Bioorganic & Medicinal Chemistry Letters 21 (2011) 3395–3398

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Two methionine aminopeptidases from *Acinetobacter baumannii* are functional enzymes

Hai Yuan [a], Sergio C. Chai [a], Christopher K. Lam [b], H. Howard Xu [b], Qi-Zhuang Ye [a,*]

[a] Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[b] Department of Biological Sciences, California State University, Los Angeles, CA 90032, USA

ARTICLE INFO

Article history:
Received 27 February 2011
Revised 30 March 2011
Accepted 31 March 2011
Available online 7 April 2011

Keywords:
Metalloenzyme
Drug resistance
Gram-negative bacteria
Drug discovery
Antibiotics
Metal activation

ABSTRACT

Drug resistance in Gram-negative bacteria, such as *Acinetobacter baumannii*, is emerging as a significant healthcare problem. New antibiotics with a novel mechanism of action are urgently needed to overcome the drug resistance. Methionine aminopeptidase (MetAP) carries out an essential cotranslational methionine excision in many bacteria and is a potential target to develop such novel antibiotics. Two putative MetAP genes were identified in *A. baumannii* genome, but whether they actually function as MetAP enzymes was not known. Therefore, we established an efficient *E. coli* expression system for their production as soluble and metal-free proteins for biochemical characterization. We demonstrated that both could carry out the metal-dependent catalysis and could be activated by divalent metal ions with the order Fe(II) ≈ Ni(II) > Co(II) > Mn(II) for both. By using a set of metalloform-selective inhibitors discovered on other MetAP enzymes, potency and metalloform selectivity on the *A. baumannii* MetAP proteins were observed. The similarity of their catalysis and inhibition to other MetAP enzymes confirmed that both may function as competent MetAP enzymes in *A. baumannii* and either or both may serve as the potential drug target.

Acinetobacter baumannii is an aerobic Gram-negative bacillus, which exists ubiquitously in nature (soil, water and animals).[1] It has emerged globally as one of the most troublesome pathogens, especially over the last 20 years.[2,3] The bacterium can infect various parts of the body including lung, urinary tract and bloodstream and cause both community and nosocomial infections.[4] *A. baumannii* infections have also been identified in American soldiers injured in Afghanistan and the Iraq/Kuwait region.[5] The significant capacity of this bacterium to survive in hospital environment for a long period makes the infection even more problematic, because the transmission rate between patients is high.[4] Treatment of infection caused by this bacterium is often difficult, because of the widespread resistance of this bacterium to most classes of antibiotics in use. Only a few 'abandoned' antibiotics, such as colistin and polymyxin B, are effective, but they can also cause severe side-effects. New drugs to treat this deadly infection with a novel mechanism of action are in urgent need to overcome the drug resistance.[3]

Methionine aminopeptidase (MetAP) is ubiquitously found in both prokaryotic and eukaryotic cells.[6] It is responsible for removing the N-terminal methionine from 50–70% of nascent proteins, which is required for localization, activation and degradation.[6] There are two subtypes of MetAP (type 1 and type 2). Most bacteria and achaea have a single MetAP gene (either type 1 or 2), while eukaryotic cells express both type 1 and type 2 MetAP enzymes. Lethal deletion of this gene has been shown in *Escherichia coli*[7] and *Salmonella typhimurium*.[8] Therefore, MetAP is a potential target for developing novel antibiotics. We demonstrated that inhibitors of *E. coli* MetAP (*Ec*MetAP) displayed antibacterial activity on *E. coli*, *Bacillus megaterium*, and *Bacillus subtilis*.[9–11] Liu and colleagues also reported that inhibitors of *Mycobacterium tuberculosis* MetAPs displayed antitubercular activity,[12] and inhibitors of *Plasmodium falciparum* MetAPs displayed antimalarial activity.[13,14]

Multiple putative MetAP genes have been identified in a small number of bacteria, when their genomic sequences became known. For example, in genome of *A. baumannii* ATCC 17978,[15] two genes for putative *A. baumannii* MetAP (*Ab*MetAP) were annotated. To investigate whether they code for functional MetAP enzymes and whether the MetAPs can serve as drug targets, we cloned and expressed the two MetAP proteins from *A. baumannii* ATCC 17978 in *E. coli* and named them as *Ab*MetAPx (gene locus A1S_1940) and *Ab*MetAPy (gene locus A1S_2324). All bacterial MetAPs, such as *Ec*MetAP, belong to type 1 MetAP that contains only a catalytic domain. Both *Ab*MetAPx (264 residues) and *Ab*MetAPy (275 residues) are either identical to or slightly longer than *Ec*MetAP (264

Abbreviations: MetAP, methionine aminopeptidase; *Ab*MetAP, *A. baumannii* methionine aminopeptidase; *Ec*MetAP, *E. coli* methionine aminopeptidase; 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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doi:10.1016/j.bmcl.2011.03.116

residues) in length, and when the three sequences were aligned, high sequence homology was observed, with 119 identical residues (Fig. 1). Importantly, all five residues (D97, D108, H171, E204, and E235 in *Ec*MetAP) for ligation to two catalytic metal ions[16] are conserved in both *Ab*MetAPs, as well as the catalytically critical residues C70,[17] H79,[18] and H178[19] in *Ec*MetAP. *Ab*MetAPy is longer than the other two by nine residues, and an insert of six residues (GRPAFQ) is possibly located in a loop region (between P66 and K67 in *Ec*MetAP), according to the structure-based sequence alignment. The existence of every key structural element for structure and function in the two *Ab*MetAP sequences suggests that both may be functional MetAP enzymes.

The genes for *Ab*MetAPx and *Ab*MetAPy were amplified by PCR, using primers designed with ligation independent cloning (LIC) specific 5′ overhangs (EMD, Gibbstown, NJ) and according to the gene sequences in *A. baumannii* ATCC 17978 genome (795 nucleotides and 828 nucleotides, respectively). The PCR fragments were cloned into pET-30Ek/LIC vector using LIC procedures with a His-tag at the N-terminus. The recombinant plasmids were transformed into *E. coli* BL21(DE3) strain for an IPTG-inducible expression. For purification of *Ab*MetAPx, the *E. coli* strain was cultured in LB media, containing kanamycin, and expression of recombinant *Ab*MetAPx was induced by adding IPTG. The harvested cells were lysed by passing through a French press. The supernatant of the lysate was separated on an HisTrap column. The protein was treated with Chelex-100 resin to remove divalent metal ions and concentrated to 33.5 mg/mL. *Ab*MetAPy was prepared in a similar manner, except that the Chelex 100 resin treatment was replaced by EDTA. The purified *Ab*MetAPy was concentrated to 11.0 mg/mL. The constructed *E. coli* expression systems produced both *Ab*MetAP proteins in high yield in soluble form (65 mg *Ab*MetAPx or 45 mg *Ab*MetAPy per 500 mL of *E. coli* cell culture), and both were purified as metal-free preparations. Although the proteins were not analyzed for metal content after treatment with either Chelex-100 (for *Ab*MetAPx) or EDTA (for *Ab*MetAPy), *Ab*MetAPx showed only very weak residual activity, and *Ab*MetAPy showed no detectable activity, indicating most of the metal ions were removed.

All MetAPs studied so far require divalent metal ions for catalysis as purified enzymes,[16] but the actual metal ion used by MetAP in cells is largely unknown.[9,20] We have previously shown the importance of inhibiting the MetAP enzyme in its native metalloform for antibacterial activity[9] and discovered MetAP inhibitors that can distinguish different metals at the enzyme active site (i.e., metalloform-selective inhibitors).[10,21] Therefore, we used the two metal-free *Ab*MetAPs to study their activation by divalent metal ions and inhibition by a set of metalloform-selective inhibitors.

Metal ions directly participate in MetAP catalysis,[22] and *Ec*MetAP can be activated by Co(II), Mn(II), Fe(II), Ni(II) and Zn(II).[23–25] To test activation of the *Ab*MetAP proteins by different divalent metal ions, we used a fluorescence assay with the fluorogenic methionyl aminomethylcoumarin (Met-AMC) as the substrate.[26] The formation of 7-amino-4-methylcoumarin (AMC) was monitored continuously for 30 min at room temperature by fluorescence (λ_{ex} = 360 nm, λ_{em} = 460 nm). While no activation was observed for Zn(II) in this assay, Co(II), Mn(II), Fe(II) and Ni(II) were found to activate both *Ab*MetAPs. The metal activation was instant, and the fluorescence increased linearly for at least 15 min once the metal-free proteins were mixed with the metals. Activity of *Ab*MetAPx or *Ab*MetAPy (both at 1 μM) increased along with increasing amounts of metals until peak values were reached. Inhibition of the enzymatic activity by excessive metal ions at high concentrations was observed, which has been observed in other MetAP enzymes as well.[25,27,28] For *Ab*MetAPx, Ni(II) and Fe(II) were the best activators (Fig. 2A). Both Co(II) and Mn(II) showed much weaker but detectable activation. The metal activation profile of *Ab*MetAPy was similar, except that Fe(II) was found to be the best, and the activation by Fe(II) required much lower metal concentration than that by Ni(II) (Fig. 2B). Using the observable activity as an indication of metal binding to the *Ab*MetAP proteins, we determined the affinity of the activating metals [Co(II), Mn(II), Fe(II) and Ni(II)] to either *Ab*MetAPx or *Ab*MetAPy (Table 1), by monitoring the activity with increasing amounts of the metal, and calculated the K_d values.[29] The affinities distributed within a narrow range of 0.14 μM–1.84 μM, in a good agreement with reported affinities for *Ec*MetAP [0.3 μM, 0.2 μM and 6 μM for Co(II), Fe(II) and Mn(II), respectively].[30,31] The K_d value for Ni(II) binding to *Ab*MetAPy at 1.84 μM is significantly higher than other K_d values for the two *Ab*MetAP proteins, and the weak binding is consistent with the shift of Ni(II) activation profile to higher metal concentrations for *Ab*MetAPy (Fig. 2B).

Conversion of Met-AMC to fluorescent AMC is easily monitored kinetically by fluorescence, and we used this fluorescence assay to investigate the catalytic efficiencies of *Ab*MetAPx and *Ab*MetAPy, when they were activated by Co(II), Mn(II), Fe(II), and Ni(II). The kinetic constants k_{cat} and K_m were determined at the optimal concentration of activating metals (for *Ab*MetAPx: 2.5 μM of $FeCl_2$, 5 μM of $NiCl_2$, 20 μM of $CoCl_2$, 20 μM of $MnCl_2$; for *Ab*MetAPy: 2.5 μM of $FeCl_2$, 20 μM of $NiCl_2$, 20 μM of $CoCl_2$, 20 μM of $MnCl_2$) (Table 1). For *Ab*MetAPx, the Ni(II)-form was the most efficient metalloform, followed by the Fe(II)-form. The Co(II) and Mn(II) forms were relatively less efficient. The efficiencies of different metalloforms of *Ab*MetAPy are similar to those of *Ab*MetAPx, except that the Fe(II)-form is noticeably more efficient than the Ni(II)-form. These parameters are consistent with the metal titration curves (Fig. 2).

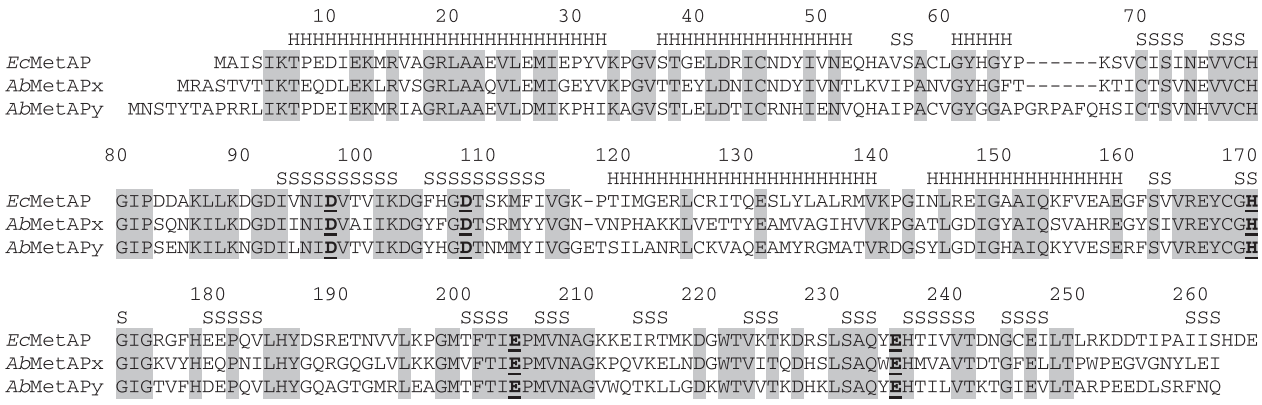


Figure 1. Sequence alignment between *Ec*MetAP, *Ab*MetAPx and *Ab*MetAPy. The secondary structures (H, helix; S, sheet) are based on our *Ec*MetAP structure (1XNZ)[21] and shown above the sequences. Identical residues are shaded, and the five conserved metal-coordinating residues are in bold face and underlined.

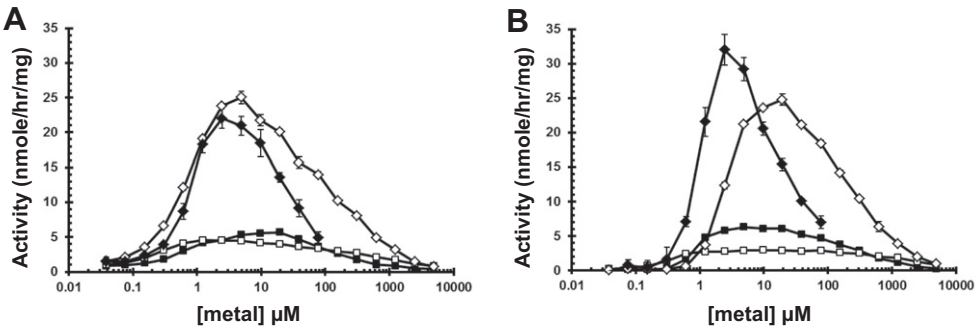


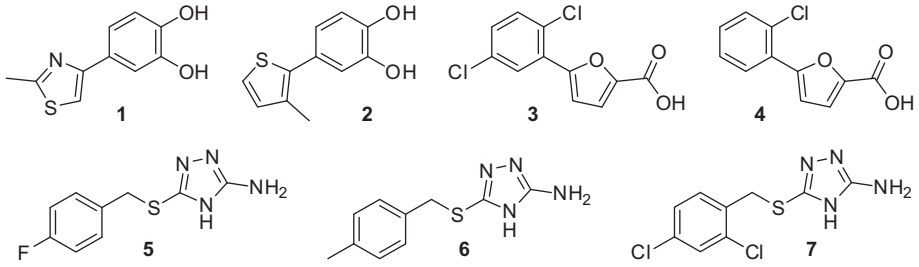
Figure 2. Activation of *Ab*MetAPx (A) and *Ab*MetAPy (B) by divalent metals. Co(II), closed squares; Mn(II), open squares; Fe(II), closed diamonds; and Ni(II), open diamonds.

Table 1
Binding and kinetic parameters of *Ab*MetAPx and *Ab*MetAPy[a]

	*Ab*MetAPx				*Ab*MetAPy			
	Co(II)	Mn(II)	Fe(II)	Ni(II)	Co(II)	Mn(II)	Fe(II)	Ni(II)
K_d, μM	0.45	0.57	0.14	0.26	0.39	0.33	0.54	1.84
K_m, μM	168	154	155	111	130	115	161	144
k_{cat}, $s^{-1} \times 10^4$	1.59	0.508	2.63	3.05	1.37	0.550	4.49	3.12
k_{cat}/K_m, $M^{-1}s^{-1}$	0.948	0.330	1.70	2.75	1.05	0.479	2.78	2.17

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

Table 2
Inhibition of *Ab*MetAPx and *Ab*MetAPy by metalloform-selective inhibitors[a,b]



Compounds	*Ab*MetAPx				*Ab*MetAPy			
	Co(II)	Mn(II)	Fe(II)	Ni(II)	Co(II)	Mn(II)	Fe(II)	Ni(II)
1	39	79	32	72	24	34	0.95	56
2	33	22	0.65	27	21	30	0.51	55
3	494	7.1	>500	251	>500	6.1	355	286
4	394	13	358	188	381	0.92	288	186
5	0.88	303	>500	23	0.64	34	>500	2.2
6	2.1	145	>500	44	3.8	103	>500	4.4
7	0.78	64	128	4.9	0.38	14	94	0.40

[a] IC_{50} values are expressed in μM.
[b] Purified metal-free *Ab*MetAP proteins were activated by the metals at the optimal activation concentration [for *Ab*MetAPx: 2.5 μM Fe(II), 5 μM Ni(II), 20 μM Co(II) or Mn(II); for *Ab*MetAPy: 2.5 μM Fe(II); 20 μM Co(II), Ni(II) or Mn(II)].

To inhibit the growth of *A. baumannii*, it is important to know the native metal used by *Ab*MetAPs in cells and to effectively inhibit *Ab*MetAPs in their physiologically relevant metalloform. We have discovered unique metalloform-selective inhibitors of *Ec*MetAP[10,21] and a *M. tuberculosis* MetAP,[28] and we selected a few of them and tested them on the newly purified *Ab*MetAPs for metalloform-selective inhibition. Based on our previous studies, the catechol compounds **1** and **2** were the Fe(II)-form selective, **3** and **4** were the Mn(II)-form selective, and **5**–**7** were the Co(II)- and Ni(II)-forms selective. When tested against *Ab*MetAPs, the test compounds showed both good potency on some of the metalloforms and similar metalloform selectivity as we have observed before (Table 2).[10,21,28] The metalloform-selective inhibition by these inhibitors further confirms the structural and functional similarity of the two *Ab*MetAPs to other known MetAP enzymes. The similarity of their catalysis and inhibition to other MetAP enzymes confirms that both may function as competent MetAP enzymes in *A. baumannii*.

MetAP exists in every cell. *Mycoplasma genitalium* has one of the smallest genomes with only 521 genes, and MetAP gene is one of its 382 essential genes.[32] The recently synthesized 1.08 Mbp *Mycoplasma mycoides* JCVI-syn1.0 genome has the MetAP gene.[33] The ubiquitous presence of this enzyme, coupled with the essential nature revealed in the gene deletion studies,[7,8,34] suggests that MetAP is a potential target for discovering broad-spectrum antibacterial agents, and its novelty as a drug target may help to develop antibiotics that can overcome the current problematic drug-resistance in bacteria.

Most bacteria have only a single MetAP, but *A. baumannii* is among a small number of bacteria that have multiple MetAP genes. Two homologous MetAP isozymes in *B. subtilis* were isolated and investigated.[35] Although both showed enzymatic activity, only one of them was shown to be essential for growth, and the other one was concluded as non-essential for reason of low expression.[35] Protozoan parasite *Plasmodium falciparum* has four MetAP proteins, and inhibitors were discovered and characterized on one of the four and showed antimalarial activity.[13] Therefore, although both *Ab*MetAPx and *Ab*MetAPy are competent MetAP enzymes, their relative biological significance in *A. baumannii* remains to be elucidated, and either or both may serve as the drug target.

Bacterial MetAPs, including *Ab*MetAPx and A*b*MetAPy, are also homologous to human type 1 MetAP, and to a lesser extent, to human type 2 MetAP. Because of the significant role of human MetAPs in cell proliferation and angiogenesis,[36,37] potentially, antibacterial MetAP inhibitors may exhibit toxicity due to inhibition of human MetAPs. However, the redundant human MetAPs have overlapping substrate specificity,[38] which may alleviate some of the potential toxicity of *Ab*MetAP inhibitors. In addition, *Ec*MetAP was found to use Fe(II) as the native cofactor,[9] and other bacterial MetAPs may use Fe(II), too, due to its availability. On the other hand, Fe(II) is tightly regulated in mammalian cellular environment. The ability for human hosts to sequester iron is a defense mechanism against bacterial infection, and the amount of free iron (10^{-18} M) is well below the levels required to support the growth of most bacteria (10^{-6}–10^{-7} M).[39,40] It was reported that human type 2 MetAP uses Mn(II) as its physiological metal,[20] and the native metal for human type 1 MetAP is currently unknown but could be Mn(II) due to the low Fe(II) abundance. Therefore, our metalloform-selective inhibitors, especially the Fe(II)-form selective inhibitors, could be the promising leads with such selectivity for bacterial MetAPs.

Acknowledgements

This work was supported by National Institutes of Health Grants R01 AI065898 and R56 AI065898 (to Q.-Z.Y.), and SC3 GM083686 (to H.H.X.). Additional support was from Indiana University School of Medicine (BRG) and Indiana University and Purdue University at Indianapolis (RSFG).

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