

Catalysis and Inhibition of *Mycobacterium tuberculosis* Methionine Aminopeptidase[†]

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Methionine aminopeptidase (MetAP) carries out an important cotranslational N-terminal methionine excision of nascent proteins and represents a potential target to develop antibacterial and antitubercular drugs. We cloned one of the two MetAPs in *Mycobacterium tuberculosis* (*MtMetAP1c* from the *mapB* gene) and purified it to homogeneity as an apoenzyme. Its activity required a divalent metal ion, and Co(II), Ni(II), Mn(II), and Fe(II) were among activators of the enzyme. Co(II) and Fe(II) had the tightest binding, while Ni(II) was the most efficient cofactor for the catalysis. *MtMetAP1c* was also functional in *E. coli* cells because a plasmid-expressed *MtMetAP1c* complemented the essential function of MetAP in *E. coli* and supported the cell growth. A set of potent *MtMetAP1c* inhibitors were identified, and they showed high selectivity toward the Fe(II)-form, the Mn(II)-form, or the Co(II) and Ni(II) forms of the enzyme, respectively. These metalloform selective inhibitors were used to assign the metalloform of the cellular *MtMetAP1c*. The fact that only the Fe(II)-form selective inhibitors inhibited the cellular *MtMetAP1c* activity and inhibited the *MtMetAP1c*-complemented cell growth suggests that Fe(II) is the native metal used by *MtMetAP1c* in an *E. coli* cellular environment. Finally, X-ray structures of *MtMetAP1c* in complex with three metalloform-selective inhibitors were analyzed, which showed different binding modes and different interactions with metal ions and active site residues.

Introduction

Tuberculosis (TB) is a deadly disease caused by mycobacterial infection, and *Mycobacterium tuberculosis* is the major pathogen for TB in humans. 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^a) is a ubiquitous enzyme found in both prokaryotic and eukaryotic cells and carries out an important posttranslational modification of newly synthesized proteins. Therefore, MetAP is a promising target for developing novel drugs against bacterial infection, including TB-causing drug-resistant bacteria.²

Every protein is expressed with a methionine at its N-terminus, and about 50–70% of nascent proteins undergo N-terminal methionine excision, which is required for localization, activation, and degradation.³ Eukaryotic cells express two MetAPs, type 1 and type 2. The two subtypes of MetAP are homologous, and type 2 MetAP has an extra insert in the catalytic domain. In contrast, prokaryotic cells usually only express one MetAP. Eubacteria only have a type 1 MetAP, while archaea only have a type 2 MetAP. Multiple MetAPs are rare in bacteria, but with more genomic sequences reported, two or more putative MetAP genes have been identified in a small number of bacteria. So far, 20 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 (*EcMetAP*). 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 *MtMetAP1c*, was purified, and its structures in apo-form and in complex with methionine were reported.⁴ The structural analysis revealed a SH3 binding motif at its N-terminus, and potential interaction with ribosome through the motif to facilitate cotranslational methionine excision was proposed.⁴ The other MetAP (from *mapA* gene) of *M. tuberculosis*, named *MtMetAP1a*, is shorter at the N-terminus and has no such SH3 binding motif.

MetAP is one of the dinuclear metallohydrolases,^{5,6} and divalent metal ions play a key role in the hydrolysis catalyzed by MetAP. When purified as an apoenzyme, MetAP can be activated by several divalent metals, including Co(II), Mn(II), and Fe(II).^{7,8} Initially, MetAP was believed to be a Co(II) enzyme because Co(II) is among the best activators, and early X-ray structures of MetAP all contained two Co(II) ions at the active site.⁹ 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 MetAP inhibitors with high potencies on purified enzymes failed to show any significant antibacterial activity.^{10–12} We confirmed that inhibitors of the Co(II)-form may not inhibit other metalloforms of MetAP.^{8,13} There are many reasons that an *in vitro* active compound may be

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^a Abbreviations: MetAP, methionine aminopeptidase; *EcMetAP*, *E. coli* methionine aminopeptidase; *MtMetAP1c*, *M. tuberculosis* methionine aminopeptidase type 1c; Met-AMC, methionyl aminomethylcoumarin.

inactive *in vivo*, such as absorption or metabolism. However, one explanation for the lack of antibacterial activities may be a disparity between the metalloform tested 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,^{13,14} and these inhibitors can inhibit either Co(II)-, Mn(II)- or Fe(II)-form of *EcMetAP* with both high potency and selectivity. We used these metalloform-selective MetAP inhibitors to characterize inhibition of purified *EcMetAP* and inhibition of cellular MetAP in *E. coli*. The fact that only 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.¹⁵

Characterization of catalysis and inhibition of mycobacterial MetAPs is a necessary step in discovery and development of their inhibitors. However, enzymatic activity of *MtMetAP*A1a and *MtMetAP*1c was reported only recently by over-expressing the proteins in *E. coli*.¹⁶ Both of the recombinant *M. tuberculosis* MetAP enzymes were soluble and active when purified, and the activity was enhanced by Co(II) and inhibited by Cu(II), Fe(II) and Ni(II).¹⁶ Apparently, the MetAP proteins were purified with metal already incorporated. We independently expressed *MtMetAP*1c in *E. coli* and purified it to homogeneity as an apoenzyme. Contrary to what was reported,¹⁶ we found that Ni(II) and Fe(II) were excellent activators of the purified apoenzyme. Further, we demonstrated that it was a functional MetAP in *E. coli* cells. No inhibitors have been described for mycobacterial MetAPs. Here, we report the characterization of several metalloform-selective inhibitors for inhibition of both purified *MtMetAP*1c and the enzyme in an *E. coli* cellular environment and present the first inhibited structures of *MtMetAP*1c in complex with three metalloform-selective inhibitors.

Results

Expression and Purification of *MtMetAP*1c Protein. The expression plasmid for *MtMetAP*1c was constructed by PCR amplification of *mapB* gene in *M. tuberculosis* H37Rv strain as an *Nhe*I and *Eco*RI fragment and insertion of the fragment into an expression vector pGEMEX1 for a T7-promoter controlled expression in *E. coli*. Only three extra amino acid residues MAS were added to its N-terminus for cloning purposes. No attempt was made to remove the extra sequence, although the terminal methionine is often processed cotranslationally by MetAP in *E. coli* cells.³¹ The protein was expressed at a high level as a soluble and active protein, and it was purified easily to homogeneity as an apoenzyme in high yield (20 to 30 mg/liter cell culture). The apoenzyme showed no activity when tested by the fluorogenic substrate Met-AMC and could be activated by divalent metals instantly.

Activation of *MtMetAP*1c Apoenzyme by Divalent Metals. It is common for a purified metalloenzyme to be activated by several different metal ions. For example, *EcMetAP* can be activated by Co(II), Ni(II), Mn(II), Fe(II) and Zn(II).⁷ Similar sizes of these metal ions make this isomorphous replacement possible. We tested *MtMetAP*1c for activation by divalent metals, including Co(II), Ni(II), Mn(II), Fe(II) and Zn(II) (Figure 1). The bell-shaped metal activation curves were observed, and similar curves were observed

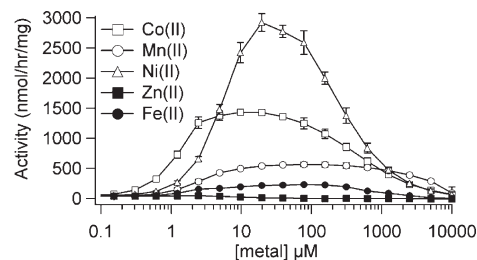


Figure 1. Activation of *MtMetAP*1c apoenzyme by divalent metals.

Table 1. Binding and Activation of *MtMetAP*1c by Different Metals^a

	Fe(II)	Ni(II)	Co(II)	Mn(II)
K_d , μM	0.15	1.41	0.13	0.76
K_m , μM	349	141	232	192
k_{cat} , s^{-1}	0.015	0.093	0.054	0.020
k_{cat}/K_m , $\text{M}^{-1} \text{s}^{-1}$	41.6	659	232	107

^a K_d is the dissociation constant. K_m and k_{cat} are the Michaelis–Menten constants, and they were obtained with Fe(II) at 50 μM , Ni(II) and Mn(II) at 20 μM , and Co(II) at 10 μM .

previously with *EcMetAP*.⁸ High concentrations of a metal often inhibit MetAP enzymatic activity. Co(II) activated *MtMetAP*1c effectively, starting at a low concentration less than 1 μM , consistent with its activation for many MetAPs.⁸ What is unique and surprising is the observed strong activation of *MtMetAP*1c by Ni(II), because this has not been observed in *EcMetAP* or other MetAPs, and this contradicts the belief that bacterial MetAPs use Fe(II) as their native cofactor.^{7,15} This raises the question whether Ni(II) is the native cofactor for *MtMetAP*1c. Mn(II) and Fe(II) also showed activation, while no activation was observed for Zn(II).

Kinetic Characterization of Metal Binding and Activation of *MtMetAP*1c. To further understand the metal binding and activation of *MtMetAP*1c, we carried out detailed kinetic studies with the activating metal ions. The hydrolysis of Met-AMC can be conveniently monitored by fluorescence from the released aminomethylcoumarin. Holding the amount of *MtMetAP*1c apoenzyme and 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, taking into consideration the amount of functional enzyme.²⁰ It is apparent that Co(II) and Fe(II) bound to *MtMetAP*1c the tightest with the lowest K_d values, followed by Mn(II) (Table 1). Ni(II) was shown to have the weakest affinity, which is about 10-fold weaker than Fe(II) or Co(II). At the optimal activating metal concentrations, Michaelis–Menten constants were calculated. Interestingly, Ni(II)-activated *MtMetAP*1c was the most efficient among the metalloforms tested in catalyzing the hydrolysis of the substrate, with the lowest K_m and the fastest k_{cat} , consistent with the metal titration curve (Figure 1).

Complementation of Function of *EcMetAP* by *MtMetAP*1c in *E. coli* Cells. A functional MetAP enzyme is essential for *E. coli* growth,³² and the *EcMetAP* gene cannot be removed. However, an *E. coli* strain with an amber mutation at its chromosomal *EcMetAP* gene was constructed.^{18,21} This *E. coli* strain carries a plasmid with a pBAD-regulated amber suppressor tRNA gene, and expression of the tRNA suppresses the lethal effect of the amber mutation.¹⁸ The *E. coli* cells grow in the presence of arabinose, because expression of

the tRNA is induced, and a functional *EcMetAP* is produced. On the other hand, the cells do not grow in the presence of glucose, because no such tRNA is expressed and a truncated and inactive *EcMetAP* is produced due to the amber mutation not being suppressed. Function of the chromosomally expressed *EcMetAP* can be complemented with a functional MetAP expressed from a plasmid. To test whether *MtMetAP1c* can function in an *E. coli* cellular environment, we constructed such a plasmid by putting the *MtMetAP1c* gene behind an IPTG-inducible *tac* promoter in pFLAGCTC plasmid as pFLAGCTC-*MtMetAP1c* and transformed the *E. coli* cells. As controls, parent plasmid pFLAGCTC, which expresses no MetAP, and the plasmid pFLAGCTC-*EcMetAP*, which expresses *EcMetAP*, were also used for the transformation. On agar plates, we observed that the cells transformed with pFLAGCTC grew in the presence of arabinose and did not grow in the presence of glucose (Figure 2A), confirming the requirement for a functional MetAP for growth. The cells with either pFLAGCTC-*EcMetAP* or pFLAGCTC-*MtMetAP1c* grew on the agar plate in the absence of arabinose and presence of glucose (Figure 2A),

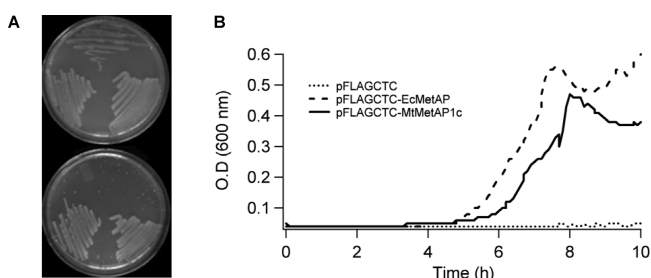


Figure 2. Complementation of *EcMetAP* function by *MtMetAP1c*. (A) *E. coli* cells carrying an amber mutation in chromosomal *EcMetAP* gene were streaked on agar plates with glucose (bottom plate) or with arabinose (top plate). Each plate displays cells containing pFLAGCTC (top), pFLAGCTC-*MtMetAP1c* (bottom left), or pFLAGCTC-*EcMetAP* (bottom right). (B) Growth of the *E. coli* cells in liquid medium supplemented with glucose.

which suggests that *EcMetAP* or *MtMetAP1c* expressed from the plasmids was functional and complemented the function of chromosomally expressed *EcMetAP* to support the growth. We also tested the growth of the transformed *E. coli* strains in a liquid medium supplemented with glucose and without arabinose. The cells with either pFLAGCTC-*EcMetAP* or pFLAGCTC-*MtMetAP1c* showed robust growth, while the *E. coli* cell with the parent plasmid pFLAGCTC did not grow under the same conditions (Figure 2B). These results confirm that *MtMetAP1c* expressed from a plasmid in *E. coli* was a functional enzyme and supported the growth of *E. coli* cells when *EcMetAP* was absent.

Metalloform-Selective Inhibition of Purified *MtMetAP1c* and the Enzyme in an *E. coli* Cellular Environment. A set of inhibitors (Table 2) with known metalloform-selectivity for *EcMetAP* were used to evaluate inhibition of purified *MtMetAP1c* enzyme and the same enzyme in an *E. coli* cellular environment, to provide clues for the metal cofactor used by *MtMetAP1c* in cells. The catechol compounds **1** and **2** inhibited selectively the Fe(II)-form of *EcMetAP*,¹⁴ while **3** and **4** were shown to be highly selective inhibitors for the Mn(II)-form.¹³ Triazole inhibitors were shown to interact directly with the catalytic metal ions through nitrogen atoms,³³ and we predicted their preference for the Co(II) and Ni(II) forms of MetAP because of their chelation through nitrogen atoms.³⁴ We selected four such triazole compounds, **5–8**, for this study. All of the eight MetAP inhibitors were first tested with purified *MtMetAP1c* apo-enzyme activated by Co(II), Ni(II), Mn(II), or Fe(II). Indeed, all of these inhibitors showed potent and metalloform-selective inhibition at low micromolar or submicromolar concentrations (Table 2). **1** and **2** were selective for the Fe(II)-form, **3** and **4** were selective for the Mn(II)-form, and **4–8** were selective for the Ni(II) and Co(II) forms. Subsequently, we tested some of them for their ability to inhibit the cellular enzymatic activity of *MtMetAP1c* in permeabilized *E. coli* cells. Inclusion of Ca(II) at 5 mM made these cells permeable to substrates and inhibitors, and Ca(II)

Table 2. Inhibition of Enzymatic Activities of Purified and Cellular *MtMetAP1c* and Inhibition of Cell Growth of *MtMetAP1c*-Complemented *E. coli* by Metalloform-Selective Inhibitors^a

compd	purified enzyme ^b				cellular enzyme ^c	bacterial growth ^d
	Fe(II)	Ni(II)	Co(II)	Mn(II)		
1	3.6	104	76	37	20	208
2	1.4	60	38	14	35	89.5
3	> 500	> 500	> 500	14	623	> 1000
4	> 500	> 500	> 500	16	ND ^e	ND ^e
5	> 500	1.3	0.74	18	121	> 1000
6	> 500	2.5	0.69	26	281	> 1000
7	> 500	0.58	2.0	143	ND ^e	ND ^e
8	40	0.24	0.26	2.0	ND ^e	ND ^e

^a IC₅₀ values are expressed in μM. ^b Purified enzymes were reconstituted by activating the apo-enzyme with different divalent cations [Fe(II), 50 μM; Ni(II) and Mn(II), 20 μM; Co(II), 10 μM]. ^c Cellular enzyme was the recombinant *MtMetAP1c* expressed in *E. coli* cells lacking endogenous *EcMetAP*. ^d Growth of *E. coli* cells with the recombinant *MtMetAP1c* was monitored. ^e Not determined.

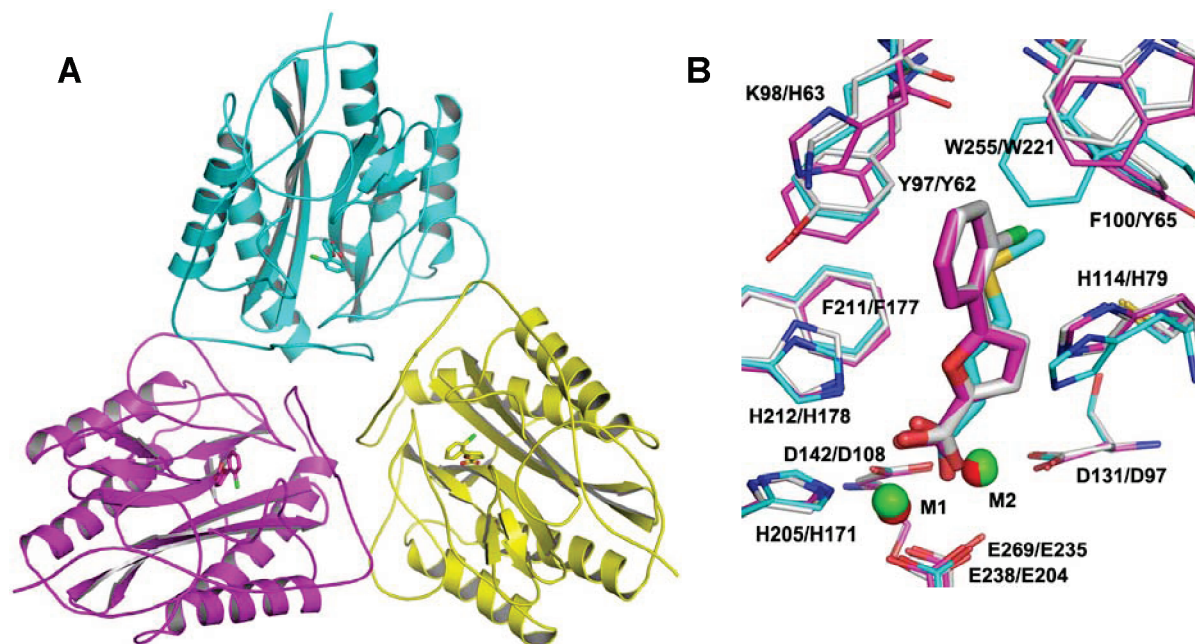


Figure 3. Structure of *MtMetAP1c* in the Mn(II)-form in complex with the Mn(II)-form selective inhibitor **4**. (A) Trimeric arrangement in crystal. Inhibitor **4** at the active site is shown as sticks. The three molecules of *MtMetAP1c* were colored cyan, magenta, and yellow, respectively. (B) Overlay of this structure with *EcMetAP* in complex with the same inhibitor (carbon magenta, PDB code 1XNZ) and with the same protein in complex with methionine (carbon cyan, PDB code 1YJ3). Only residues (thin sticks) surrounding the ligands (thick sticks) at the active site are shown. Noncarbon atoms are colored: oxygen, red; nitrogen, blue; sulfur, yellow; chlorine, green. Mn(II) (green) and Co(II) (red) ions are shown as spheres. For residue labeling, the first is for *MtMetAP1c* and the second for *EcMetAP*.

had no effect on MetAP activity.¹⁵ Clearly, the highest inhibition of the cellular *MtMetAP1c* activity was achieved by the two Fe(II)-form selective inhibitors **1** and **2** (Table 2), suggesting that *MtMetAP1c* present in the live *E. coli* cells used Fe(II), not Ni(II) or Co(II), as the cofactor for catalysis.

Growth Inhibition of *MtMetAP1c*-Complemented *E. coli* Cells. The N-terminal methionine excision is an important cotranslational process, and a lethal phenotype was observed when the single gene coded for *EcMetAP* was deleted in *E. coli*,³² demonstrating its essentiality for bacterial survival. In our construct, *MtMetAP1c* complements the essential function of *EcMetAP* in *E. coli* cells. Therefore, effective inhibition of cellular *MtMetAP1c* will conceivably inhibit the growth of the *E. coli* cells. We tested the metalloform-selective inhibitors on the *E. coli* cells with *MtMetAP1c* complementation and observed that only the Fe(II)-form selective inhibitors **1** and **2** proved to arrest bacterial cell growth (Table 2). In contrast, inhibitors **3**, **5**, and **6** showed no inhibition at the highest concentration (1 mM) tested, indicating that they could not inhibit the cellular *MtMetAP1c*. Although it is still possible that **3**, **5**, and **6** could not penetrate the permeabilized cell walls, the observed inhibition by **1** and **2** is consistent with the conclusion that the functional metalloform of cellular *MtMetAP1c* exists as the Fe(II)-form. We have previously demonstrated that **1** and **2** prevented *E. coli* growth by directly targeting *EcMetAP*, as observed by monitoring the retention of the N-terminal methionine in the biomarker glutathione-*S*-transferase.¹⁵

X-ray Structures of *MtMetAP1c* in Complex with Inhibitors. Structural information for mycobacterial MetAPs is lacking, and only two X-ray structures of *MtMetAP1c* either as an apoenzyme or in complex with product methionine were reported.⁴ With the confirmed inhibition of *MtMetAP1c* by our metalloform selective inhibitors, we embarked on crystallization and structural analysis to elucidate their

binding mode at the active site of *MtMetAP1c*. Three structures of such enzyme–inhibitor complexes with **4**, **7**, or **8** were obtained independently. All of the structures were folded in the “pita-bread” shape commonly seen in MetAP structures.⁹ Electron density for two metal ions was clearly observed at the dinuclear metal site, and the inhibitors bound in the shallow and mostly hydrophobic active site pocket.

It is interesting that all of our three structures showed $P6_3$ space group in the crystal packing instead of the common $P2_1$ space group for other MetAPs. This symmetry indicates a trimeric arrangement in the crystals (Figure 3A), and indeed, the calculation on the PISA server (protein interfaces, surfaces, and assemblies service; http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html)³⁵ showed that there are large contact surfaces between the protein molecules, and a trimeric form is energetically favored. However, similar analysis for the previous *MtMetAP1c* structures (PDB codes 1Y1N and 1YJ3, with $P2_1$ space group) yielded no specific interactions between protein molecules, and a monomeric solution structure was predicted. The previously reported *MtMetAP1c* has a longer N-terminus with a His-tag present,⁴ and it is not known whether the extra sequence, although not visible in the X-ray structures, prevented its packing in crystals as a trimer. The His-tag is not located near the contact surfaces. The question is whether *MtMetAP1c* is trimeric in solution. We eluted our *MtMetAP1c* (31 kDa predicted molecular weight) in 50 mM Tris, pH 7.5, with 150 mM NaCl through a Superdex 75 size-exclusion column, with blue dextran (2000 kDa), aldolase (153 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease (13.7 kDa) as molecular weight standards. *MtMetAP1c* was eluted between ovalbumin and ribonuclease, suggesting a monomeric state in the solution condition. Disagreement in oligomer states between crystal packing and solution has been noted,³⁶ and it is possible that *MtMetAP1c*

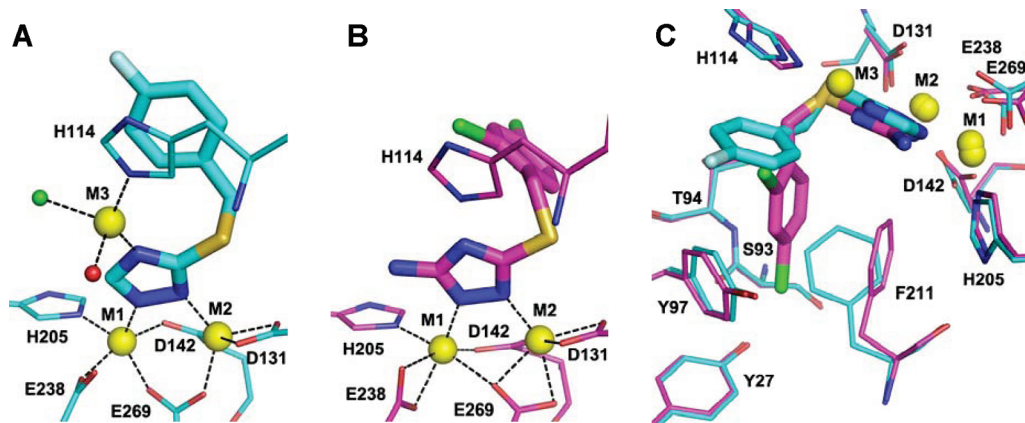


Figure 4. Structures of *MtMetAP1c* in the Ni(II)-form in complex with the Co(II)-form and Ni(II)-form selective inhibitors **7** and **8**. (A) Trimetalated active site with **7** bound. Inhibitor is shown as thick sticks, and the protein residues are shown as thin sticks (carbon, cyan; oxygen, red; nitrogen, blue; sulfur, yellow; fluorine, pale cyan). Ni(II) ions (yellow) are shown as large spheres, and water (red) and chlorine ion (green) are shown as small spheres. Metal coordination is shown as dashed lines. (B) Dimetalated active site with **8** bound. Color scheme is the same as for part A except carbon is magenta and chlorine is green. (C) Comparison of the bound conformations of **7** and **8**. For clarity, only selected protein residues are shown.

exists in a trimeric form in some crystals, while it is monomeric in solution.

X-ray Structure of *MtMetAP1c* in Complex with **4 in the Mn(II)-Form.** The structure was solved to 1.4 Å resolution, and two molecules of **4** were fitted to the structure. One of the inhibitor molecules occupied the active site, and the other took a position on the opposite side of the protein molecule, 10.9 Å away from the active site inhibitor. The structure of *EcMetAP* in complex with the same inhibitor was solved before (PDB code 1XNZ), and only one inhibitor molecule was identified.¹³ When these two structures were overlaid, the active site inhibitor showed the same binding mode with a noncoplanar conformation between its two aromatic rings (Figure 3B). Occupation of **4** at the active site is likely sufficient for MetAP inhibition because the second molecule of **4** was not observed in the *EcMetAP* structure, and **4** inhibited both *MtMetAP1c* and *EcMetAP* potently at 16 and 0.24 μM,¹³ respectively. *EcMetAP* has a longer C-terminus, and the binding of a second **4** is spatially incompatible with residues R251, D253, and D254 in *EcMetAP*.

The previous *MtMetAP1c* structure (PDB code 1YJ3) is in the Co(II)-form, and the ligand used is also different. However, superimposing the two structures by aligning all main chain atoms from residue R4 to the end residue L285 gave a rmsd of 0.301 Å, indicating very similar structures. Because of different ligands, the active site residues showed movements to accommodate the different sizes of the ligands. The most significant movements were H114, which moved by 1.4 Å and is a conserved residue in *EcMetAP* that plays an important role in catalysis,³⁷ and W255, which moved the most by 2.2 Å.

X-ray Structures of *MtMetAP1c* in Complex with **7 or **8** in the Ni(II)-Form.** Triazoles were reported as potent inhibitors of the Co(II)-form of *Staphylococcus aureus* MetAP (IC₅₀ = 43.7 nM),³³ and X-ray structures of enzyme–inhibitor complexes were reported. Here, we described the inhibition of *MtMetAP1c* by similar triazole compounds with high potency at submicromolar concentrations and selectivity for the Co(II)-form and Ni(II)-form. We crystallized two of the triazole inhibitors (**7** and **8**) with *MtMetAP1c* in a unique Ni(II)-form and solved the complexes to 1.85 and 1.75 Å resolution, respectively. Although both inhibitors have the

same triazole moiety, they bound differently at the active site. While **8** bound at the active site as a dimetalated structure (Figure 4B), **7** acquired an additional Ni(II) ion to form a trimetalated structure (Figure 4A). The extra ion was tetracoordinated with ligation to the conserved H114 mentioned before and to a water molecule and a chlorine ion as the third and fourth coordination points. Trimetalated MetAP enzymes in complex with other types of inhibitors have been observed before,^{12,34,38} and their formation requires specific spatial arrangement of coordinating heteroatoms. However, it is interesting to note that **7** formed a trimetalated structure, while **8** formed a dimetalated structure with small structural differences. These two structures also differ significantly at the dinuclear metal site. M1 and M2 were both pentacoordinated in the complex with **7**, and they became hexacoordinated in the complex with **8**. The distances from M1 to the two oxygen atoms of E238 are 2.0 and 3.2 Å in the complex with **7**; therefore, E238 provided only one oxygen atom for coordination. In contrast, the distances are 2.1 and 2.2 Å in the other complex, and both oxygen atoms coordinated with M1. For M2 coordination, one of the oxygen atoms of E269 shifted from a monodentate mode to M1 in complex with **7** to a bridging bidentate mode to both M1 and M2 in complex with **8**, providing the additional coordination point for M2.

A unique structural feature of the complex with **8** is the bound conformation of the inhibitor. Although both **7** and **8** have the core benzylthiotriazole structure, **8** adapted a bound conformation, with its benzyl group turning into a pocket formed by rotation of F211 by 90° (Figure 4C). This binding pocket identified by this structure has not been seen in any other MetAP structures and provides additional interactions for MetAP inhibitor design.

Discussion

Both MetAPs in *M. tuberculosis* were active as enzymes when purified, and their mRNA transcripts were analyzed and showed different levels in the log phase and the stationary phase.¹⁶ The *MtMetAP1a* gene (*mapA*) expressed more in the log phase, while *MtMetAP1c* gene (*mapB*) showed a higher level in the stationary phase. It was concluded that the two MetAPs may perform important functions in different growth

phases of *M. tuberculosis*.¹⁶ The special characteristics of the mycobacterial life cycle may require more than one MetAP enzyme to carry out the important cotranslational modification. Although the majority of bacteria have only one MetAP gene, two or more MetAP genes were identified in a small number of bacteria, and most of them have not been characterized enzymatically. Two homologous type 1 MetAP isozymes in *Bacillus subtilis* were isolated and investigated.³⁹ Although both showed enzymatic activity, only one of them was essential for growth, and the other was concluded to be nonessential because of low expression.³⁹ Two MetAP genes were also identified in *Acinetobacter baumannii*, but none of them have been demonstrated as a functional enzyme. The protozoan parasite *Plasmodium falciparum* has four MetAP sequences, and inhibitors discovered and characterized on one of the four showed antimalarial activity.⁴⁰ Deletion of the single MetAP gene from *E. coli*³² or *Salmonella typhimurium*⁴¹ is lethal. *Saccharomyces cerevisiae* has two copies of MetAP enzymes, a type 1 and a type 2. Deletion of one of them showed a slow growth phenotype, and deletion of both was required to produce lethality.⁴² *MtMetAP1a* and *MtMetAP1c* both belong to type 1 MetAP, and an alignment of their protein sequences showed 33% identity.⁴³ Because *MtMetAP1a* and *MtMetAP1c* are very homologous enzymes, it is likely that the function of one can be complemented by the other, and inhibitors of one will inhibit the other as well. Therefore, both *MtMetAP1a* and *MtMetAP1c* are potential drug targets, and inhibition of one or both is likely required to show antimycobacterial activity.

We purified *MtMetAP1c* to homogeneity as an apoenzyme and demonstrated its enzymatic function not only as a purified enzyme but also in live *E. coli* cells. Divalent metals Co(II), Mn(II), Ni(II), and Fe(II) all showed instant activation of the purified apoenzyme. Co(II) and Fe(II) had higher affinities, and Ni(II) bound more weakly but showed the highest catalytic efficiency. Zhang et al.¹⁶ described Fe(II) and Ni(II) as inhibitors of *MtMetAP1c* activity, contradicting some of our results. However, their enzyme showed high activity before the metal ion was added, indicating that a metalated enzyme was used instead of an apoenzyme. The enzyme was purified as a His-tagged protein, and no procedure was described for metal removal. Their observed inhibitory effect of Fe(II) or Ni(II) was probably due to competition of the metal added with the active site metal already in place. Another possibility is that higher metal concentrations (in addition to the metal already in place) were used in their experiments, and our metal activation profiles (Figure 1) showed that a metal can also inhibit MetAP activity at high concentrations. It is interesting to note that a MetAP enzyme was purified from *Mycobacterium smegmatis* mc²155 strain, and its enzymatic activity was enhanced by Mg(II) and Co(II) and inhibited by Fe(II) and Cu(II).⁴⁴ However, the enzyme was purified by following the hydrolysis of a MetAP substrate, and its identity as a mycobacterial MetAP was not confirmed by sequencing.

Many dinuclear metallohydrolases⁵ play key roles in physiological and pathological processes and often are targets for therapeutics, for instance, MetAP in protein cotranslational modification, dicer in RNA interference,⁴⁵ HIV reverse transcriptase in AIDS,⁴⁶ and protein phosphatase-1 in cell cycle regulation.⁴⁷ Assignment of their physiologically relevant metalloform is often difficult and confusing, but it is critically important for the discovery and development of inhibitors that are effective against cellular enzymes. Initially

from high throughput screening, we discovered several classes of unique MetAP inhibitors that can distinguish different metal ions at the enzyme active site.^{13,14} These metalloform-selective inhibitors are valuable research tools for the assignment, and here, we present an example of their application to clarification of the native metalloform of *MtMetAP1c* in an *E. coli* cellular environment. With confirmed inhibitory potency and selectivity on the metalloforms of the purified *MtMetAP1c*, we characterized these inhibitors for inhibition of MetAP activity from the recombinant *MtMetAP1c* in live *E. coli* cells. Only the Fe(II)-form selective inhibitors inhibited the cellular *MtMetAP1c* activity and inhibited the growth of *MtMetAP1c*-complemented *E. coli* cells, leading to the conclusion that *MtMetAP1c* is in the Fe(II)-form in an *E. coli* cellular environment. It is intriguing why *MtMetAP1c* utilizes Fe(II) for catalysis when Ni(II) offers higher catalytic efficiency. One possibility is the higher affinity and easier availability of Fe(II). Expression of *MtMetAP1c* in *E. coli* probably does not swap the intracellular type of metal utilized by *MtMetAP1c* in *M. tuberculosis*. For instance, it was demonstrated that *E. coli* peptide deformylase uses Fe(II) as the native metal cofactor, but the *Borrelia burgdorferi* counterpart utilizes Zn(II) after heterologous expression in *E. coli*.⁴⁸ Nevertheless, the native metalloform of *MtMetAP1c* in *M. tuberculosis* remains to be confirmed. Our approach of using *MtMetAP1c* in *E. coli* cells is an attractive alternative to the direct manipulation of pathogenic organisms for drug discovery that would be accessible to only a selected few because of the dangers they pose.

We presented the first group of small molecule inhibitors for a mycobacterial MetAP enzyme and elucidated their binding characteristics at the enzyme active site. They showed not only potency but also selectivity for different metalloforms and are initial lead compounds for the development of inhibitors of mycobacterial MetAPs as novel anti-TB drugs. Although metalloform selectivity may not be required for effective inhibition of cellular MetAPs, selective inhibition of different metalloforms may be advantageous. Fe(II) is likely the metal used by MetAPs in bacteria such as *E. coli* and *Bacillus*.¹⁵ In contrast, human type 2 MetAP uses Mn(II) as its physiologically relevant metal cofactor.⁴⁹ It is unknown which metal human type 1 MetAP uses for its catalysis, but it is probably not Fe(II) because free Fe(II) concentration in mammalian cells is low and sequestering iron is a defense mechanism against bacterial infection.^{50,51} Metalloform-selectivity may provide a viable strategy for selective inhibition of bacterial MetAP enzymes.

Experimental Section

Materials. The fluorogenic substrate, methionyl aminomethylcoumarin (Met-AMC), was purchased from Bachem Bioscience (King of Prussia, PA). Fluorescent dye resazurin was obtained from Acros Organics (Morris Plains, NJ). MOPS-based rich defined medium¹⁷ was acquired from Teknova (Hollister, CA). The *E. coli* strain with an amber mutation in the chromosomal *EcMetAP* gene¹⁸ was a generous gift from Prof. Frederick Blattner at University of Wisconsin. The genomic DNA of *M. tuberculosis* H37Rv was kindly provided by Prof. Scott Franzblau at University of Illinois at Chicago. Triazole inhibitors were purchased from ChemBridge (San Diego, CA) with purity of >95%, and their purity and identity were confirmed by LC-MS and NMR.

Preparation of *MtMetAP1c* Protein. The *MtMetAP1c* gene (locus_tag Rv2861c) was cloned by PCR from the genomic

DNA of *M. tuberculosis* H37Rv, using primers 5'-ggatcaccaGC-TAGCatgctagctgctaccg-3' (forward) and 5'-agcactcGAA-TTctacagacaggtcag-3' (reverse) (restriction sites are in capital letters). The DNA fragment was digested with *NheI* and *EcoRI* and cloned into an *E. coli* expression vector pGEMEX-1 (Promega, Madison, WI) as pGEMEX1-*MtMetAP1c* for expression under the control of a T7 promoter. The final recombinant protein *MtMetAP1c* has three residues MAS added to its N-terminus. The recombinant plasmid was introduced into the *E. coli* BL21(DE3) strain for expression. A single colony was grown overnight in 5 mL of LB medium with 100 $\mu\text{g}/\text{mL}$ ampicillin at 37 °C, and the overnight culture was used to inoculate 1 L of LB medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin. The culture grew at 37 °C, and protein expression was induced by adding IPTG when OD₆₀₀ reached 0.6–0.8. Afterward, cells were shaken at 16 °C for 20 h before harvesting by centrifugation. A resuspension buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA) was used to suspend cell pellets, and the cell suspension was stored at –20 °C. Frozen cells after thawing were broken by three consecutive passes through a French press at 1000 psi. The supernatant was collected from centrifugation at 20 000 rpm for 30 min and loaded to a Q-Sepharose column, which was equilibrated with buffer A (50 mM Tris, pH 8.0). The proteins were eluted with a linear gradient of NaCl from 0 to 1 M. *MtMetAP1c* appeared in the flow-through fractions. Ammonium sulfate was added into the combined fractions in a stepwise manner. The majority of *MtMetAP1c* was precipitated when ammonium sulfate was 40% saturated. The pellet was resuspended in buffer A, and the mixture was loaded to a desalting column (5 mL HiTrap) to remove ammonium sulfate. Chelex-100 resin (BioRad, Hercules, CA) was added to the combined active fractions, and the mix was shaken at 4 °C for 2 h. The sample was filtered to remove the resin, loaded to a desalting column, and eluted with buffer B (50 mM Tris, pH 8.0, 150 mM NaCl, which was pretreated with Chelex-100). The *MtMetAP1c* in apoform was verified by hydrolysis of Met-AMC in the presence and absence of divalent metals, and no activity was detected before metal was added.

Assays and Inhibition of *MtMetAP1c* as a Purified Enzyme. Enzymatic activity of *MtMetAP1c* was monitored by fluorescence ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$) on a SpectraMax Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA), following hydrolysis of Met-AMC at room temperature as described.^{8,19} All kinetic experiments were carried out on 384-well plates. Each well contained 80 μL of assay mixture with 50 mM MOPS, pH 7.5, 100 μM Met-AMC, 0.5 μM apoenzyme, and metal ions (50 μM FeCl₂ with 100 μM ascorbic acid, 10 μM CoCl₂, 20 μM MnCl₂, or 20 μM NiCl₂). For IC₅₀ determination, the inhibitors were tested at six or more serially diluted concentrations. The IC₅₀ values were calculated from nonlinear regression curve fitting of percent inhibitions as a function of inhibitor concentrations. For metal titration, each well contained 80 μL of assay mixture with 50 mM MOPS, pH 7.5, 100 μM Met-AMC, 0.5 μM apoenzyme, and increasing concentrations of metal ions (FeCl₂ with twice the concentration of ascorbic acid, CoCl₂, MnCl₂, NiCl₂, or ZnCl₂). The initial velocity values were converted to specific activity values and plotted against increasing concentrations of the metal. For determination of kinetic parameters, the 80 μL assay mixture per well contained 50 mM MOPS, pH 7.5, 0.5 μM apoenzyme, metal ions (50 μM FeCl₂ with 100 μM ascorbic acid, 10 μM CoCl₂, 20 μM MnCl₂, or 20 μM NiCl₂), and increasing concentrations of Met-AMC. The initial rates were plotted with the corresponding substrate concentrations, and the curve was fitted with the Michaelis–Menten equation to obtain K_{m} and k_{cat} . We used an improved model of the multiple independent binding sites to calculate the binding affinity K_{d} , taking into consideration the amount of functional enzyme, which we described recently.²⁰ Briefly,

the initial rate of hydrolysis was plotted against increasing concentrations of Co(II) at two *MtMetAP1c* protein concentrations (20 and 0.5 μM) and fit with the model via an iterative process to obtain an accurate protein concentration. For K_{d} determination of the various divalent metals to *MtMetAP1c*, the titration curves were generated using the calculated apoenzyme of 0.54 μM in 50 mM MOPS, pH 7.5, 200 μM Met-AMC, and increasing concentrations of CoCl₂, MnCl₂, NiCl₂, or FeCl₂. In the case of FeCl₂, ascorbic acid was added at double the concentration of FeCl₂. An iterative process was allowed to proceed until K_{d} and functional enzyme concentration values converged after a few cycles.

Complementation of the Essential Function of *EcMetAP* with *MtMetAP1c* in *E. coli*. To construct a plasmid with expression of the *MtMetAP1c* gene under the control of an IPTG-inducible *tac* promoter, an *NdeI/BamHI* fragment was cut from pGEMEX1-*MtMetAP1c* and cloned into pFLAGCTC (Sigma, St. Louis, MO) as pFLAGCTC-*MtMetAP1c*. Similarly, the *EcMetAP* gene was cloned into the pFLAGCTC plasmid as pFLAGCTC-*EcMetAP*. Both the parent plasmid pFLAGCTC and the plasmid pFLAGCTC-*EcMetAP* were used as controls. The plasmids were introduced into the *E. coli* cells with an amber mutation in the chromosomal *EcMetAP* gene. A special MOPS-based rich defined medium^{18,21} was used in combination with kanamycin, ampicillin, L-arabinose, D-glucose, and IPTG. The cells were cultured in the liquid medium or on agar plates prepared with the medium.

Inhibition of Cellular *MtMetAP1c* Activity. The above-mentioned pFLAGCTC-*MtMetAP1c*-transformed *E. coli* cells (*MtMetAP1c*-complemented cells) were used to establish the cellular *MtMetAP1c* activity assay. Bacterial cells were allowed to grow to the exponential phase, harvested, and washed twice with water. The final cell pellet was resuspended in 10 mM CaCl₂, 100 mM Tris, pH 7.5, and then an equal volume of glycerol was added. The cell suspension was aliquoted and kept at –80 °C for storage. For the cellular *MtMetAP1c* activity assay, the cell suspension was diluted with 10 mM CaCl₂ and 100 mM Tris, pH 7.5. The cells, substrate Met-AMC, and inhibitor at 12 serially diluted concentrations were combined in wells of a 384-well plate. The final assay volume was 80 μL with 150 μM Met-AMC, 5 mM CaCl₂, and 50 mM Tris, pH 7.5. Increase of the fluorescent product was monitored via fluorescence ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$) at room temperature every 2 min for 6–8 h. The IC₅₀ values were calculated from the rate of substrate hydrolysis.¹⁵

Inhibition of *MtMetAP1c*-Complemented *E. coli* Cell Growth. Inhibition of bacterial growth was carried out by using the *MtMetAP1c*-complemented *E. coli* cells. The experiments were performed in a similar way as previously reported with minor modifications.^{14,15} The assay was carried out on 384-well opaque plates containing 12 serially diluted concentrations for each inhibitor (40 μL per well) with the highest final concentration of 1 mM in the assay. A suspension of bacterial cells was prepared from agar plates containing rich defined media with 0.2% glucose, 50 $\mu\text{g}/\text{mL}$ kanamycin, and 100 $\mu\text{g}/\text{mL}$ ampicillin grown to the exponential phase, which was used to inoculate a second culture batch. This ensured that the survival of the cells was due to *MtMetAP1c* complementation rather than residual endogenous *EcMetAP*. The suspension was adjusted to 0.5 McFarland optical density²² and then further diluted by 1000-fold in the same medium containing 100 mM Tris, pH 7.5, and 225 μM resazurin. Cells were dispensed into the microplate (40 μL per well) by a Multidrop Combi reagent dispenser (Thermo Scientific, Waltham, MA). The conversion from resazurin to resofurin was monitored kinetically by fluorescence ($\lambda_{\text{ex}} = 530 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$) using a SpectraMax Gemini XPS plate reader. Fluorescence kinetic experiments were carried out for 10 h at 37 °C, with readings taken every 5 min. Signal intensities at time points along the exponential phase of the growth curve corresponding to 50–85% of total intensity

Table 3. X-ray Data Collection and Refinement Statistics

	inhibitor		
	4	7	8
inhibitor code	FCD	T03	T07
PDB code	3IU7	3IU8	3IU9
metal ion	2 Mn(II)	3 Ni(II)	2 Ni(II)
Cell Parameters			
space group	<i>P</i> 6 ₃	<i>P</i> 6 ₃	<i>P</i> 6 ₃
<i>a</i> (Å)	106.4	105.7	106.2
<i>b</i> (Å)	106.4	105.7	106.2
<i>c</i> (Å)	50.4	50.4	50.8
α (deg)	90	90	90
β (deg)	90	90	90
γ (deg)	120	120	120
X-ray Data Collection			
resolution range (Å) ^a	50–1.40 (1.42–1.40)	50–1.85 (1.88–1.85)	50–1.75 (1.78–1.75)
collected reflections	642 578	304 665	301 490
unique reflections	64 180	27 549	32 971
completeness (%) ^a	99.9 (100)	99.5 (90.5)	99.5 (92.2)
<i>I</i> / <i>σ</i> (<i>I</i>) ^a	43.8 (9.4)	23.3 (3.8)	59.8 (15.7)
<i>R</i> _{merge} (%) ^a	4.5 (21.5)	15.6 (71.4)	7.8 (17.5)
Refinement Statistics			
<i>R</i> (%)	17.1	16.5	16.4
<i>R</i> _{free} (%)	19.0	20.0	19.5
rmsd bonds (Å)	0.031	0.028	0.028
rmsd angles (deg)	2.52	1.97	2.31
no. of solvent molecules	249	162	193
⟨B⟩ protein (Å ²)	11.0	14.4	14.1
⟨B⟩ inhibitor (Å ²)	9.5	21.4	12.5
⟨B⟩ water (Å ²)	18.6	19.2	19.7

^a Values given in parentheses correspond to the outer shell of data.

of an uninhibited sample were averaged and converted to percent inhibitions to calculate IC₅₀ values by nonlinear regression curve fitting.

Crystallization and Data Collection. Crystals of the enzyme–inhibitor complexes were obtained independently by a hanging-drop vapor-diffusion method at room temperature. Each of the inhibitors (**4**, **7**, and **8**; 100 mM in DMSO) was added to concentrated metalated enzyme (10 mg/mL, 0.32 mM protein, 2 mM metal) in 50 mM Tris, pH 8.0, and 150 mM NaCl, and the molar ratio of inhibitor to *MtMetAP1c* was 5:1 or 10:1. The enzyme/inhibitor mixture was mixed with a reservoir buffer in a 1:1 ratio. The reservoir buffer was 100 mM Bis-Tris, pH 5.5, 1.1 M NH₄SO₄, and 50 mM NaCl for **4**; 100 mM Bis-Tris, pH 5.5, 1.4 M NH₄SO₄, and 15% glycerol for **7**; and 100 mM Bis-Tris, pH 5.5, 1.3 M NH₄SO₄, and 15% glycerol for **8**. Diffraction data were collected at the Advanced Photon Source, Argonne National Laboratory (beamline 19BM) and processed with HKL3000.^{23,24} All of the crystals belong to space group *P*6₃. One molecule is in the asymmetric unit.

Structural Solution and Refinement. The structures were solved by molecular replacement with MolRep²⁵ in CCP4²⁶ with CCP4i interface,²⁷ using the previously published *MtMetAP1c* structure (PDB code 1YJ3)⁴ as the search model. The structure was refined with REFMAC5²⁸ with iterative model building using WinCoot.²⁹ The refinement was monitored with 5% of the reflections set aside for *R*_{free} factor analysis throughout the whole refinement process. Electron density was clear for all residues except a few residues at the N-terminus, and residues from the second (P2) in the native protein to the end (L285) were modeled. Comparison of structures and generation of structural drawings were carried out by using PyMOL.³⁰ Statistic parameters in data collection and structural refinement are shown in Table 3. Atomic coordinates and structure factors for the three structures were deposited in the Protein Data Bank.

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