be the measure of their direct binding

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author. Tel.: +1 701 231 7831. Fax: +1 701 231 8324, dk.srivastava@ndsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

affinities (K_d 's), and such assumption may not be true for all metalloenzymes and their cognate metal ions. The identity of physiological metal ions for pathogenic metalloenzymes is important not only from the point of deducing their mechanistic details but also for designing their potent inhibitors as therapeutic agents. It has been frequently observed that the inhibitors developed against metalloenzymes harboring non-physiological metal ions (via in vitro enzymatic screening), are ineffective under in vivo conditions [2,3].

In pursuit of investigating the catalytic and inhibitory features of the recombinant forms of both *E. coli* and human methionine aminopeptidases [4], we sought to investigate the relative binding affinities of selected metal ions to gain insights their mode of bindings to the enzyme. However, this endeavour appeared challenging due to the lack of sensitive spectroscopic and/or thermal signals to quantitatively probe the occupancy of the metal ions at the enzyme sites. In the quest of exploring highly sensitive electronic spectroscopic signals for probing the binding of various metal ions to *EcMetAP*, we recognized that the luminescent intensity of Eu^{3+} is markedly enhanced upon binding to the enzyme, and it is diminished upon competitive displacement by different metal ions. An even fortuitous discovery was the activation of apo-*EcMetAP* by Eu^{3+} . As will be elaborated in the following sections, by using Eu^{3+} as the luminescent probe, we have been able to determine the binding affinities of different types of metal ions for the enzyme and provide a molecular rationale as to the nature of the metal ions in promoting the *EcMetAP* catalysed reaction. It should be pointed out that prior to this study, the direct binding affinities of Mn^{2+} and Co^{2+} to *EcMetAP* have been previously determined via isothermal titration calorimetry [5,6]

2. Experimental

2.1 Cloning, expression and purification of *EcMetAP*

The original *EcMetAP* clone was received from Dr. Brian Matthews from University of Oregon, Eugene. This clone contained a His tag and thrombin cleavage site. The latter was replaced with a TEV cleavage site by re-cloning into the pLIC-His vector provided by Dr. Stephen Bottomley of Monash University, Australia [7]. For re-cloning, the oligonucleotides 5'-CCAGGGAGCAGCCTCGATGGCTATCTCAATCAAGACC-3' and 5'-GCAAAGCACCGGCTCGTTATTCGTCGTGCGAGATTATCG-3' were used as the forward and reverse primers respectively, and the overall process was accomplished via the ligation independent cloning method as described by Cabrita et al [7]. The vector was then transformed into BL21-Gold® DE3 cells. The expression and purification of MetAP was carried out as described by Lowther et al [8] with slight modification. Luria-Bertani broth containing 0.1 mg/ml ampicillin was inoculated with a starter culture of the transformed cells and incubated at 37° C in a shaker for 4 h. The expression of *EcMetAP* was induced by 1 mM IPTG. The cells were harvested after overnight incubation and lysed by sonication in lysis buffer (50 mM HEPES, pH 8, containing 0.5 M KCl, 0.1% Triton X-100, 10% glycerol, 5 mM imidazole and 1 mM PMSF). The crude extract was loaded onto a Ni-IDA column, washed with wash buffer (lysis buffer without Triton X-100, glycerol and PMSF), and the elution of the enzyme was carried out by using a gradient of 5 – 100 mM imidazole in the above buffer. The eluted fractions of MetAP were pooled in the presence of 10 mM EDTA, and the fractions showing single bands on SDS-PAGE were pooled and concentrated. The purified MetAP was treated with His-tagged TEV in 50 mM Tris-HCl pH 8, 0.5 mM EDTA and 1 mM DTT for overnight at 4°C to remove the N-terminal His tag from the enzyme. The cleaved enzyme was re-purified by passing it through the Ni-IDA column as described above. The eluted fractions (load flow through) containing MetAP were pooled in the presence of 10 mM EDTA and dialyzed against 25 mM HEPES, pH 7.5, containing 100 mM NaCl to obtain apo-MetAP. The purity of MetAP was confirmed by SDS-PAGE, and the protein concentration was determined by the Bradford method. The

enzyme was stored at -70°C in the presence of 30% glycerol in the storage buffer (25 mM HEPES, pH 7.5, containing 100 mM NaCl).

2.2 Enzyme activity assay

The activity of various metalloforms of *EcMetAP* was measured in an assay system containing Met-7-amino-4-methylcoumarin (Met-AMC) as the fluorogenic substrate [9]. The enzyme assay was performed at RT in 50 mM HEPES buffer, pH 7.5, containing 100 mM NaCl, 400 μM substrate, and varying concentrations of metal salts (total volume = 200 μl) in 96 well plates (on a Molecular Devices Gemini EM plate reader). The enzymatic reaction was initiated by addition of appropriately diluted MetAP, and the reaction progress was monitored for 30 min at 460 nm ($\lambda_{\text{ex (excitation)}} = 360 \text{ nm}$). The slope of the linear region of the reaction trace was taken as the measure of the initial rate of the enzyme catalyzed reaction. The activation constants (K_a 's of metal ions for the enzyme were determined from the plots of the initial rates of the enzyme catalysis as a function of metal concentrations, and the data were analyzed by a complete solution of the underlying quadratic equation as described by Wang et al. [10]:

$$y = C * \left(\frac{([M] + K_d + n[E]) - \sqrt{([M] + K_d + n[E])^2 - 4n[E][L]}}{2} \right) \quad \text{Eq. 1}$$

Where y, C, M, and n represent the signal (initial rate), change in signal (maximal increase in rate at saturating concentration of the metal ions), metal ion concentration, and stoichiometry of the enzyme-metal complex, respectively. The steady-state kinetic parameters of the MetAP catalyzed reaction were determined by performing the experiment on 384 well plates with 1 μM apo-*EcMetAP*, 10 μM of different metal ions (40 μM in case of Eu^{3+}) and 5 – 600 μM substrate, in the above buffer (total volume = 75 μl). The reaction rates were calculated from the linear portions of the time dependent reaction traces and the fluorescence units were translated to the concentration of the product formed using AMC as the standard. The steady-state kinetic data were analyzed by the Michaelis-Menten equation using Origin 7.0 software.

2.3 Luminescence Spectrometry

The luminescence experiments involving Eu^{3+} were carried out on a PTI (Photon Technology International) life-time spectrofluorometer (Quantamaster), configured with both pulsed Xenon and LED as excitation sources. The Eu^{3+} luminescence spectra were acquired using the pulsed Xenon as the excitation source with $\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em (emission)}} = 525\text{--}625 \text{ nm}$, and a high bandpass cutoff filter of 340 nm. To eliminate the fluorescence signal of the protein, a 150 μs of “gate time” was used, and the signal was integrated for 2150 μs . All experiments were carried out in the assay buffer containing 3.6 μM *EcMetAP*. The binding affinity of Eu^{3+} to the enzyme was measured by titrating EuCl_3 into apo-*EcMetAP* and plotting the increase in luminescence intensity ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 614 \text{ nm}$) as a function of the metal ion concentration. A control experiment was performed in the absence of *EcMetAP*.

The binding affinities of various metal ions to *EcMetAP* were determined by titrating the enzyme- Eu^{3+} complex (3.6 μM *EcMetAP* and 50 μM EuCl_3) with different metal ions and measuring the decrease in the luminescence intensity (due to competitive displacement of Eu^{3+} from the enzyme's active site) as a function of the metal ion concentration. The data were analyzed by a modified form of the competitive binding model of Eq. 2 as described by Banerjee et al [11]:

$$L = \left(\left(\frac{L_c}{[E_t]} \right) * [E_t] - \frac{([E_t] + [M] + K_d + \left(\frac{K_d [Eu]}{K'_d} \right)) - \sqrt{([E_t] + [M] + K_d + \left(\frac{K_d [Eu]}{K'_d} \right))^2 - 4[E_t][M]}}{2} \right) + \text{offset}$$

Eq. 2

Where L_c is the total change in luminescence signal (L) upon complete displacement of Eu^{3+} from the active site, $[E_t]$, $[M]$ and $[Eu]$ are the concentrations of the enzyme, the displacing metal ion, and Eu^{3+} respectively, while K_d and K'_d are the dissociation constants of displacing metal ion and Eu^{3+} respectively.

3. Results and Discussion

Due to weaker binding of metal ions to different MetAPs, the identity of its physiological metal ion has been the subject of considerable debate in recent years [12–18]. The latter has been important since the binding affinities of inhibitors to the enzyme (as putative antibiotics) have been shown to be dependent on the nature of the metal ion present at the active site pocket of the enzyme [3,19]. Previous steady-state kinetic data for the activation of *EcMetAP* have revealed that Co^{2+} is the most potent metal ion for activating the enzyme, and the potency of activation by different metal ions conforms to the following order: $\text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+}$ [13,19]. However, although Co^{2+} serves as the most efficient metal ions for activating the enzyme, some of the most potent inhibitors identified for the Co^{2+} harbouring MetAP have been found to be ineffective antibiotic agents [19,20]. In view of the observation that Fe^{2+} substituted *EcMetAP* is inhibited (both under in vitro as well as in vivo conditions) by Fe^{2+} -selective inhibitors [21,22], it has been surmised that Fe^{2+} serves as the physiological metal ion of the enzyme.

Although there has been controversy of whether MetAP catalysis involves one [24–26] or two [27–29] metal ions at the active site pocket of the enzyme, the structural data of the Mn^{2+} -substituted *EcMetAP* clearly shows a single metal ion at the enzyme's active site pocket, and the metal ion is coordinated by one His (H171), three carboxyls (D108, E204 and E235) and a water molecule (Figure 1) [23]. It has been proposed that the coordination geometry of the metal ion is increased from five to six upon binding of the substrate and during the hydrolytic reaction. In view of this feature, added with our interest of determining the direct binding affinities of different metal ions for MetAP, we sought to identify metal ions which would serve as cofactors of the enzyme as well as provide spectroscopic signatures upon binding to the enzyme site. Fortuitously, we recognized that Eu^{3+} serves both as a cofactor of *EcMetAP* and its luminescence emission spectrum is significantly enhanced upon binding to the enzyme. Figure 2 shows the luminescence spectrum of 15 μM Eu^{3+} ($\lambda_{\text{ex}} = 295 \text{ nm}$) in the absence and presence of 15 μM *EcMetAP*.

To eliminate the contribution of the protein's fluorescence, we performed the spectral acquisition of Figure 2 using a gate time of 150 μs and the signal to noise ratio was enhanced by using the integration time of 2150 μs as well as by averaging 10 spectra. As apparent from the spectral data of Figure 2, while the emission maximum of Eu^{3+} remains practically unchanged upon binding to the enzyme site, the emission intensity is enhanced by about 300 %. The latter is presumably due to the decrease in the oscillator strength of the

solvated Eu^{3+} (in aqueous solution) upon exchange of the coordinated water molecules by the cognate groups/residues of the enzyme within the active site pocket of the enzyme [24]. By using the luminescent signal of *EcMetAP*- Eu^{3+} complex, we could titrate a fixed concentration of the enzyme (3.6 μM) by increasing concentration of Eu^{3+} ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 614 \text{ nm}$) (Figure 2 inset). The solid smooth line is the best fit of the data for the hyperbolic dependence of the increase in the luminescence signal as a function of Eu^{3+} concentration for the dissociation constant of the *EcMetAP*- Eu^{3+} complex as being equal to $25 \pm 5 \mu\text{M}$.

During a preliminary experiment, we confirmed that the unique luminescent spectral feature of the enzyme-bound Eu^{3+} is abolished by Ca^{2+} presumably due to competitive binding of these metal ions to the enzyme. This feature prompted us to determine the binding affinities of selected metal ions for *EcMetAP* by competitively displacing the enzyme bound Eu^{3+} by other metal ions. Figure 3 shows the decrease in the luminescence intensity of *EcMetAP*- Eu^{3+} complex ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 614 \text{ nm}$) upon titration with Ca^{2+} and Fe^{3+} . Due to the fact that Eu^{3+} exhibits a fairly weak affinity for *EcMetAP* (Figures 2 and 4) as compared to most other metal ions, we could analyze the overall binding isotherm by a complete solution of quadratic (rather than cubic) equation (Eq. 2) as previously described [11]. The solid smooth lines are the best fit of the data for the apparent K_d values of the enzyme- Ca^{2+} and Fe^{3+} complexes as being equal to 1.6 and 0.6 μM , respectively. Since a major fraction (about 80%) of FeCl_3 is rapidly converted into insoluble $\text{Fe}(\text{OH})_3$ in aqueous solution (see discussion below; [28–29]), the real K_d value of the *EcMetAP*- Fe^{3+} complex was recalculated to be 0.12 μM . By employing the above approach, we determined the binding affinities of other metal ions, and their K_d values are summarized in Table 1. A casual perusal of the data of Table 1 reveals that the binding affinity of metal ions decrease from Fe^{2+} to Cd^{2+} in the following order: $\text{Fe}^{2+} > \text{Co}^{2+}$, $\text{Fe}^{3+} > \text{Ni}^{2+} > \text{Cu}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} > \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Cd}^{2+}$. To the best of our knowledge, the data of Table 1 represent the first comparative binding affinity of different metal ions for *EcMetAP*, and such outcome became possible due to our discovery that Eu^{3+} serves as an efficient luminescent probe for determining the binding affinities of different metal ions to the enzyme. It should be pointed out that prior to this study, Holz and his collaborators [5,6] determined the direct binding affinities of Mn^{2+} and Co^{2+} to *EcMetAP* via isothermal titration calorimetry and found their magnitudes to be 3 and 6.4 μM , respectively [5,6]. Of these, whereas the above binding affinity of Mn^{2+} is comparable to that value obtained by the Eu^{3+} displacement method (0.7 μM ; Table 1), the binding affinity of Co^{2+} is about 30 fold higher than that reported herein (0.2 μM ; Table 1). At this stage, we offer no explanation of this discrepancy. However, we note that under our experimental condition, the activation constants (K_a value of Table-1; determined via the steady-state kinetic method) of Mn^{2+} (0.6 μM) and Co^{2+} (0.5 μM) are similar to their corresponding K_d values determined via the Eu^{3+} displacement method.

Aside from serving as a luminescent probe, we further observed that Eu^{3+} serves as a metal cofactor in activating *EcMetAP* and participating in overall catalysis. We note that this feature is similar to the activation of certain metalloenzymes by Eu^{3+} [24]. To ascertain the effectiveness of Eu^{3+} in the *EcMetAP* catalysed reaction, we performed the steady-state kinetic experiment in the presence of a fixed concentration of substrate (300 μM) and increasing concentrations of Eu^{3+} (Figure 4). The experimental data reveals that as the concentration of Eu^{3+} increases, the rate of the enzyme catalysis increases and finally attains a zero-order plateau. The analysis of the data (for the hyperbolic dependence of the reaction velocity as a function of Eu^{3+} concentration) yields the activation constant (K_a) of the enzymatic reaction as being equal to $15 \pm 5 \mu\text{M}$. Note that the K_a value derived from the steady-state experiment is comparable to the dissociation constant (K_d) of *EcMetAP*- Eu^{3+} of $25 \pm 5 \mu\text{M}$ (see Figure 2 inset), suggesting that the binding of Eu^{3+} to the enzyme and its activating role conform to a common macroscopic pathway. To ascertain the relative

effectiveness of Eu^{3+} in activating *EcMetAP* vis a vis that given by the most potent metal ion (Co^{2+}) of the enzyme, we performed a parallel experiment in the presence of increasing concentration of Co^{2+} (Figure 4) as well as other activating metal ions (data in Table 1). The analysis of the experimental data yielded the K_a value of Co^{2+} as being equal to 0.50 ± 0.25 μM . Clearly, Eu^{3+} is not as effective metal ion as Co^{2+} in promoting the enzyme catalysis. The binding affinities of Co^{2+} and Fe^{2+} as determined from enzyme activation have been reported previously as 0.3 and 0.2 μM respectively [25] and these are very similar to the values reported here. Figure 5 summarizes the relative effectiveness of different metal ions in activating the *EcMetAP* catalysed reaction under our experimental conditions. The data of Figure 5 reveals that despite their avidity for the enzyme site, several metal ions (viz., Fe^{3+} , Cu^{2+} , Mg^{2+} , Ca^{2+} , Zn^{2+} , Cd^{2+}) do not activate *EcMetAP*. Other metal ions, which activate *EcMetAP*, exhibit their effectiveness in the following order: $\text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+} > \text{Eu}^{3+}$. However, the data of Figure 5 reveals that even at sub-saturating concentration of Eu^{3+} ($K_a = 25$ μM) it is as effective in activating *EcMetAP* as Ni^{2+} , and it is only 5 fold less effective than the most potent metal ion (Co^{2+}) for the enzyme. The trend in relative activation of *EcMetAP* reported here ($\text{Co}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$) is in agreement with that reported by D'souza and Holz [13]. Unlike the previous report [13], we found that Ni^{2+} was also able to activate the enzyme, and we believe this difference is presumably due to the different types of substrate (Met-Gly-Met-Met vs MetAMC) used by Holz's and our group. We further note that although *EcMetAP* has been reported to be activated by Zn^{2+} [13], we did not observe any activation while using Met-AMC as the enzyme substrate, which is in accord with the findings from other laboratories [9,19].

We further investigated the influence of Eu^{3+} and Co^{2+} on the steady-state kinetic parameters of the enzyme for the cleavage of Met-AMC substrate. We determined the K_m for the substrate and $V_{\max}$ value of the enzyme in the presence of saturating concentrations of Eu^{3+} and Co^{2+} . The solid smooth lines are the best fit of the data for the K_m value of the substrate as being equal to 91 ± 21 and 196 ± 39 μM in the presence of Eu^{3+} and Co^{2+} respectively. Note that the K_m value of the substrate is about 2 fold lower in the presence of Eu^{3+} as compared to that obtained in the presence of Co^{2+} , suggesting that Eu^{3+} enhances the apparent binding affinity of the substrate to the enzyme. The above trend is observed even in the presence of sub-saturating concentration of Eu^{3+} (as it is often difficult to use high concentration of Eu^{3+} during the steady-state reaction due to solubility problem). We further determined the K_m value of the substrate in the presence of Ni^{2+} and Mn^{2+} and these data (along with those of Figure 6) are summarized in Table 2.

Of different metal ions utilized herein, Fe^{3+} has been known to exhibit unusual properties in aqueous solution as its salts undergo hydrolysis in a biphasic manner. The first (fast) phase involves the conversion of Fe^{3+} salts to insoluble $\text{Fe}(\text{OH})_3$, which complexes with its parent salt, and the latter undergo further hydrolysis during the second (slow) phase. The rate and extent of such hydrolysis are dependent on the salt type, concentration, temperature, pH, and other anionic components of the mixture [26–28]. To circumvent such a problem, typical biochemical experiments involving Fe^{3+} are performed in the presence of chelating agents, such as citrate or NTA [29,30]. While this approach is suitable for proteins/enzymes that bind Fe^{3+} with very high affinity, it was not applicable to *EcMetAP* due to its relatively poor binding affinity with the metal ion. Moreover, the interaction of such chelating agents with Eu^{3+} was found to significantly perturb the luminescence spectra of the lanthanide ion, further complicating the experimental set up and data analysis. Hence, the recourse was made to determine the effective concentration of Fe^{3+} , i.e., the metal ion which remained unhydrolyzed (during the fast phase) upon dilution of acidic FeCl_3 in the buffer solution, by monitoring the time dependent changes in the absorption spectra. The experimental data revealed that 20% of FeCl_3 remained unhydrolyzed (i.e. 80% hydrolysed) during the course of our experiment (data not shown), which is in agreement with the literature value [26].

With this information in hand, we recalculated the binding affinity of Fe^{3+} to *EcMetAP* from the data of Fig. 3, and found its magnitude to be $0.12 \mu\text{M}$ (noted in Table 1).

Given the information of binding affinities and catalytic efficiencies of different metal ions for *EcMetAP*, it is tempting to rationalize the physical determinants of metal ions in eliciting differential binding affinities to the enzyme as well as their activation properties. It has been interesting to note that on the basis of their binding affinities, different metal ions fall in two classes: the metal ions with $K_d < 1 \mu\text{M}$ (class I) and those with $K_d > 1 \mu\text{M}$ (class II). Of these, except for Eu^{3+} , all class II metal ions are catalytically inactive. Among class I metal ions, except for Fe^{3+} and Cu^{2+} , all exhibit catalytic activity. Although Fe^{2+} does not show maximal catalytic efficiency, it has the highest binding affinity for *EcMetAP*, and thus the Fe^{2+} -bound *EcMetAP* may be the prevalent enzyme species in the physiological system. This is consistent with the demonstration that the enzyme is potently inhibited (under in vivo condition) by the compounds which preferentially interact with Fe^{2+} [21].

The question arose why some metal ions serve as activators of *EcMetAP* but others do not, and this feature is not reflected in their relative binding affinities. In order to explicate this selectivity, we considered the role of the metal ions in the catalytic reaction of *EcMetAP*. As proposed for the mono-metalated enzyme [23], the metal ion coordination increases from 5 to 6 on binding of the substrate via the carbonyl oxygen. The metal-bound water acts as a nucleophile and it attacks at the carbonyl group of the substrate forming a tetrahedral intermediate. Thus the catalytic effectiveness of the active site metal might be envisioned to be controlled by two factors: (i) the ability of the metal ion to undergo transition from pentacoordinate to hexacoordinate geometry to allow for the binding of the substrate and (ii) the degree of Lewis acidity of the metal ion being just appropriate to allow for the bound water to serve as a strong nucleophile.

In view of our cumulative experimental data, it appears evident that both “softness” of metal ions (as described by Pearson’s HSAB theory [31]) and the coordination geometry are the major determinants of the catalytic functionality of the metal ions. Metal ions with “borderline soft” acidity and higher coordination states serve as desirable cofactors in catalysing the *EcMetAP* dependent reaction. The borderline soft acidic characteristic of metal ions facilitates the deprotonation of the bound water molecule to serve as the nucleophile toward attacking the carbonyl carbon of the substrate during catalysis. Thus despite its strong binding affinity, Fe^{3+} does not show the catalytic activity as it falls in the category of hard acid and thermodynamically stabilizes the bound hydroxide anion, and thus obviating the enzyme catalysed hydrolytic cleavage of the peptide bond. Furthermore, metal ions with higher coordination states serve as activators of the enzyme since they stabilize the putative ground and transition states during catalysis. Thus despite its strong binding affinity to the enzyme, Cu^{2+} does not exhibit catalytic activity since it prefers the “undesirable” square planar geometry within the active site pocket of the enzyme.

In view of the above features, the ligand binding and activation role of Eu^{3+} during *EcMetAP* catalysed reaction can be justified. The higher coordination geometry of Eu^{3+} is expected to promote the stabilization of both ground and transition states during the enzyme catalysis. Despite its hard acid characteristics, Eu^{3+} exhibits the potential to rapidly exchange the coordinated water molecules (two to three orders of magnitude higher than other catalytically active metal ions) [32] and provide nucleophilic hydroxide anion for attacking the carbonyl carbon of the substrate during catalysis.

However, despite their structural-functional features in promoting the *EcMetAP* catalysed reaction, we note that the binding data of different metal ions to the enzyme do not adhere to the Irving-Williams series. This is not surprising as many other metalloenzymes exhibit

similar behaviour [33–35] presumably because of strong influence of the microenvironment of the enzyme's active site pocket in discriminating the avidity of different metal ions. This is in marked contrast to the binding of metal ions to small molecular weight chelates which adheres to the Irving-Williams series [36].

4. Conclusions

In conclusion, our discovery that the luminescent intensity of Eu^{3+} is significantly enhanced upon binding to *EcMetAP* has allowed us to reliably and quantitatively determine the direct binding affinity of different metal ions to the enzyme. On comparing the metal dependent activation profile of the enzyme, it appears evident that the metal ions fall in two classes (Class I – $K_d < 1 \mu\text{M}$ and Class II – $K_d > 1 \mu\text{M}$), and except for Eu^{3+} , all Class II metal ions are catalytically inactive. Due to pronounced micro-environmental effect of the enzyme's active site pocket, the binding of metal ions to *EcMetAP* does not adhere to the Irving-Williams series. The selectivity of *EcMetAP* towards the metal ions reported in this study suggests that higher coordination states, borderline soft Lewis acidity and rapid water exchange features of the metal ions are important for catalytic activation of the enzyme.

Abbreviations

<i>EcMetAP</i>	Escherichia coli methionine aminopeptidase
TEV	Tobacco etch virus
IPTG	Isopropyl β -D-1-thiogalactopyranoside
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PMSF	Phenylmethylsulfonyl fluoride
NTA	Nitrilotriacetic acid
DTT	Dithiothreitol
Met-AMC	Methionine-7-amino-4-methyl coumarin
HSAB	Hard and soft (Lewis) acids and bases

Acknowledgments

This work was supported by the NIH grants CA113746 and CA132034 to DKS. Molecular graphics images were produced using the UCSF Chimera package (supported by the NIH grant P41 RR-01081).

References

1. Wagner, FW. Methods in Enzymology. Riordan, JF.; Vallee, BL., editors. Academic Press; 1988. p. 21-32.
2. Farkas E, Katz Y, Bhusare S, Reich R, Roschenthaler G-V, Konigsmann M, Breuer E. J. Biol. Inorg. Chem. 2004; 9:307–315. [PubMed: 14762707]
3. Schiffmann R, Heine A, Klebe G, Klein CDP. Angew. Chem. Int. Ed. Engl. 2005; 44:3620–3623. [PubMed: 15880695]
4. Haldar MK, Scott MD, Sule N, Srivastava DK, Mallik S. Bioorg. Med. Chem. Lett. 2008; 18:2373–2376. [PubMed: 18343108]
5. D'souza VM, Swierczek SI, Cospier NJ, Meng L, Ruebush S, Copik AJ, Scott RA, Holz RC. Biochemistry. 2002; 41:13096–13105. [PubMed: 12390038]
6. Copik AJ, Swierczek SI, Lowther WT, D'souza VM, Matthews BW, Holz RC. Biochemistry. 2003; 42:6283–6292. [PubMed: 12755633]
7. Cabrita L, Dai W, Bottomley S. BMC Biotechnol. 2006; 6:12. [PubMed: 16509985]

8. Lowther WT, McMillen DA, Orville AM, Matthews BW. *Proc. Natl. Acad. Sci. USA*. 1998; 95:12153–12157. [PubMed: 9770455]
9. Yang G, Kirkpatrick RB, Ho T, Zhang G-F, Liang P-H, Johanson KO, Casper DJ, Doyle ML, Marino, Thompson SK, Chen W, Tew DG, Meek TD. *Biochemistry*. 2001; 40:10645–10654. [PubMed: 11524009]
10. Wang Z-X, Ravi Kumar N, Srivastava DK. *Anal. Biochem*. 1992; 206:376–381. [PubMed: 1443609]
11. Banerjee AL, Swanson M, Roy BC, Jia X, Haldar MK, Mallik S, Srivastava DK. *J. Am. Chem. Soc.* 2004; 126:10875–10883. [PubMed: 15339172]
12. Ben-Bassat A, Bauer K, Chang SY, Myambo K, Boosman A, Chang S. *J. Bacteriol*. 1987; 169:751–757. [PubMed: 3027045]
13. D'souza VM, Holz RC. *Biochemistry*. 1999; 38:11079–11085. [PubMed: 10460163]
14. Jørgensen AT, Norrby P-O, Liljefors T. *J. Comput. Aid. Mol. Des*. 2002; 16:167–179.
15. Walker KW, Bradshaw RA. *Protein Sci*. 1998; 7:2684–2687. [PubMed: 9865965]
16. Meng L, Ruebush S, D'souza VM, Copik AJ, Tsunasawa S, Holz RC. *Biochemistry*. 2002; 41:7199–7208. [PubMed: 12044150]
17. Wang J, Sheppard GS, Lou P, Kawai M, Park C, Egan DA, Schneider A, Bouska J, Lesniewski R, Henkin J. *Biochemistry*. 2003; 42:5035–5042. [PubMed: 12718546]
18. Leopoldini M, Russo N, Toscano M. *J. Am. Chem. Soc.* 2007; 129:7776–7784. [PubMed: 17523636]
19. Li J-Y, Chen L-L, Cui Y-M, Luo Q-li, Li J, Nan F-J, Ye Q-Z. *Biochem. Biophys. Res. Commun*. 2003; 307:172–179. [PubMed: 12849997]
20. Ye Q-Z, Xie S-X, Huang M, Huang W-J, Lu J-P, Ma Z-Q. *J. Am. Chem. Soc.* 2004; 126:13940–13941. [PubMed: 15506752]
21. Wang W-L, Chai SC, Huang M, He H-Z, Hurley TD, Ye Q-Z. *J. Med. Chem*. 2008; 51:6110–6120. [PubMed: 18785729]
22. Chai SC, Wang W-L, Ye Q-Z. *J. Biol. Chem*. 2008; 283:26879–26885. [PubMed: 18669631]
23. Ye Q-Z, Xie S-X, Ma Z-Q, Huang M, Hanzlik RP. *Proc. Natl. Acad. Sci. USA*. 2006; 103:9470–9475. [PubMed: 16769889]
24. Horrocks WD, Sudnick DR. *Accounts Chem. Res*. 1981; 14:384–392.
25. D'souza VM, Bennett B, Copik AJ, Holz RC. *Biochemistry*. 2000; 39:3817–3826. [PubMed: 10736182]
26. Mellor, JW. *A Comprehensive Treatise on Inorganic and Theoretical Chemistry*, Longmans. New York: Green and Co.; 1935.
27. Lamb AB, Jacques AG. *J. Am. Chem. Soc.* 1938; 60:967–981.
28. Cotton, FA.; Murillo, CA.; Bochmann, M.; Grimes, RN. *Advanced Inorganic Chemistry*. 6th Edition, 6th ed.. Wiley-Interscience; 1999.
29. Weaver KD, Gabricević M, Anderson DS, Adhikari P, Mietzner TA, Crumbliss AL. *Biochemistry*. 2010; 49:6021–6032. [PubMed: 20496864]
30. Gabricević M, Anderson DS, Mietzner TA, Crumbliss AL. *Biochemistry*. 2004; 43:5811–5819. [PubMed: 15134455]
31. Pearson RG. *J. Am. Chem. Soc.* 1963; 85:3533–3539.
32. Caravan P, Merbach AE. *Chem. Commun*. 1997:2147–2148.
33. Ray WJ. *J. Biol. Chem*. 1969; 244:3740–3747. [PubMed: 4979769]
34. Tse P, Scopes RK, Wedd AG. *J. Am. Chem. Soc.* 1989; 111:8703–8706.
35. Williams, RJP. *A Study of Enzymes: Mechanism of Enzyme Action*. Kuby, SA., editor. CRC Press; 1991. p. 90-91.
36. Irving H, Williams RJP. *J. Chem. Soc.* 1953:3192–3210.

Figure 1. Cartoon of pentacoordinated Mn^{2+} at the active site pocket of *EcMetAP* [13] The image was generated by Chimera [14]. Note that Mn^{2+} is coordinated by one histidine, three monodentate carboxyls and one water molecule.

Figure 2.

Changes in the luminescence profile of Eu^{3+} upon binding to the apo form of *EcMetAP*. The experiments were performed at RT in 25 mM Hepes buffer at pH 7.5, containing 100 mM NaCl. The excitation wavelength and gate time were maintained at 295 nm and 2150 μs , respectively. $[\text{apo-}EcMetAP] = 15 \mu\text{M}$; $[\text{Eu}^{3+}] = 15 \mu\text{M}$. Note the luminescence spectrum of Eu^{3+} (dotted line) is significantly enhanced upon binding to *EcMetAP* (solid line). The inset shows the binding isotherm of Eu^{3+} for *EcMetAP*. The increase in the luminescence intensity ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 615 \text{ nm}$) of Eu^{3+} upon binding to the enzyme is plotted as a function of the metal ion concentration. The solid smooth curve is the best fit of the data for the K_d value of the enzyme- Eu^{3+} complex as being equal to $25 \pm 5 \mu\text{M}$.

Figure 3.

Competitive displacement of *EcMetAP*-bound Eu^{3+} by Ca^{2+} and Fe^{3+} . The changes in the luminescence intensity of *MetAP*- Eu^{3+} complex ($[\text{EcMetAP}] = 3.65$; $[\text{Eu}^{3+}] = 50 \mu\text{M}$) in the presence of increasing concentrations of Ca^{2+} (filled circle) and Fe^{3+} (open circle) are shown. Other conditions are same as in Figure 2. The solid smooth lines are the best fit of the data giving K_d values of Ca^{2+} and Fe^{3+} to be 1.6 ± 0.2 and $0.6 \pm 0.1 \mu\text{M}$, respectively. However the latter value decreases to $0.12 \mu\text{M}$ on the basis that only 20% of the FeCl_3 remains unhydrolyzed under the experimental conditions.

Figure 4.

Representative profiles for the activation of apo-*EcMetAP* by Eu^{3+} and Co^{2+} . The enzyme activity of was measured in the presence of increasing concentrations of the above metal ions (in 25 mM HEPES buffer, pH 7.5) and plotted as a function of their concentrations. [Substrate] = 300 μM , [*EcMetAP*] = 1 μM . The smooth lines represent the best fit of the data for the activation constants (K_a values) of Co^{2+} (●) and Eu^{3+} (○) being equal to 0.50 ± 0.25 μM and 15 ± 5 μM , respectively.

Figure 5.

Activation of *EcMetAP* by different metal ions. [apo-*EcMetAP*] = 1 μ M; [M^{2+}] = 10 μ M ([Eu³⁺] = 50 μ M). The activity of the enzyme (determined by the time dependent cleavage of Met-AMC) was measured over 30 min time period, and normalized with respect to the enzyme's activity containing Co²⁺ as the cofactor (taken as 100%).

Figure 6.

Steady-state kinetics for the *EcMetAP* catalyzed reaction as a function of the substrate (Met-AMC) concentration in the presence of 40 μM Eu³⁺ and 10 μM Co²⁺. The solid smooth lines represent best fit of the data for the K_m of Met-AMC and V_{max} values for the Co²⁺ and Eu³⁺ substituted enzymes as follows: Eu³⁺-MetAP (○) $K_m = 91 \pm 20$ μM; $V_{max} = 4.12 \pm 0.35$ nM/min; Co²⁺-MetAP (●) $K_m = 196 \pm 39$ μM, $V_{max} = 1.03 \pm 0.07$ nM/min.

Table 1 K_d and K_a values of various metals for MetAP.^a

Metal	K_d (μ M)	K_a (μ M)
Class I		
Mn ²⁺	0.7 $\pm$ 0.1	0.6 $\pm$ 0.4
Fe ²⁺	0.020 $\pm$ 0.004	0.4 $\pm$ 0.2 ^b
Fe ³⁺	0.12 $\pm$ 0.10 ^c	inactive
Co ²⁺	0.2 $\pm$ 0.1	0.50 $\pm$ 0.25
Ni ²⁺	0.30 $\pm$ 0.06	0.300 $\pm$ 0.004
Cu ²⁺	0.4 $\pm$ 0.2	inactive
Class II		
Mg ²⁺	2.0 $\pm$ 0.7	inactive
Ca ²⁺	1.6 $\pm$ 0.2	inactive
Zn ²⁺	1.9 $\pm$ 0.8	inactive
Cd ²⁺	3.5 $\pm$ 0.4	inactive

^a K_d and K_a values of various metals for MetAP were determined by the Eu³⁺ displacement and the kinetics of enzyme activation, respectively.

The luminescence intensity of *Ec*MetAP-bound Eu³⁺ was measured on titrating the enzyme with competing metal ions, and the dissociation constant (K_d) of the competing metal ions was determined by a modified form of the competitive binding model [*Ec*MetAP] = 3.65 μ M, [Eu³⁺] = 50 μ M. The activity of apo-*Ec*MetAP was assayed at a fixed substrate (Met-AMC) concentration as a function of metal ion concentrations added to the assay, and the activation constant (K_a) was determined by a complete solution of the underlying quadratic equation [*Ec*MetAP] = 1 μ M, [Met-AMC] = 300 μ M. All experiments were carried out at RT in 25 mM HEPES buffer at pH 7.5 containing 100 mM NaCl. Experiments involving Fe²⁺ were carried out in the presence of 2 mM TCEP and with N₂ purging to prevent oxidation.

^bThe affinity of Fe²⁺ determined by enzyme activation was significantly higher than that determined by Eu³⁺ displacement and we attribute this discrepancy to the unavoidable oxidation of Fe²⁺ during the assay in the 96 well microtiter plate.

^cThe binding affinity of Fe³⁺ was calculated using the adjusted concentration of FeCl₃, i.e. by taking into consideration that 80% of the metal salt is rapidly hydrolyzed in the aqueous solution to form insoluble Fe(OH)₃.

Table 2Metal dependent K_m for substrate and V_{max} values of *EcMetAP*.^b

Metal ion	K_m (μM)	$V_{max} \times 10^{-3}$ ($\mu\text{mol/min}$)
Co^{2+}	196 ± 39	4.12 ± 0.35
Mn^{2+}	193 ± 39	1.91 ± 0.16
Ni^{2+}	131 ± 40	2.07 ± 0.24
Eu^{3+}	91 ± 21	1.03 ± 0.07

^b The K_m and V_{max} values for various metalloforms of *EcMetAP* were determined by measuring the activity of the enzyme in the presence of different metal ions as a function of varying substrate concentrations, and analyzing the data by the Michealis-Menten equation. The enzyme activity was assayed at RT in 25 mM HEPES buffer at pH 7.5, containing 100 mM NaCl. [*EcMetAP*] = 1 μM , [metal ion] = 10 μM (except [Eu^{3+}] = 40 μM), [Met-AMC] = 5 – 600 μM .