The Methionyl Aminopeptidase from *Escherichia coli* Can Function as an Iron(II) Enzyme†

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ABSTRACT: The identity of the physiologically relevant metal ions for the methionyl aminopeptidase (MetAP) from *Escherichia coli* was investigated and is suggested to be Fe(II). The metal content of whole cells in the absence and presence of expression of the type I MetAP from *E. coli* was determined by inductively coupled plasma (ICP) emission analysis. The observed change in whole cell concentrations of cobalt, cadmium, copper, nickel, strontium, titanium, and vanadium upon expression of MetAP was negligible. On the other hand, significant increases in the cellular metal ion concentrations of chromium, zinc, manganese, and iron were observed with the increase in iron concentration being 4.4 and 6.2 times greater than that of manganese and zinc, respectively. Activity assays of freshly lysed BL21(DE3) cells containing the pMetAAP plasmid revealed detectable levels (>2 units/mg) of MetAP activity. Control experiments with BL21(DE3) without the MetAP plasmid showed no detectable enzymatic activity. Since MetAP is active upon expression, these data strongly suggest that cobalt is not the in vivo metal ion for the MetAP from *E. coli*. The MetAP from *E. coli* as purified was found to be catalytically inactive (≤2 units/mg). ICP emission analysis of the as-purified enzyme revealed no catalytically relevant metal ions. Both the Co(II)− and Fe(II)−MetAP enzymes are susceptible to autoxidation, so strict care must be taken to remove all dissolved oxygen. Enzymatic assays performed under anaerobic conditions indicated that of the di- and trivalent metal cations tested to date, only Co(II) (37.3 units/mg), Fe(II) (29.7 units/mg), Mn(II) (7.0 units/mg), and Zn(II) (3.3 units/mg) provided detectable levels of enzymatic activity. In each case, excess metal ions were found to be inhibitory. The observed specific activity of Co(II)−MetAP is more than 3 times greater than that previously reported for the MetAP from *E. coli* [Ben-Bassat, A., et al. (1987) *J. Bacteriol. 169*, 751−757]. This increase in activity is likely due to the strict exclusion of air from reaction samples. Oxidation of either the Fe(II) or Co(II) form of the enzyme resulted in the complete loss of catalytic activity. The substrate binding constants (K_m) for Met-Gly-Met-Met binding to the Co(II)- or Fe(II)-substituted MetAP enzymes, under anaerobic conditions, were found to be 3.16 and 1.95 mM, respectively. The combination of these data suggests that the in vivo metal ions for the MetAP enzyme from *E. coli* are likely Fe(II) ions.

In the cytosol of eukaryotes, the translation of all proteins is initiated with an N-terminal methionine residue, whereas in prokaryotes, mitochondria, and chloroplasts, translation of proteins is initiated with an *N*-formylmethionine (*1*−*4*). The *N*-formyl group is typically removed cotranslationally by a deformylase that leaves the N-terminal methionine with a free NH_2 group (*2*). In both eukaryotic and prokaryotic cells, methionyl aminopeptidases (MetAPs)[1] selectively cleave methionine residues from the N-termini of polypeptide chains (*5*). Removal of methionine is essential for protein maturation as well as for the post-translational modification of the N-termini of proteins, such as N-acetylation or N-myristoylation (*2*). The compositions of mature N-termini play important roles in the directed degradation and cellular targeting of proteins (*1*, *3*, *4*). Examples include hemoglobin as well as proteins involved in signal transduction, protein trafficking, cancer cell growth, and both bacterial and viral infections (*6*, *7*). Deletion of the gene encoding MetAP is lethal to *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*; therefore, MetAPs are essential for cell growth and proliferation (*8*−*10*). The importance of understanding the mechanism of action of MetAPs is underscored by the recent observation that MetAPs are the target for the anti-angiogenesis drugs ovalicin and fumagillin (*11*−*15*). Therefore, the inhibition of methionyl aminopeptidase activity in malignant tumors is critically important in preventing tumor vasculature formation and, thus, the growth and proliferation of carcinoma cells.

Several MetAPs have been purified and sequenced from a variety of bacterial and mammalian sources, including humans (*16*−*22*). The enzymes from *E. coli*, *S. cerevisiae*, *Sa. typhimurium*, *Pyrococcus furiosus*, porcine liver, and

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[1] Abbreviations: MetAP, methionyl aminopeptidase; AMPP, aminopeptidase P; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactoside; TFA, trifluoroacetic acid; PMSF, phenylmethanesulfonyl fluoride; ICP, inductively coupled plasma; *v*, velocity; V_{max}, maximal velocity; K_m, Michaelis constant; [S], substrate concentration.

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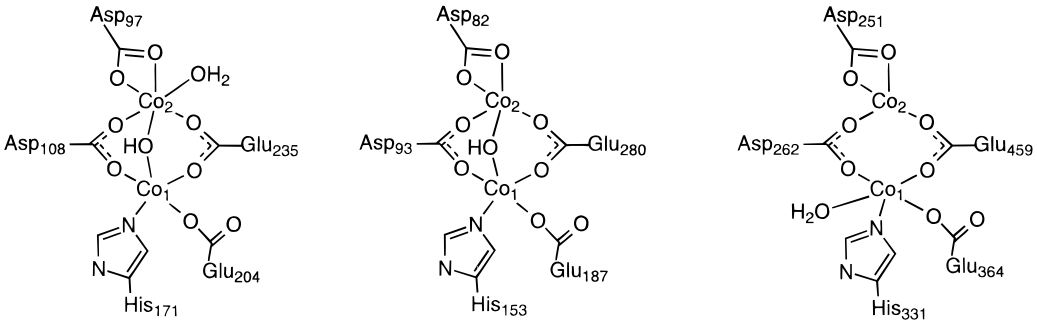

FIGURE 1: Schematic representations of the dinuclear cobalt active sites of the three crystallographically characterized methionyl aminopeptidases.

humans have been the most extensively studied. Three MetAPs have been crystallographically characterized, and all three have a central antiparallel pleated β-sheet flanked by two α-helical segments (*15*, *23*, *24*). The MetAP from *E. coli* contains an internal pseudo-2-fold symmetry that relates both halves of the molecule to one another from a structural standpoint. This symmetry is lost for the *P. furiosus* and human enzymes due to a 62-amino acid insert, which distinguishes type II MetAPs from type I MetAPs (*3*). At this time, the functional role of this 62-amino acid insert remains unknown, but the homologous relationship between all MetAPs has been suggested to indicate an evolutionary separation of considerable distance (*3*). A dinuclear cobalt active site is located at roughly the center of the molecule in the concave face of the β-pleated sheet. The two cobalt ions in each of the three structurally characterized MetAPs contain identical ligands that are made up of side chain Asp, Glu, and His residues (Figure 1). The two cobalt ions reside in a distorted five-coordinate environment in the human and *P. furiosus* structures but were reported to be five- and six-coordinate in the *E. coli* structure (*15*, *23*−*25*). The *P. furiosus* and *E. coli* structures revealed a bridging water/hydroxide group, whereas the human enzyme exhibits only a terminal water/hydroxide molecule on the Co_1 center. This difference in terminal water/hydroxide versus bridging water/hydroxide may be the result of variations in pH during crystallization conditions.

All of the purified and sequenced MetAPs from prokaryotes, eukaryotes, and archaebacteria exhibit strict catalytic specificity for methionine and exhibit an identical metal binding motif (*3*, *5*). Moreover, each of the MetAPs purified to date is inactivated by the addition of complexing agents such as EDTA and 1,10-phenanthroline, establishing the necessity for divalent metal ions in catalysis. However, none of these enzymes have been screened for their native metal ion content, and therefore, the in vivo metal ions remain unknown. In an effort to identify the in vivo metal ions for MetAPs, we have examined the metal content of whole cells with and without the expression of the type I MetAP from *E. coli*. We have also re-examined the levels of MetAP activity observed under anaerobic conditions, with various di- and trivalent metal ions, including Mg(II), Mn(II), Fe(II), Fe(III), Co(II), Co(III), Ni(II), Zn(II), and Cd(II). The substrate (Met-Gly-Met-Met) binding constants for both the Co(II)- and Fe(II)-substituted MetAP enzymes were also determined under anaerobic conditions. The combination of these data suggest that the in vivo metal ions for the MetAP enzyme from *E. coli* are likely Fe(II).

MATERIALS AND METHODS

Enzyme Purification. All reagents used in this study were purchased commercially and were of the highest quality available. Recombinant *E. coli* MetAP was purified from a stock culture kindly provided by B. W. Matthews and W. T. Lowther (*14*). MetAP was purified according to the previously published protocol with minor modifications (*14*). Briefly, 5 L fermentation cultures of *E. coli* BL21(DE3) (pMetAAP) in LB-Kanamycin broth were grown. The temperature was maintained at 37 °C until an OD_{600} of 1.0 was reached, upon which the temperature was decreased to 30 °C and overexpression of MetAP was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). After 3 h, the cells were harvested by centrifugation and resuspended in 100 mL of +T/G buffer [50 mM Hepes (pH 7.9), 10% glycerol, 0.1% Triton X-100, and 0.5 M KCl], 4 mg of DNase, 100 μL of 1 M $MgCl_2$, 1 mL of 100 mM PMSF, and 0.22 g of methionine. The cells were then lysed with a French press and centrifuged at 18 000 rpm for 45 min. The resulting solution was filtered through a 0.45 μm filter and loaded onto a 10 mL NTA−agarose column that had been equilibrated with +T/G buffer. After extensive washing with +T/G buffer followed by −T/G buffer (same as +T/G but without Triton X-100 and glycerol), MetAP was eluted with −T/G buffer containing 60 mM imidazole. The active fractions were pooled, and EDTA was added immediately to a concentration of 5 mM. MetAP was then dialyzed against three changes of 25 mM Hepes buffer (pH 7.9) containing 150 mM KCl and 15 mM methionine at 4 °C that had been passed through a Chelex-100 column to remove any trace metals. Aggregation of protein was observed with the use of nonchelexed buffer. At this point, MetAP is pure as determined by SDS−polyacrylamide gel electrophoresis.

Removal of the His tag from MetAP was accomplished by incubation of 100 mg aliquots with 0.125 unit/mg biotinylated thrombin at 15 °C for 18−20 h. This mixture was then filtered through a 0.45 μm filter and loaded onto a 10 mL NTA−agarose column that had been pre-equilibrated with −T/G buffer. The resulting MetAP-containing fractions were pooled and concentrated to 1.5−2 mL and loaded onto a pre-equilibrated [25 mM Hepes (pH 7.5), 150 mM KCl, and 15 mM methionine] Superdex 75 Hi-load prep-grade

16/60 gel filtration column. Removal of the poly-His tail was confirmed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectroscopy. Via elimination of any divalent metal ions in the last column step, apo-MetAP was obtained as shown by inductively coupled plasma (ICP) emission analysis. The protein was washed free of methionine using chelexed methionine-free buffer [25 mM Hepes (pH 7.5) and 150 mM KCl] prior to all kinetic assays. Samples of MetAP were routinely concentrated by microfiltration using a Microcon-10 concentrator (Millipore Corp.) and stored in liquid nitrogen until they were needed.

Protein concentrations were determined by the absorption at 280 nm using an extinction coefficient of 16 445 M^{-1} cm^{-1}. This value was determined by the Edelhoch method using a 1:8:7 molar ratio mixture of *N*-acetyl-L-tryptophanamide, Gly-Tyr-amide, and L-cysteine to model MetAP (*26*, *27*). The molar absorptivity determined from this method (ϵ_{280} = 16 445 M^{-1} cm^{-1}) for MetAP solubilized in 6 M guanidine hydrochloride is in good agreement (ϵ_{280} = 16 350 M^{-1} cm^{-1}) with that reported by Lowther et al. (*14*). All electronic absorption spectra were recorded on a Shimadzu UV-3101 PC spectrophotometer equipped with a constant-temperature cell holder and a Haake (type 423) constant-temperature circulating bath.

Preparation of Cell Extracts for ICP Metal Analysis. All glassware that was used was soaked in concentrated nitric acid and rinsed in chelexed nanopure water. Three 250 mL cultures of BL21(DE3) cells containing the expression plasmid for *E. coli* MetAP were grown in Luria-Bertani broth with kanamycin (50 mg/L) at 30 °C in a shaker. Overexpression of MetAP was induced by the addition of IPTG to a concentration of 1 mM at an OD_{600} of 1.0. The cells were grown at 25 °C for 3 h and harvested by centrifugation at 8000 rpm for 15 min. These cells were washed extensively with buffer [25 mM Hepes (pH 7.5) and 150 mM KCl]. Overexpression of MetAP was verified by SDS−PAGE. Three control experiments with BL21(DE3) cells without the expression plasmid were also carried out under identical conditions and the cells extensively washed with buffer. Cells (0.5 g wet weight) from each flask were resuspended in 5 mL of buffer [25 mM Hepes (pH 7.5) and 150 mM KCl]. Following the addition of 4 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mg of lysozyme to each sample, the cell suspensions were incubated at 4 °C for 3 h. The cells were lysed by sonication (Heat Systems-Ultrasonics, Inc., three pulses with a duration of 30 s at 5 min intervals) and the lysates centrifuged at 14 000 rpm for 45 min. Concentrated nitric acid was added to the supernatants to a final concentration of 1 M and the mixture left for overnight digestion. The flocculent precipitates were removed by microfuging at 14 000 rpm for 15 min and the supernatants diluted 1:5 in chelexed nanopure water. Concentrations of metal ions were determined by inductively coupled plasma (ICP) emission analysis. The buffer used for resuspension of cell cultures was also shown to be metal free by ICP metal analysis. Three cell cultures of MetAP cells not bearing the vector carrying the gene for MetAP, carrying the vector but not overexpressing MetAP, and carrying the vector which were subsequently induced to overexpress MetAP were grown in Minimal Media to which 1 μM $CoCl_2$ had been added. After identical workup, ICP analysis indicated that no cobalt uptake was observed, thus indicating that cobalt is not limiting in our experiments.

Enzymatic Assay of MetAP. MetAP was assayed using the tetrapeptide substrate Met-Gly-Met-Met and an HPLC system (Shimadzu LC-10A Class-VP5) with a C8 column (Phenomenex, Luna; 5 μm, 4.6 mm × 25 cm) to directly detect the product of hydrolysis Gly-Met-Met. All assays were performed under strict anaerobic conditions in an inert atmosphere glovebox in a dry bath incubator maintained at 30 °C. All substrate stock solutions were prepared anaerobically in buffer [25 mM Hepes (pH 7.5) and 150 mM KCl] to eliminate oxidation of the methionine component of the substrate. Metal insertion in apo-MetAP was effected by the addition of 3 equiv of a divalent metal chloride salt to 20 μM enzyme, where the divalent metal ion was Co, Fe, Ni, Zn, Cd, or Mn (≥99.999% pure, Strem Chemicals, Newburyport, MA, or Aldrich) followed by a 30 min incubation period at 30 °C. A typical assay involved the addition of 4 μL of M(II)−MetAP to a 16 μL reaction mixture which contained 8 mM substrate in 25 mM Hepes (pH 7.5) and 150 mM KCl and was incubated at 30 °C for 1 min. The reaction was quenched by the addition of 20 μL of a 0.1% trifluoroacetic acid solution. The reaction mixture was filtered through a 0.2 μm filter, and 10 μL samples were applied to a reverse-phase HPLC C8 column. The eluting solvents were as follows: (i) mobile phase A being 98% water, 2% acetonitrile, and 0.1% trifluoroacetic acid and (ii) mobile phase B being 100% acetonitrile and 0.1% trifluroacetic acid. The applied sample was resolved into substrate and product peaks during a 15 min run of mobile phase A at a flow rate of 1.5 mL/min. Mobile phase B was then used to elute any residual material before returning to initial conditions. The kinetic parameters v (velocity) and K_m (Michaelis constant) were determined at pH 7.5 by quantifying the tripeptide Gly-Met-Met at 215 nm in triplicate. One unit was defined as the amount of enzyme that releases 1 μmol of Gly-Met-Met at 30 °C in 1 min.

RESULTS

Whole Cell Metal Analyses. To determine which metal ions are potentially the in vivo metal ions for the MetAP from *E. coli*, cell paste samples were collected both before and after the induction of MetAP synthesis. After collection, the cells were washed extensively with buffer, lysed, and digested with nitric acid. Once the cell debris was removed, the supernatant was subjected to metal analysis using ICP emission spectroscopy. Since MetAP is expressed at high levels after induction (Figure 2), comparison of the whole cell metal concentrations in the presence and absence of MetAP expression should reflect the metal ion content of MetAP (Table 1). The observed changes in whole cell concentrations of cobalt, cadmium, copper, nickel, strontium, titanium, and vanadium upon expression of MetAP were negligible. On the other hand, detectable increases in the cellular metal ion concentrations of chromium, zinc, manganese, and iron were observed with the increase in iron concentration being 4.4 and 6.2 times greater than that of manganese and zinc, respectively. Activity assays of freshly lysed BL21(DE3) cells containing the pMetAAP plasmid revealed detectable levels of MetAP activity. Control experiments with BL21(DE3) cells without the MetAP plasmid had no detectable enzymatic activity.

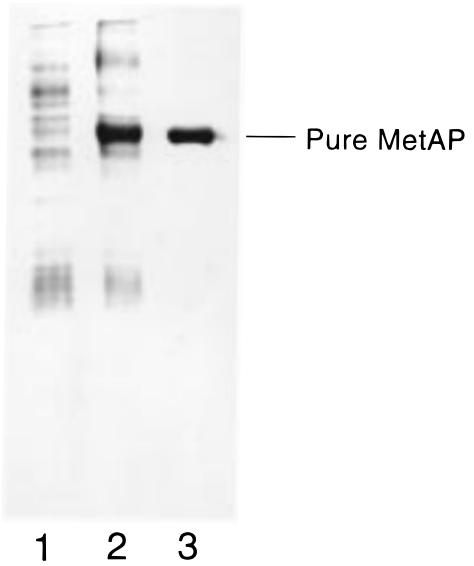

FIGURE 2: SDS−polyacrylamide gel of (lane 1) BL21(DE3) cells, without the plasmid (pMetAAP) that contains the MetAP gene, 3 h after induction with IPTG, (lane 2) BL21(DE3) cells containing the pMetAAP plasmid that contains the MetAP gene, 3 h after induction with IPTG, and (lane 3) purified MetAP from *E. coli*. The molecular weight of the MetAP from E. coli was found to be 29 630 using matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

Table 1: Total Metal Ion Concentrations of Whole Cells before and after Overexpression of the Methionyl Aminopeptidase from *E. coli*

metal ion	cellular metal ion concentration without MetAP expression (ppm)[a]	cellular metal ion concentration with MetAP expression (ppm)[a]	Δconc (ppm)
Cd	0.00	0.00	0.00
Co	0.00	0.00	0.00
Cr	0.22	0.35	0.13
Cu	0.15	0.13	−0.02
Fe	0.42	0.90	0.48
Mn	0.09	0.20	0.11
Ni	0.03	0.01	−0.02
Sr	0.00	0.01	0.01
Ti	0.00	0.00	0.00
V	0.03	0.04	0.01
Zn	0.58	0.66	0.08

[a] Values are the average of three individual experiments. Each value has an experimental error of ±0.06.

Activation of MetAP by Divalent Metal Ions. The MetAP from *E. coli* as purified was found to be catalytically inactive (≤2 units/mg). ICP emission analysis of the as-purified enzyme revealed no catalytically relevant metal ions. Therefore, MetAP as purified is in the apo form, which is likely the consequence of incubation with EDTA during the initial stages of purification. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy provided a molecular weight of 29 633 ± 2.9 for apo-MetAP. This molecular weight corresponds to the MetAP enzyme and the four amino acid residues (Leu-Val-Pro-Arg) which are attached to the C-terminus prior to the engineered thrombin cleavage site and six-histidine tail (*14*). In a similar experiment, the molecular weight of the poly-His-MetAP enzyme was determined and found to be 30 869.7 ± 2.5. The molecular weight difference of 1236.4 ± 2.7 corresponds to the four amino acid residues (Gly-Ser-Leu-Glu) to the C-terminal side of the thrombin cleavage site and the six-histidine tail. The molecular weight of 29 633 for the MetAP from *E. coli* determined by mass spectroscopy is in excellent agreement (29 630 and 29 333) with those previously reported for the MetAP from *E. coli* (*14*, *17*).

Table 2: Specific and Relative Activities of the MetAP from *E. coli* with Various Divalent Cations

metal ion	specific activity[a] (units/mg)	relative activity (%)
none	≤2	≤5
Mg^{2+}	≤2	≤5
Mn^{2+}	7.0 ± 2	19
Fe^{2+}	27.5 ± 3	74
Fe^{2+} and GSH[b]	29.7 ± 3	80
Fe^{3+}	≤2	≤5
Co^{2+}	36.9 ± 3	99
Co^{2+} and GSH[b]	37.3 ± 3	100
Co^{3+}	≤2	≤5
Ni^{2+}	≤2	≤5
Zn^{2+}	2.3 ± 2	6
Zn^{2+} and GSH[b]	3.3 ± 2	9
Cd^{2+}	≤2	≤5

[a] One unit of activity equals 1 μmol of tripeptide Gly-Met-Met produced per minute. [b] Assayed in the presence of 5 mM glutathione (GSH).

Strict care must be taken to remove all dissolved oxygen from MetAP samples in the presence of Co(II) or Fe(II) since the coordinated divalent metal ions are susceptible to autoxidation. To ensure homogeneous enzyme solutions, each metal-substituted MetAP sample was prepared under anaerobic conditions by adding 3 equiv of MCl_2 salts to apo-MetAP, followed by incubation for 30 min at 30 °C. Only 3 equiv of metal ion was used because excess metal was found to be inhibitory. To determine the activity of the Co(III)− and Fe(III)−MetAP enzymes, the divalent metal-containing enzyme samples were allowed to sit in air for several days and minutes, respectively, at pH 7.5. Alternatively, Co(II)-substituted MetAP was treated with 1 mM hydrogen peroxide to effect the conversion of Co(II) to Co(III). Prior to activity measurements, the hydrogen peroxide-treated enzyme was washed extensively with 25 mM Hepes (pH 7.5), 150 KCl buffer. Oxidation of Fe(II)−MetAP to Fe(III)−MetAP was confirmed by EPR spectroscopy.

We have re-examined the levels of MetAP activity in the presence of several different metal ions under anaerobic conditions. To date, we have tested MetAP with Mg(II), Mn(II), Fe(II), Fe(III), Co(II), Co(III), Ni(II), Zn(II), and Cd(II) (Table 2). Of the di- and trivalent cations that were tested, only Co(II) (37.3 units/mg), Fe(II) (29.7 units/mg), Mn(II) (7.0 units/mg), and Zn(II) (3.3 units/mg) provided detectable levels of enzymatic activity (Table 2). The reaction time of 1 min used for all activity assays is well within the linear region of the progress curve, indicating that under our reaction conditions, we are in the steady state regime (Figure 3). The observed activity level for Co(II)−MetAP is more than 3 times higher than that previously reported for the MetAP from *E. coli* (*17*). This increase in activity is likely due to the strict exclusion of air from reaction samples. Interestingly, Fe(II)−MetAP provides a MetAP enzyme that is ∼80% as active as the Co(II)-substituted form of MetAP, while Zn(II)-substituted MetAP exhibits activity levels ∼10-fold lower than those of Co(II)− or Fe(II)−MetAP. Activity measurements performed under anaerobic conditions in the presence of reduced glutathione (5 mM), which mimics the conditions of the cellular cytoplasmic space, showed no

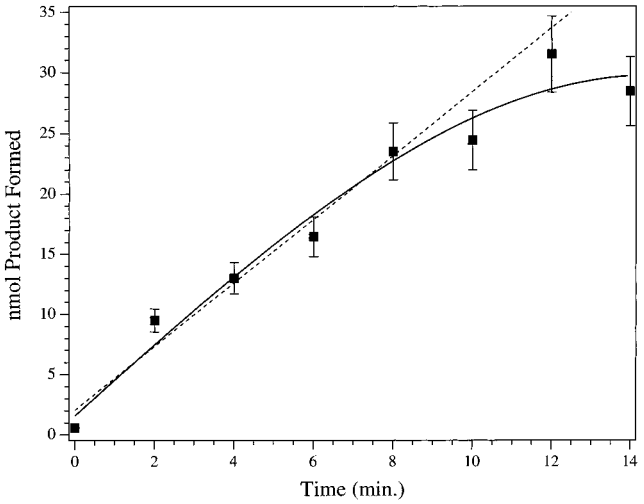

FIGURE 3: Plot of the rate of product formation vs time (minutes). Co(II)−MetAP was assayed using the tetrapeptide substrate Met-Gly-Met-Met and an HPLC system equipped with a C8 column to directly detect the product of hydrolysis, Gly-Met-Met. All assays were performed under strict anaerobic conditions in an inert atmosphere glovebox in a dry bath incubator maintained at 30 °C. All enzyme and substrate stock solutions were prepared anaerobically in 25 mM Hepes buffer (pH 7.5) containing 150 mM KCl.

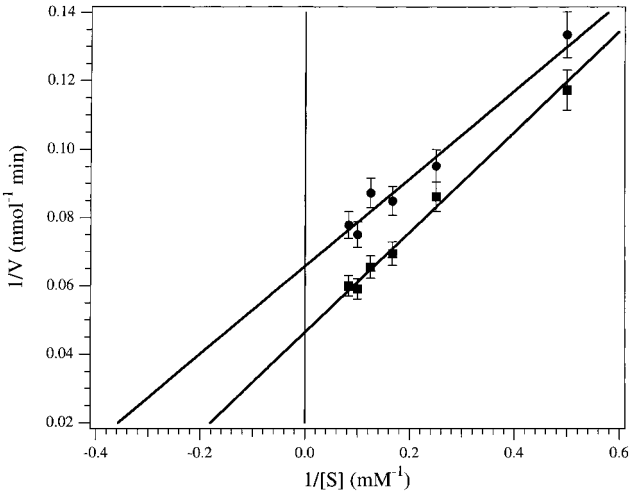

FIGURE 4: Plot of velocity vs substrate (Met-Gly-Met-Met) concentration for Co(II)−MetAP (■) and Fe(II)−MetAP (●). The lines result from a fit of the entire data set for each metal-substituted MetAP enzyme to the Michaelis−Menten equation. All assays were performed under strict anaerobic conditions in an inert atmosphere glovebox in a dry bath incubator maintained at 30 °C. All enzyme and substrate stock solutions were prepared anaerobically in 25 mM Hepes buffer (pH 7.5) containing 150 mM KCl.

detectable change in activity for Co(II)−, Fe(II)−, or Zn(II)−MetAP. In these experiments, apo-MetAP was incubated with 5 mM glutathione for 30 min at 30 °C followed by the addition of 3 equiv of divalent metal ion and incubation for an additional 30 min at 30 °C. For each catalytically active metal-substituted MetAP sample, ICP emission analysis was performed to establish the metal content of the sample. In all cases, no detectable metal ion impurities were observed.

Determination of the K_m Value for Met-Gly-Met-Met. The strength of Met-Gly-Met-Met binding to the active site of MetAP (K_m) was determined for both Fe(II)− and Co(II)−MetAP. Metal-substituted MetAP samples were prepared by adding 3 equiv of the appropriate divalent metal ion to a 20 μM buffered solution of apo-MetAP, and the mixture was incubated at 30 °C for 30 min. The initial rates of hydrolysis of Met-Gly-Met-Met were monitored as a function of Gly-Met-Met concentration in 25 mM Hepes buffer (pH 7.5) and 150 mM KCl. Triplicate activity assay determinations at six concentrations of Met-Gly-Met-Met (0−12 mM) were made, and the results are shown in Figure 4 as a plot of $1/V$ versus 1/[S]. From fits of these data, the Michaelis constant (K_m) for Met-Gly-Met-Met binding to Co(II)−MetAP (■) was determined to be 3.16 ± 0.34 mM, and for Fe(II)−MetAP (●), the K_m was found to be 1.95 ± 0.44 mM. Combination of these data with k_{cat} values for Co(II)− and Fe(II)−MetAP provides the catalytic efficiencies (k_{cat}/K_m) of each enzyme which are 350 ± 40 and 410 ± 40 mM^{-1} min^{-1}, respectively.

DISCUSSION

Until recently, all MetAPs studied to date had been reported to be cobalt-dependent metalloproteases (*3*, *5*, *7*). However, Walker and Bradshaw have suggested that the type I MetAP from yeast is a Zn(II)-dependent enzyme (*28*). Several Zn(II)-containing metalloproteases with active site ligands similar to those of MetAPs can be substituted with Co(II) in vitro, and in nearly all cases, active and even hyperactive enzymes are obtained (*29*, *30*). The previous conclusion that MetAPs are cobalt-dependent enzymes arose from the reproducible observation that MetAPs exhibit high activity in the presence of cobalt when compared to the activity levels of other metal ions. However, the identity of the metal ion in vivo has not been established, and in all studies to date, Co(II) concentrations were artificially increased to the millimolar range during purification. To gain insight into the identification of the in vivo metal ions for the MetAP from *E. coli*, we examined the metal content and enzymatic activities of whole cell extracts in the absence and presence of MetAP expression.

Since the MetAP from *E. coli* is overexpressed at high levels, any change in cellular metal content should reflect the divalent metal ion coordinated to MetAP. The only significant increases in cellular metal ion concentrations were observed for chromium, zinc, manganese, and iron. The increase in iron concentration was 4.4 and 6.2 times greater than that in either manganese and zinc, respectively (Table 1). The cellular cobalt concentration did not change upon overexpression of MetAP (Table 1), suggesting that either MetAP is expressed in the apo form or cobalt is not the in vivo metal ion for the MetAP from *E. coli*. Whole cell extracts from cells where MetAP was overexpressed exhibited reproducibly detectable enzymatic activity (>2 units/mg), while no activity was detected in whole cell extracts without MetAP overexpression. These data indicate that cobalt is not coordinated to MetAP upon overexpression. Since MetAP is active upon expression, these data strongly suggest that cobalt is not the in vivo metal ion for the MetAP from *E. coli*. On the basis of whole cell metal analyses, manganese, iron, and zinc are the most likely candidates for the in vivo metal ion of MetAP.

The MetAP from *E. coli* is only active toward tripeptides or larger substrates (*17*), so commercially available L-amino acid−*p*-nitroanilide and −*p*-nitrophenol substrates were not cleaved. Therefore, an HPLC method was developed to directly detect the major product of hydrolysis, Gly-Met-

Met. This has allowed us to routinely and accurately measure the specific activity of purified MetAP. The specific activity of Co(II)−MetAP was typically found to be 37.3 units per mg of enzyme, more than 3 times the level reported by Ben-Bassat et al. (11.3 units/mg) (*17*). An interesting finding in this study is the fact that the Co(II) ions in MetAP are susceptible to autoxidation at pH 7.5 and are oxidized to the Co(III) form upon prolonged exposure to air. On the other hand, the Fe(II)−MetAP enzyme is oxidized to the Fe(III) form rapidly upon exposure to air. On the basis of our results, both the Co(III)− and Fe(III)−MetAP enzymes are enzymatically inactive. The lack of activity for Co(III)-bound MetAP is not surprising since Co(III) ions are kinetically inert (*29, 30*). Similarly, Fe(III) ions form very thermodynamically stable Fe(III)−hydroxide complexes so if a metal-bound hydroxide existed in Fe(III)−MetAP, it must be unreactive toward the hydrolysis of peptides (*29*). Since all of the activity measurements reported for MetAPs to date have been taken in air, the low levels of enzymatic activity reported for Co(II)-substituted MetAP are likely the result of partial oxidation of Co(II) to Co(III). Moreover, the lack of reported activity for the Fe(II)-bound form of MetAP is likely the consequence of very rapid oxidation of Fe(II) to Fe(III) resulting in activity measurements being taken on the inactive Fe(III) form of MetAP.

The reproducibility of the HPLC assay described herein has allowed us to readdress the ability of divalent cations to stimulate catalytic turnover for MetAP. Of the divalent metal cations tested to date, only Co(II), Fe(II), Mn(II), and Zn-(II) provide detectable levels of enzymatic activity. In each case, excess metal ions were found to be inhibitory. This is similar to results reported for several different MetAPs (*28*). Since MetAPs are found in the cellular cytoplasmic space (*3*), activity measurements of glutathione-treated apo-MetAP enzyme that was then activated with either Co(II), Fe(II), or Zn(II), under anaerobic conditions, were also performed. Under these conditions, no detectable change in the catalytic activity was observed. The addition of 5 mM reduced glutathione to Co(III)− or Fe(III)−MetAP did not provide active enzyme. The observed activity levels of Zn(II)−MetAP were approximately 10-fold lower than those observed for Co(II)−MetAP or Fe(II)−MetAP under similar conditions. Zn(II) ions were recently shown to activate the type I MetAP from *S. cerevisiae*, in the presence of 5 mM glutathione (*28*). The role of glutathione, a cellular reductant, in the Zn(II)-substituted MetAP from yeast was not discussed, but it may potentially bind excess Zn(II) ions since excess Zn(II) was shown to be inhibitory. Alternatively, glutathione may be required to reduce active site Cys residues which are conserved for nearly all type I MetAPs (*24*). However, this alternative is less likely since no change in activity is observed for the MetAP from *E. coli* in the presence or absence of glutathione.

The substrate binding constants (K_m) for Met-Gly-Met-Met at pH 7.5 for the Co(II)− and Fe(II)−MetAP enzymes from *E. coli* were found to be 3.16 and 1.95 mM, respectively. Moreover, the catalytic efficiencies (k_{cat}/K_m) for Co(II)− and Fe(II)−MetAP are 350 and 410 mM^{-1} min^{-1}, respectively. These data indicate that Fe(II)−MetAP is a better catalyst than Co(II)−MetAP. Thus, the suggestion that Fe(II) is likely the in vivo metal ion for the MetAP from *E. coli* is supported by these data since substrate binds to the Fe(II) enzyme 1.6 times more tightly than to the Co(II) enzyme. The K_m values obtained for the MetAP from *E. coli* are similar to those obtained for the type I MetAP enzyme from yeast, assayed in the presence of 0.5 mM Co(II), which was reported to have a K_m value of 6.6 mM for Met-Gly-Met-Met (*10*). Moreover, the K_m value for the MetAP from porcine liver, assayed in the presence of 0.1 mM Co(II), has been found to be in the millimolar range (*31*). On the other hand, the K_m value of the human homologue of a rat initiation factor-2 associate protein (p^{67}) was found to be 0.65 mM toward Met-Gly-Met-Met when assayed in the presence of 0.5 mM Co(II) (*22*). In addition, the K_m value of the type I MetAP enzyme from yeast toward the octapeptide Met-Ser-Ser-His-Arg-Trp-Asp-Trp was found to be 0.019 mM (*28*). Clearly, the chain length of the peptide substrate affects the strength at which substrate binds to MetAPs.

It has been proposed that the K_m values obtained for MetAPs in vitro may not be reflective of the K_m value in vivo which may be lower due to the association of MetAP with the ribosome. Support for this hypothesis is provided by N-terminal extensions that include zinc finger domains in the type I MetAP enzyme from yeast (*10*) and stretches of highly charged amino acids at the N-terminal region of the type II MetAP enzyme from yeast, and the human homologue of rat p^{67} (*22*). These N-terminal extensions have been proposed to assist in the association of these MetAPs with the ribosome where they are positioned to perform N-terminal methionine processing on nascent peptides emerging from the ribosome. The MetAP from *E. coli* appears to lack these extensions, so the mode of its association with the ribosome is unclear at this time.

The fact that (i) no cobalt ions are detected in whole cells containing overexpressed MetAP but significant iron was detected, (ii) MetAP activity is observed in the presence of MetAP overexpression but not in control cells, (iii) Fe(II)−MetAP is a better catalyst than Co(II)−MetAP, and (iv) the Fe(II) enzyme is nearly 10 times more active than Zn(II)-substituted MetAP suggests that Fe(II) is likely the physiologically relevant metal ion. These data suggest that the related enzymes, aminopeptidase P from *E. coli* (AMPP) and prolidase, might also be activated in vitro by Fe(II) ions. These three families of enzymes are related on the basis of sequence comparisons which show that the unusual protein fold observed in MetAPs is also observed in AMPP and prolidase (*32*). AMPP has been crystallographically characterized, revealing active site ligands that are identical to those of MetAPs and AMPP's active site is fully superimposable with those of MetAPs (*33*). Since all MetAPs and AMPPs have fully conserved active site ligands, similar metal ion requirements might be expected. For the AMPP from *E. coli*, it was recently shown that maximal catalytic activity was obtained in the presence of Mn(II) (*34*). AMPP is also nearly equally active in the presence of Co(II) ions but was reported to be inactive in the presence of Zn(II) and Fe(II). No structural data are currently available for prolidase, but catalytic activity measurements show that the prolidase from *P. furiosus* is activated by Co(II) and Mn(II) but not by Fe(II) or Zn(II) (*35*). However, all of the enzymatic activity measurements for both AMPP and prolidase were performed aerobically, so it is likely that the Fe(II) and possibly Co(II) ions had fully or partially oxidized to inactive Fe(III) and Co(III) forms, respectively. Additional studies with AMPP

and prolidase performed under anaerobic conditions with the Fe(II) forms of the enzymes are needed to fully compare activities between these three families of enzymes.

In conclusion, the combination of whole cell metal analyses and activity measurements with in vitro activity measurements and substrate binding constants strongly suggests that the MetAP from *E. coli* is an Fe(II) enzyme. Moreover, iron is the most prevalent cellular metal ion, and MetAPs are found in the cytoplasm where conditions are highly reducing. The catalytic activity of MetAPs in the presence of Fe(II) has not been previously observed because all activity measurements performed on MetAPs to date have been taken under aerobic conditions, causing the Fe(II) ions to quickly oxidize to the inactive Fe(III) form. Hydrolytic enzymes that utilize Fe(II) ions for catalytic activity are not particularly uncommon. The peptide deformylase from *E. coli*, which is the enzyme prior to the MetAP in the methionine cycle, has also been shown to be an Fe(II) enzyme (*36*). Since the cellular functions of these enzymes are tied to one another, Fe(II) may play a cellular regulatory role in protein maturation. Other examples of iron-containing hydrolases include the purple acid phosphatases and the protein phosphatases 2B such as calcineurin (*37*). In addition, several non-heme diiron enzymes, including methane monooxygenase, ribonucleotide reductase, and rubrerythrin, have been crystallographically characterized and exhibit active site structures remarkably similar to the MetAP from *E. coli* (*38*−*40*). Since MetAPs appear to be angiogenic drug targets, characterization of the in vivo metal ion and the catalytic mechanism is critical for the future development of new anti-cancer drugs. Studies that address the roles of each Fe(II) atom in catalysis are currently underway in our laboratory.

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