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Expression and characterization of *Mycobacterium tuberculosis* methionine aminopeptidase type 1a

Jing-Ping Lu, Qi-Zhuang Ye *

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

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ABSTRACT

Methionine aminopeptidase (MetAP) carries out the cotranslational N-terminal methionine excision and is essential for bacterial survival. *Mycobacterium tuberculosis* expresses two MetAPs, *Mt*MetAP1a and *Mt*MetAP1c, at different levels in growing and stationary phases, and both are potential targets to develop novel antitubercular therapeutics. Recombinant *Mt*MetAP1a was purified as an apoenzyme, and metal binding and activation were characterized with an activity assay using a fluorogenic substrate. Ni(II), Co(II) and Fe(II) bound tightly at micromolar concentrations, and Ni(II) was the most efficient activator for the MetAP-catalyzed substrate hydrolysis. Although the characteristics of metal binding and activation are similar to *Mt*MetAP1c we characterized before, *Mt*MetAP1a was significantly more active, and more importantly, a set of inhibitors displayed completely different inhibitory profiles on the two mycobacterial MetAPs in both potency and metalloform selectivity. The differences in catalysis and inhibition predicted the significant differences in active site structure.

Mycobacterium tuberculosis is the major pathogen for tuberculosis (TB) in human. Now, multidrug-resistant and extensively drug-resistant TB is happening at an alarming rate.[1] To overcome the drug resistance, new antibiotics with novel mechanisms of action are urgently needed. Methionine aminopeptidase (MetAP) is a ubiquitous enzyme found in both prokaryotic and eukaryotic cells and carries out an important cotranslational modification of newly synthesized proteins. Therefore, MetAP is a promising target for developing novel drugs against bacterial infection, including TB-causing drug resistance bacteria.[2]

MetAP removes N-terminal methionine from nascent proteins, which takes place in about 50–70% of proteins and is required for localization, activation and degradation.[3] The importance of this modification is underscored by the lethality of gene deletion in *Escherichia coli*,[4] *Salmonella typhimurium*,[5] and *Saccharomyces cerevisiae*.[6] Twenty genomes of mycobacteria have been sequenced, and putative MetAP proteins in each mycobacterial genome, ranging from two to four, were identified by sequence analysis. For example, *M. tuberculosis* has two MetAP genes (*mapA* and *mapB* in H37Rv genome and *map_1* and *map_2* in CDC1551 genome), and both belong to type 1 MetAP with high homology to *E. coli* MetAP (*Ec*MetAP). For most of these putative MetAPs, little is known about their biochemical properties beyond their sequences. The protein from *mapB* gene of *M. tuberculosis*, named *Mt*MetAP1c, was purified, and its structures in apoform and in complex with methionine were reported.[7] We recently further characterized this enzyme for metal binding and activation and described three X-ray structures with inhibitors bound.[8] The other MetAP (from *mapA* gene) of *M. tuberculosis*, named *Mt*MetAP1a, has not been expressed and characterized until recently.[9] Both MetAPs in *M. tuberculosis* were active as enzymes when purified, and their mRNA transcripts were analyzed and showed different levels in log phase and stationary phase.[9] *Mt*MetAP1a gene (*mapA*) expressed more in log phase, while *Mt*MetAP1c gene (*mapB*) showed a higher level in stationary phase. It was concluded that the two MetAPs may perform important function in different growth phases of *M. tuberculosis*.[9] The special characteristics of mycobacterial life cycle may require more than one MetAP enzyme to carry out the important cotranslational modification.

MetAP belongs to the family of dinuclear metallohydrolases,[10,11] and divalent metal ions play a direct role in the hydrolysis catalyzed by MetAP. When purified as an apoenzyme, *Ec*MetAP can be activated by several divalent metals, including Co(II), Ni(II), Mn(II) and Fe(II).[12,13] Initially, MetAP was believed to be a Co(II) enzyme, because Co(II) was among the best activators, and early MetAP structures all contained two Co(II) ions at the active site.[14] Most of the currently known MetAP inhibitors were discovered and characterized with MetAP in the Co(II)-form. However, it is puzzling that many current small molecule inhibitors of bacterial MetAPs with high potency on purified enzymes failed to show

Abbreviations: MetAP, methionine aminopeptidase; *Ec*MetAP, *Escherichia coli* methionine aminopeptidase; *Mt*MetAP1a, *Mycobacterium tuberculosis* methionine aminopeptidase type 1a; *Mt*MetAP1c, *M. tuberculosis* methionine aminopeptidase type 1c; Met-AMC, methionyl aminomethylcoumarin.

* Corresponding author. Tel.: +1 317 278 0304; fax: +1 317 274 4686.
E-mail address: yeq@iupui.edu (Q.-Z. Ye).

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any significant antibacterial activities.[15–17] We confirmed that inhibitors of the Co(II)-form may not inhibit other metalloforms of the same MetAP.[13,18] There are many reasons that an in vitro active compound may be inactive in vivo, such as absorption or metabolism. However, one explanation for the lack of antibacterial activities is a disparity between the metalloform tested in vitro using a purified enzyme and the one that exists in cells. It is apparent that MetAP inhibitors have to effectively inhibit the cellular MetAP to be therapeutically useful. We have developed several sets of metalloform-selective MetAP inhibitors based on high throughput screening hits,[18,19] and these inhibitors can inhibit either Co(II)-, Mn(II)- or Fe(II)-form of *Ec*MetAP with both high potency and selectivity. We used these metalloform-selective inhibitors to characterize inhibition of purified *Ec*MetAP in vitro, inhibition of the same enzyme in *E. coli* cells, and inhibition of bacterial cell growth.[20] The fact that only the Fe(II)-form selective inhibitors showed antibacterial activity on several *E. coli* and *Bacillus* strains led us to conclude that Fe(II) is the likely metal used by MetAP in *E. coli* and other bacterial cells.[20]

Characterization of the catalysis and inhibition of mycobacterial MetAPs is a necessary step in their inhibitor discovery and development. Recent studies by Zhang et al.[9] on *Mt*MetAP1a and *Mt*MetAP1c used recombinant enzymes from *E. coli*, presumably with metal ions already incorporated at the active site because no metal removal step was described. We purified *Mt*MetAP1c as an apoenzyme and characterized its metal binding and activation,[8] and we noticed significant differences in metal requirements for catalysis between the *Mt*MetAP1c apoenzyme we had and the metalated enzyme reported.[9] Now, we independently expressed *Mt*MetAP1a in *E. coli* and purified it to homogeneity as an apoenzyme. Its activation by different divalent metal ions was carefully investigated, and characteristics of the activation were compared with those reported by Zhang et al.[9] and those we reported earlier for *Mt*MetAP1c.[8] Finally, a set of MetAP inhibitors were evaluated and compared on the two mycobacterial MetAPs in different metalloforms.

Compared with *Mt*MetAP1c we expressed before,[8] *Mt*MetAP1a showed a much lower solubility when we expressed it in the same way without a His-tag, and most of the protein was not soluble. To potentially improve solubility and purification, we cloned it into pET28a plasmid, so that the protein was expressed with a His-tag at the N-terminus.[21] Still, a large portion of the protein went into insoluble fractions, but the protein was easily purified through a metal affinity column. The protein was treated with 1,10-phenathronine as a chelator to remove metal ions. Although it was not analyzed for metal content, the prepared apoenzyme showed no activity in hydrolyzing the fluorogenic substrate Met-AMC until metal was added in the assay mixture. The yield was around 2 mg per litre of culture. Considering that the His-tag can potentially affect metal binding in our metal activation experiments, we prepared a small amount of apoprotein with the His-tag removed by thrombin treatment. No significant difference in activity was observed between the apoenzymes with or without the His-tag. Therefore, the apoenzyme with the His-tag attached was used in the following experiments.

Activity of MetAP can be continuously monitored by fluorescence from the released aminomethylcoumarin during hydrolysis of the fluorogenic substrate methionyl aminomethylcoumarin (Met-AMC).[8] The metal activation was instant, and the fluorescence increased linearly for at least 15 min once the apoenzyme was mixed with the metal. When the apoenzyme concentration was held constant at 50 nM and increasing concentrations of metal were added for activation, Ni(II) was the best activator, followed by Co(II) (Fig. 1). Both Fe(II) and Mn(II) activated *Mt*MetAP1a reproducibly, while no activation was observed for Zn(II). These are very similar to the observations we reported for activation of apo-*Mt*MetAP1c by different metals[8] but are significantly different from those reported by Zhang et al.,[9] in which Co(II) was an activator, Mn(II) had no effect, and Ni(II) and Fe(II) showed inhibition instead of activation. The discrepancies are likely resulted from the proteins used because we characterized *Mt*MetAP1a and *Mt*MetAP1c in their apoforms, while Zhang and colleagues used proteins with metal ions likely already incorporated. It is clear from the bell-shaped activation curves, high concentrations of a metal ion often inhibited the enzyme activity, and therefore, inhibition could be observed for an activator when a metalated MetAP is used.

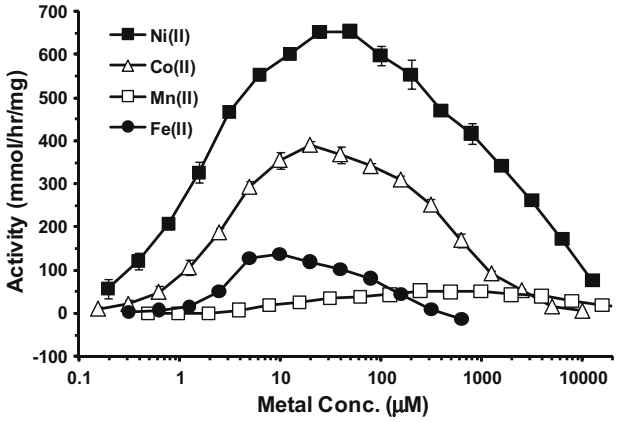


Figure 1. Activation of *Mt*MetAP1a apoenzyme by divalent metals.

To understand the metal activation better, we determined kinetic parameters of metal binding and substrate hydrolysis. Holding amounts of the *Mt*MetAP1a apoenzyme and the substrate Met-AMC constant, we calculated the affinity (K_d) for each of the activating metals by fitting a model of multiple independent binding sites.[22] It is apparent that Fe(II), Ni(II) and Co(II) all bound tightly with low micromolar K_d values, while the binding of Mn(II) was 5–10 times weaker (Table 1). At the optimal activating metal concentrations (10 μM $FeCl_2$, 20 μM $CoCl_2$, 200 μM $MnCl_2$, or 20 μM $NiCl_2$), Michaelis–Menten constants were calculated for the substrate hydrolysis. Interestingly, Ni(II)-activated *Mt*MetAP1a was the most efficient among the metalloforms tested in catalyzing the hydrolysis, with the lowest K_m and the fastest k_{cat}, consistent with the metal titration curves (Fig. 1). Co(II) was the next in the efficiency, Fe(II) followed and Mn(II) was the least efficient. It is important to note that although this order of activation is similar between *Mt*MetAP1a and *Mt*MetAP1c, *Mt*MetAP1a is a much more efficient enzyme. Previously, we showed that although Ni(II) was the best activator for *Mt*MetAP1c in vitro, *Mt*MetAP1c functioned as a Fe(II)-enzyme in an *E. coli* cellular environment.[8] It will be interesting to know which metal *Mt*MetAP1a uses in *E. coli* cells, or ultimately in *M. tuberculosis* cells. Effective inhibition of the cellular MetAP in its physiologically relevant metalloform is essential for developing MetAP inhibitors as antibacterial therapeutics.

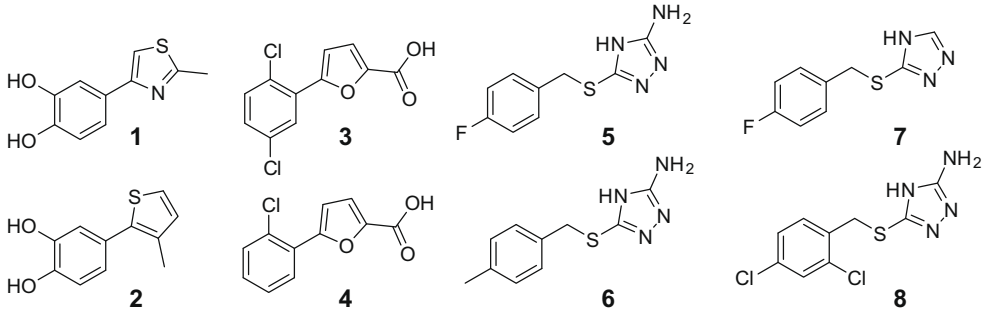
Assignment of the physiologically relevant metalloform has been difficult for a metalloenzyme, and we have developed a novel approach for the assignment using the metalloform-selective

Table 1
Binding and activation of *Mt*MetAP1a by different metals[a]

	Fe(II)	Ni(II)	Co(II)	Mn(II)
K_d (μM)	1.56	2.31	1.87	12.6
K_m (μM)	161	11	82	170
k_{cat} (s^{-1})	0.14	0.45	0.36	0.07
k_{cat}/K_m (M^{-1} s^{-1})	870	40,909	4390	412

[a] K_d is the dissociation constant. K_m and k_{cat} are the Michaelis–Menten constants.

Table 2
Inhibition of enzymatic activities of purified *Mt*MetAP1a in comparison with *Mt*MetAP1c[a]



Compd	*Mt*MetAP1a[b]				*Mt*MetAP1c[c]			
	Fe(II)	Ni(II)	Co(II)	Mn(II)	Fe(II)	Ni(II)	Co(II)	Mn(II)
1	0.19	0.70	0.031	0.11	3.6	104	76	37
2	0.07	2.3	0.53	0.10	1.4	60	38	14
3	>250	>250	>250	>250	>500	>500	>500	14
4	>250	172	20	104	>500	>500	>500	16
5	>250	>250	>250	>250	>500	1.3	0.74	18
6	>250	>250	>250	>250	>500	2.5	0.69	26
7	>250	>250	>250	>250	>500	0.58	2.0	143
8	>250	>250	205	>250	40	0.24	0.26	2.0

[a] IC_{50} values are expressed in μM.
[b] Purified *Mt*MetAP1a enzymes were reconstituted by activating the apoenzyme with different divalent cations [Fe(II), 10 μM; Co(II) and Ni(II), 20 μM; Mn(II), 200 μM].
[c] The IC_{50} values for *Mt*MetAP1c were reported previously in Ref. 8.

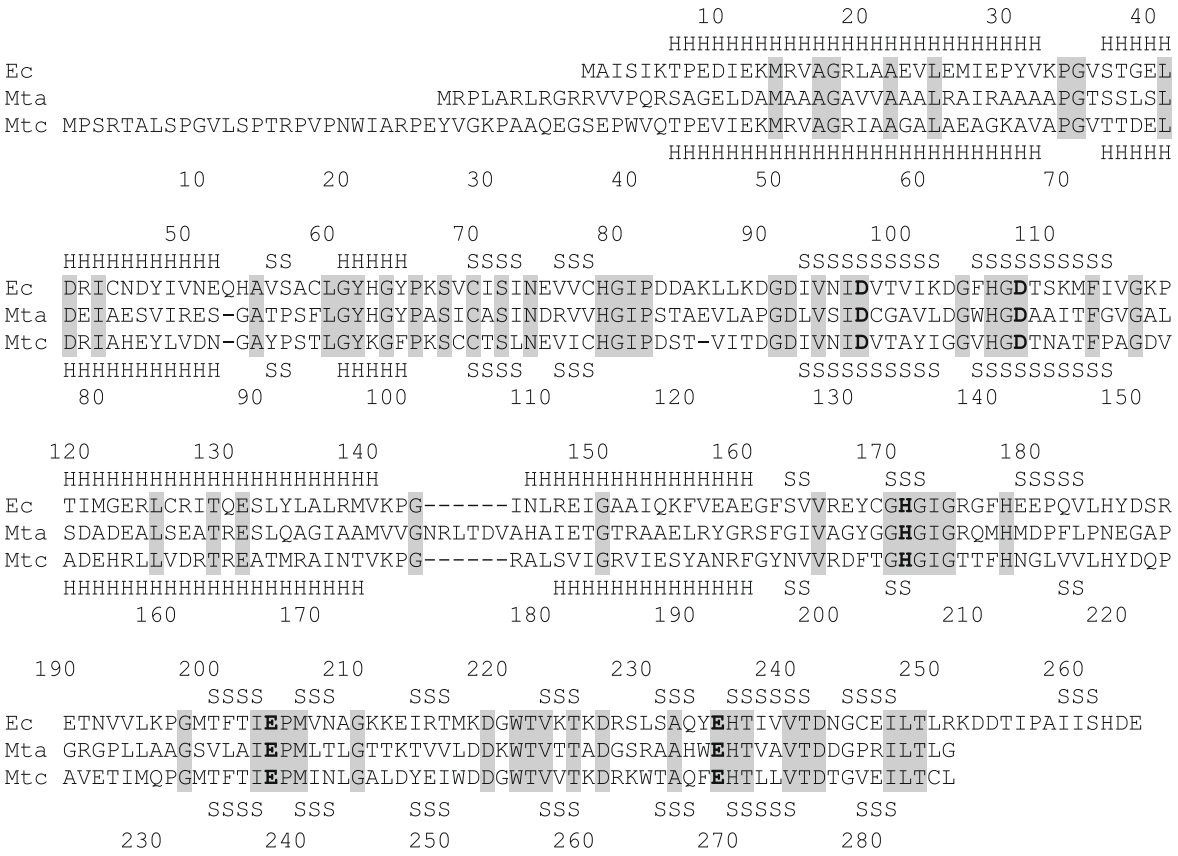


Figure 2. Structure-based sequence alignment of *Ec*MetAP, *Mt*MetAP1a and *Mt*MetAP1c (labelled as Ec, Mta and Mtc, respectively). The numbering and secondary structures of *Ec*MetAP are above the sequences, and the numbering and the secondary structures for *Mt*MetAP1c are below the sequences. 'H' stands for α-helices and 'S' stands for β-sheets. The secondary structures of *Ec*MetAP and *Mt*MetAP1c are from the coordinates of 1XNZ and 3IU7, respectively. The identical residues among all three MetAPs are highlighted by shading in grey. The five conserved metal-ligating residues are shown in bold face.

inhibitors that can distinguish different metals at the active site.[18,20] In our previous report on characterization of *Mt*MetAP1c, we tested a set of MetAP inhibitors for inhibition of different metalloforms.[8] For comparison, we tested the same inhibitors for metalloform-selective inhibition on *Mt*MetAp1a (Table 2). These inhibitors were initially identified as metalloform-selective inhibitors of *Ec*MetAP; **1** and **2** were Fe(II)-form selective,[19] **3** and **4** were Mn(II)-form selective,[18] and **5**–**7** were Co(II)- and Ni(II)-form selective.[8,18] Their metalloform selectivity was maintained on *Mt*MetAP1c.[8] However, when they were tested on *Mt*MetAP1a, **1** and **2** showed potent inhibition of all four metalloforms, and their metalloform selectivity was lost (Table 2). It was more surprising that **3**–**8** showed almost no activity, while they inhibited *Mt*MetAP1c with metalloform selectivity as expected. The differences in inhibition foretell the significant differences in structures, especially at the active site, between *Mt*MetAP1a and *Mt*MetAP1c.

*Mt*MetAP1a and *Mt*MetAP1c are highly homologous in sequence, and many active site residues are conserved (Fig. 2). For example, five residues for coordinating with two metal ions (D97, D108, H171, E204, E235 in *Ec*MetAP, and D131, D142, H205, E238, and E269 in *Mt*MetAP1c) are conserved. Two histidines, H79 and H178 in *Ec*MetAP (H114 and H212 in *Mt*MetAP1c), that were implicated in catalysis[23,24] were also conserved. The X-ray structure of *Mt*MetAP1a is not available, but the sequence alignment based on structures of *Ec*MetAP[18] and *Mt*MetAP1c[8] revealed possible structural differences, especially at the N-terminus and loop structures. It is needed to have the structures of *Mt*MetAP1a to explain the remarkable differences in inhibition of *Mt*MetAP1a by these inhibitors both in potency and metalloform selectivity.

Most bacteria have only a single essential MetAP gene, and deletion of the gene led to lethality.[4,5] *M. tuberculosis* has two MetAP genes, and they were shown to express at different levels during growing and stationary phases.[9] It is likely that both are required during the mycobacterial life cycle, and inhibition of one or both is needed for antitubercular therapy. We have previously characterized the catalysis and inhibition of *Mt*MetAP1c,[8] and now we carried out the similar experiments for *Mt*MetAP1a and compared the two *M. tuberculosis* MetAPs for differences in activation by metal ions and inhibition by small molecule inhibitors. For both *Mt*MetAP1a and *Mt*MetAP1c, the activation follow the similar order: Ni(II) > Co(II) > Fe(II) ≈ Mn(II). We showed that although Ni(II) was the best activator for *Mt*MetAP1c, Fe(II) was the metal used by *Mt*MetAP1c in an *E. coli* cellular environment. However, it is critical to clarify which metal is used by *Mt*MetAP1a and *Mt*MetAP1c in *M. tuberculosis* cells. Effectively inhibition of the mycobacterial MetAP enzymes in their native environment is the key to discover and develop MetAP inhibitors as antitubercular agents. It is interesting to note that although the two mycobacterial MetAPs are homologous in sequence, the different inhibitory profiles of the same set of inhibitors predicted the significant differences in active site structure. We are in the process of elucidating the structural differences between the two enzymes and discovering effective inhibitors as leads for novel antitubercular drugs.

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