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Probing the metal ion selectivity in methionine aminopeptidase via changes in the luminescence properties of the enzyme bound europium ion

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ABSTRACT

We report herein, for the first time, that Europium ion (Eu^{3+}) binds to the "apo" form of *Escherichia coli* methionine aminopeptidase (*Ec*MetAP), 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 that the "coordination states" and the relative softness" of metal ions are the major determinants in facilitating the *Ec*MetAP catalyzed reaction.

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 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 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 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 *Escherichia coli* and human methionine aminopeptidases [4], we sought to investigate the relative binding affinities of selected metal ions to gain insights in their mode of binding 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 *Ec*MetAP, 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-*Ec*MetAP 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 *Ec*MetAP catalyzed reaction. It should be pointed out that prior to this study, the direct binding affinities of Mn^{2+} and Co^{2+} to *Ec*MetAP have been previously determined via isothermal titration calorimetry [5,6]

2. Experimental

2.1. Cloning, expression and purification of EcMetAP

The original *Ec*MetAP 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

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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′-GCAAAGCACCGGCCTCGTTATTCGTCGTGCGAGATTATCG-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 *Ec*MetAP 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 *Ec*MetAP 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 ($\lambda_{ex\ (excitation)} = 360$ 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]:

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

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-*Ec*MetAP, 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 $\lambda_{ex} = 295$ nm, $\lambda_{em\ (emission)} = 525$–$625$ 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 *Ec*MetAP. The binding affinity of Eu^{3+} to the enzyme was measured by titrating $EuCl_3$ into apo-*Ec*MetAP and plotting the increase in luminescence intensity ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 614$ nm) as a function of the metal ion concentration. A control experiment was performed in the absence of *Ec*MetAP.

The binding affinities of various metal ions to *Ec*MetAP were determined by titrating the enzyme-Eu^{3+} complex (3.6 μM *Ec*MetAP 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]:

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

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 *Ec*MetAP 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: $Co^{2+} > Fe^{2+} > Mn^{2+} > Ni^{2+}$ [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+} harboring MetAP have been found to be ineffective antibiotic agents [19,20]. In view of the observation that Fe^{2+} substituted *Ec*MetAP 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 *Ec*MetAP 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 (Fig. 1) [23]. It