Cloning, Expression, and Characterization of Human Cytosolic Aminopeptidase P: A Single Manganese(II)-Dependent Enzyme†

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ABSTRACT: The mammalian bradykinin-degrading enzyme aminopeptidase P (AP-P; E.C. 3.4.11.9) is a metal-dependent enzyme and is a member of the peptidase clan MG. AP-P exists as membrane-bound and cytosolic forms, which represent distinct gene products. A partially truncated clone encoding the cytosolic form was obtained from a human pancreatic cDNA library and the 5′ region containing the initiating Met was obtained by 5′ rapid accumulation of cDNA ends (RACE). The open reading frame encodes a protein of 623 amino acids with a calculated molecular mass of 69,886 Da. The full-length cDNA with a C-terminal hexahistidine tag was expressed in *Escherichia coli* and COS-1 cells and migrated on SDS−PAGE with a molecular mass of 71 kDa. The expressed cytosolic AP-P hydrolyzed the X−Pro bond of bradykinin and substance P but did not hydrolyze Gly-Pro-hydroxyPro. Hydrolysis of bradykinin was inhibited by 1,10-phenanthroline and by the specific inhibitor of the membrane-bound form of mammalian AP-P, apstatin. Inductively coupled plasma atomic emission spectroscopy of AP-P expressed in *E. coli* revealed the presence of 1 mol of manganese/mol of protein and insignificant amounts of cobalt, iron, and zinc. The enzymatic activity of AP-P was promoted in the presence of Mn(II), and this activation was increased further by the addition of glutathione. The only other metal ion to cause slight activation of the enzyme was Co(II), with Ca(II), Cu(II), Mg(II), Ni(II), and Zn(II) all being inhibitory. Removal of the metal ion from the protein was achieved by treatment with 1,10-phenanthroline. The metal-free enzyme was reactivated by the addition of Mn(II) and, partially, by Fe(II). Neither Co(II) nor Zn(II) reactivated the metal-free enzyme. On the basis of these data we propose that human cytosolic AP-P is a single metal ion-dependent enzyme and that manganese is most likely the metal ion used in vivo.

Aminopeptidase P (AP-P;[1] E.C. 3.4.11.9; X−Pro aminopeptidase) is found in many different organisms including mammals, yeast, and bacteria. Mammalian AP-P was first identified in porcine kidney as an activity able to remove the N-terminal residue from peptides with a penultimate prolyl residue (*1*). It is now known that there are at least two distinct forms of mammalian AP-P, a membrane-bound form and a cytosolic form. The membrane-bound form, first purified from porcine kidney, is attached to the lipid bilayer by a glycosylphosphatidylinositol (GPI) anchor (*2*). Subsequently the cDNA encoding this form of the enzyme was isolated, sequenced, and shown to encode a protein with a cleavable N-terminal signal peptide to direct its translocation into the endoplasmic reticulum and a C-terminal GPI anchor attachment signal (*3*). The membrane-bound form of AP-P has also been purified from bovine lung (*4*) and rat lung (*5*), and the cDNA encoding the human enzyme has been isolated (*6*). This membrane-bound form of AP-P can metabolize bradykinin and substance P (*7*) and is inhibited selectively by apstatin (*8*).

The cytosolic form of AP-P has been purified from human leukocytes (*9*), human platelets (*10*), rat brain (*11*), and guinea pig brain (*12*). Cytosolic AP-P also hydrolyzes bradykinin and other peptides with a penultimate proline residue and is inhibited by chelating agents. The enzyme purified from rat brain was reported to have a molecular mass of 143 or 218 kDa under native conditions (*11*), dependent on the NaCl concentration, while the enzyme purified from human leukocytes existed as a dimer of 140 kDa (*9*). Both proteins were reported to have subunits that separated on SDS−PAGE with a molecular mass of 71 kDa. The cDNA encoding a putative cytosolic form of AP-P has been isolated from a human lymphocytic cDNA library (*13*), although the expression and subsequent functional characterization of the protein has not been reported.

On the basis of sequence and structural similarities, AP-P is classified as a member of the peptidase clan MG (*14*). This clan comprises several exopeptidases, including the methionyl aminopeptidases, both type I and type II, X−Pro aminopeptidases, and X−Pro dipeptidases (prolidases). Although the overall sequence identity between the proteins in clan MG is quite low, comparison of predicted structural motifs indicated conservation of metal binding residues and

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[1] Abbreviations: AP-P, aminopeptidase P; GPI, glycosylphosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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a similarity in the C-terminal folding, "pita-bread" fold, of the proteins (*15, 16*). The crystal structures of *Escherichia coli* methionyl aminopeptidase (*17*) and *E. coli* AP-P (*18*) revealed that the C-terminal domain has a pseudo-2-fold symmetry. Methionyl aminopeptidase is reported to contain two Co^{2+} ions that are bound by ligands on five amino acid residues, while bacterial AP-P contains two Mn^{2+} ions. These enzymes have thus been considered as dual-metal aminopeptidases, of which leucyl aminopeptidase is the unrelated prototype (*19*). However, recent data have questioned whether in fact only one metal ion is required for activity (*20*) and whether *E. coli* methionyl aminopeptidase contains Fe^{2+} in vivo (*21*).

In this study, we report the cloning, functional expression, and characterization of human cytosolic AP-P. Comparison with the membrane-bound AP-P indicates similarities with respect to substrate specificity and inhibitor profile but a difference in metal ion content. Whereas the membrane-bound form of AP-P purified from porcine kidney contained 1 mol of Zn^{2+} (*22*), the cytosolic form of human AP-P expressed in *E. coli* contained 1 mol of Mn^{2+}. Even in the presence of the reductant glutathione, only Mn^{2+} significantly caused reactivation of the metal-depleted enzyme. We therefore propose that cytosolic AP-P functions as a single Mn-dependent enzyme.

MATERIALS AND METHODS

Materials. The pancreatic adenocarcinoma (CF-PAC1) cDNA library, the nucleoprobe purification kit, and the XL1-Blue MRF′, BL21 (DE3), and SOLR cells were all purchased from Stratagene (Cambridge, U.K.). Dulbecco's modified Eagle's medium (DMEM), trypsin−EDTA, heat-inactivated fetal calf serum, penicillin/streptomycin, phosphate-buffered saline (PBS), OptiMEM (serum-free medium), and the transfection reagent (lipofectAMINE) were all purchased from Gibco−BRL (Paisley, U.K.). All other reagents were of analytical grade and were purchased from Sigma or BDH (Dorset, U.K.).

Cloning of Human Cytosolic AP-P. Primers were designed on the basis of the reported sequence for the putative human lymphocytic AP-P (*13*). Initial PCR reactions with aliquots of a human pancreatic adenocarcinoma cDNA library with the primer pairing of APPF1 (5′-GTATGTGACCGAACCGATCC-3′) and APPR1 (5′-GACCCAAGTCAAGAAGCAGG-3′) all gave products of the expected size (703 bp). One million plaque-forming units (pfu) were plated out on five plates and left to lyse a bacterial lawn. Five sublibraries were made by adding 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, and 0.01% gelatin to the plates. A sublibrary was chosen and the presence of the clone was confirmed by PCR. The 703 bp fragment generated was then used as template for a PCR reaction with the nested primer pairing of APPF2 (5′-GGAAGACTGGCTGGTGAGTGTGC-3′) and APPR2 (5′-CGGAGCTTAATTAGCCACGC-3′). The reaction gave an amplification product of the predicted size, 329 bp. This procedure was repeated twice more to create further enriched sublibraries before screening by conventional plaque hybridization techniques (*23*). A single clone (pAP-P) was isolated and fully sequenced on both strands. The 5′ region of the clone was confirmed by screening two other human cDNA libraries by 5′ RACE.

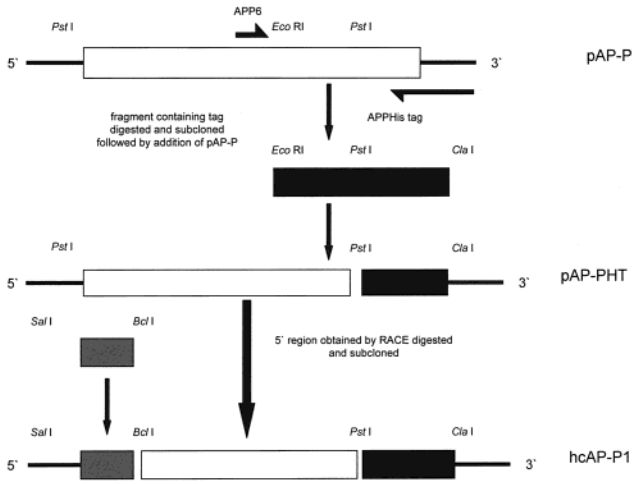

FIGURE 1: Schematic diagram showing the construction of the full-length tagged AP-P clone. A C-terminal portion of human cytosolic AP-P was amplified by use of a primer containing a hexahistidine tag (AP-PHis tag) and an upstream specific primer (APP6). This fragment containing the hexahistidine tag was subcloned (*Eco*RI/*Cla*I) before subsequent addition of the remaining coding region (pAP-P) by use of a *Pst*I digest. A fragment obtained by 5′ RACE was then added to this construct (*Sal*I/*Bcl*I) to yield the full-length tagged clone, hcAP-P1.

Automated sequencing was carried out by Taq Dye Deoxy terminator cycle sequencing in conjunction with the ABI373 A sequencing system. DNA, and protein sequences were assembled and analyzed by use of the Wisconsin-GCG suite.

Construction of the Mammalian Expression Vector. A PCR strategy was used to add a hexahistidine tag to the C-terminus of the partial clone (pAP-P) (Figure 1). pAP-P was amplified with the primers ATGpAP-P (5′-GGATCGGATATGCCTCCAAAGGTGACTTAG-3′) and APPHis tag (5′-CCATCGATTCAGTGATGGTGATGGTGATGCCTACCCTCAATATGCTGTT TGAGATGGGTTGCG-3′) and subcloned into the *Cla*I site of pBluescript SK(−). The 5′ region was added from a *Sal*I and *Bcl*I digest. Finally, the full-length clone was released following a *Sal*I/*Not*I digest and subcloned into pCIneo (Promega). This construct, pCIhcAP-P1, was then sequenced on both strands to ensure its integrity.

Transfection of Cytosolic Aminopeptidase P in COS-1 Cells. Aliquots of the expression vector pCIhcAP-P1 (2 μg) were used to transfect COS-1 cells as described (*3*) except the cells were incubated with the DNA/lipofectAMINE mix overnight before the addition of medium. The cells were left for a further 8 h before the medium was replaced with fresh medium, and the cells were then left for 36 h. Cell lysates were prepared by scraping cells into 2 mL of ice-cold 50 mM HEPES/NaOH, pH 7.4, followed by repeated freeze/thawing. Membrane and cytosolic fractions were prepared as described (*24*). Protein concentrations were determined as described (*3*).

Construction of the Bacterial Expression Vector. The full-length clone was amplified by PCR with the primers hcAP-Ps (5′-GGATCGGATATGTGGACTGACGGGCGCTAC-3′) and APPHis tag. The PCR product was digested with *Cla*I and inserted into the *Cla*I site of pBluescript SK(−). Following another *Cla*I digest, the insert was subcloned into the bacterial expression vector pHD (*25*). The insert was then fully sequenced on both strands. This vector was termed pHDhcAP-P2.

Growth and Induction of Bacterial Cells. A 50 mL starter culture was set up containing *E. coli* BL21 (DE3) and ampicillin (100 μg/mL) and left for 16 h at 30 °C. The cells were diluted to an optical density of 0.05 at 600 nm and left to grow with shaking at 30 °C. Host cells were induced at an A_{600} of 0.5 by increasing the temperature to 40 °C. The cells were then left for 3 h at this temperature and harvested by centrifugation at 150*g* for 20 min at 4 °C.

Affinity Chromatography on Ni^{2+}-NTA−Agarose. All the following steps were carried out at 4 °C. The cells were resuspended in binding buffer [20 mM Tris-HCl, pH 8.2, containing 0.5 M NaCl, 10 mM imidazole, and an EDTA-free "complete" tablet (Boehringer Mannheim)]. After sonication, the cell debris was collected by centrifugation for 20 min at 39000*g*. The supernatant was then filtered through a 0.45 μm filter and applied to a Ni^{2+}-NTA−agarose column (bed volume 4 mL) (Qiagen, Crawley, U.K.). The column was washed with binding buffer until the eluant had an A_{280} of less than 0.01. The bound protein was eluted by applying 40 mL of elution buffer (20 mM Tris-HCl, pH 8.2, 0.5 M NaCl, and 0.5 M imidazole) to the column and 2 mL fractions were collected. Fractions containing the correct product were pooled and then exchanged into 0.1 M Tris-HCl, pH 8.2, by use of protein spin concentrators (Vivascience) with a molecular weight cutoff of 10 000.

SDS−PAGE and Immunoelectrophoretic Blot Analysis. Protein expression, molecular weight determinations, and the purity of protein samples were determined by the use of 3−17% acrylamide gradient gels (*23*). Immunoelectrophoretic analysis was conducted with anti-His(C-terminal) horseradish peroxidase-conjugated antibody (Invitrogen) according to the manufacturer's guidelines. The immunoreactive proteins were visualized by chemiluminescence (ECL kit, Amersham−Pharmacia).

Enzyme Assays. Enzyme activity was assayed by high-performance liquid chromatography (HPLC), with bradykinin or substance P (final concentration 0.1 mM) as substrate, in 0.1 M Tris-HCl, pH 8.2, at 37 °C for 30 min (100 μL final volume). The reactions were terminated by heating for 5 min at 100 °C. The hydrolysis of substrate and quantitation of the products were monitored by reverse-phase HPLC as described previously (*7, 26*). For kinetic analysis, assays were set up containing a range of concentrations of bradykinin and 100 ng of the purified enzyme. After the addition of the substrate, the reactions were incubated at 37 °C for 15 min. The optimum pH for the enzyme was determined over the pH range 5.6−10 with the following buffers in their useful range (5.6−6.6, MES-HCl; 6.6−7.4, MOPS-HCl; 7.4−8.4, Tris-HCl; 8.4−9.4, Bis-tris propane hydrochloride; 9.4−10, CAPS/NaOH). To monitor the effect of inhibitors, purified AP-P (60 ng) was preincubated on ice with the inhibitor for 30 min, prior to the addition of bradykinin and incubation at 37 °C for 30 min. To examine the effect of metal ions, purified AP-P (60 ng) was preincubated on ice for 30 min with the appropriate concentration of divalent cation. Bradykinin was then added to a final concentration of 100 μM and the reaction was incubated at 37 °C for 30 min.

Chelation of Metal Ions from Cytosolic Aminopeptidase P. A solution with a molar ratio of purified AP-P to 1,10-phenanthroline of at least 1:(1×10^7) in 0.1 M Tris-HCl, pH 8.2, was left overnight on ice. The buffer was then exchanged and the protein was concentrated in spin concentrators. Purified metal-free protein (~60 ng) was preincubated on ice for 30 min in the presence of different metal ions and/or glutathione, prior to the addition of bradykinin and incubation at 37 °C for 30 min.

Inductively Coupled Plasma Atomic Emission Spectroscopy. The protein sample (6.7 mg) was prepared in distilled deionized water (4 mL) with a final concentration of nitric acid of 5%. ICP atomic emission spectroscopy was performed on a Fisons Maxim machine with an argon plasma. Metal ion content was quantified from a seven-point calibration curve.

RESULTS

Cloning and Sequence Analysis of Human Cytosolic AP-P. The full-length clone hcAP-P obtained from human cDNA libraries was fully sequenced on both strands. The cDNA sequence has been deposited in GenBank under accession number AF272981. The open reading frame comprised 1869 bp, encoding a protein of 623 amino acids with a calculated molecular mass of 69 886 Da (Figure 2). Although there are several nucleotide differences between the cDNA clone reported here and that reported previously (*13*), only one of these results in a change at the amino acid level. The previously reported sequence indicates a proline at position 332 while here the residue is an arginine. The human cytosolic AP-P displays 43% amino acid identity and 63% similarity to the human membrane-bound AP-P (Figure 2) (*6*). The most notable difference is the lack of the two hydrophobic signal sequences (the N-terminal signal peptide and the C-terminal GPI anchor addition signal) from the cytosolic protein. Sequence alignments with *E. coli* methionyl aminopeptidase (*17*) and *E. coli* AP-P (*18*) indicate conservation of the predicted catalytically important residues (see Figure 2 and ref *27*).

Expression of Human Cytosolic AP-P in COS-1 Cells. Transient expression of human cytosolic AP-P was achieved in COS-1 cells. Whole-cell lysate and membrane and cytosolic fractions were analyzed by immunoblotting with an anti-His(C-terminal) antibody. Under either reducing (data not shown) or nonreducing conditions, a single polypeptide with apparent molecular mass of 71 kDa was detected in the lysate and cytosolic fraction (Figure 3A). No immunoreactive protein was detected in the membrane fraction, consistent with this cDNA encoding the cytosolic form of AP-P. The lysates of transfected COS-1 cells, but not of nontransfected cells, were capable of hydrolyzing bradykinin, and this hydrolysis was inhibited by apstatin (data not shown).

Characterization of E. coli-Expressed Human Cytosolic AP-P. For large-scale expression of the human cytosolic AP-P, the cDNA was inserted into a bacterial expression vector and the protein was expressed in *E. coli* (see Materials and Methods). AP-P was purified by Ni^{2+}-affinity chromatography from the soluble fraction of *E. coli* expressing the construct pHDhcAP-P2. Analysis of fractions eluted from the Ni^{2+} column by SDS−PAGE indicated a major protein of apparent molecular mass 71 kDa (Figure 3B). The minor lower molecular weight bands visible on the gel may represent degradation products. When analyzed under native conditions by gel filtration and native PAGE, the protein had an approximate molecular mass of 140 kDa (data not

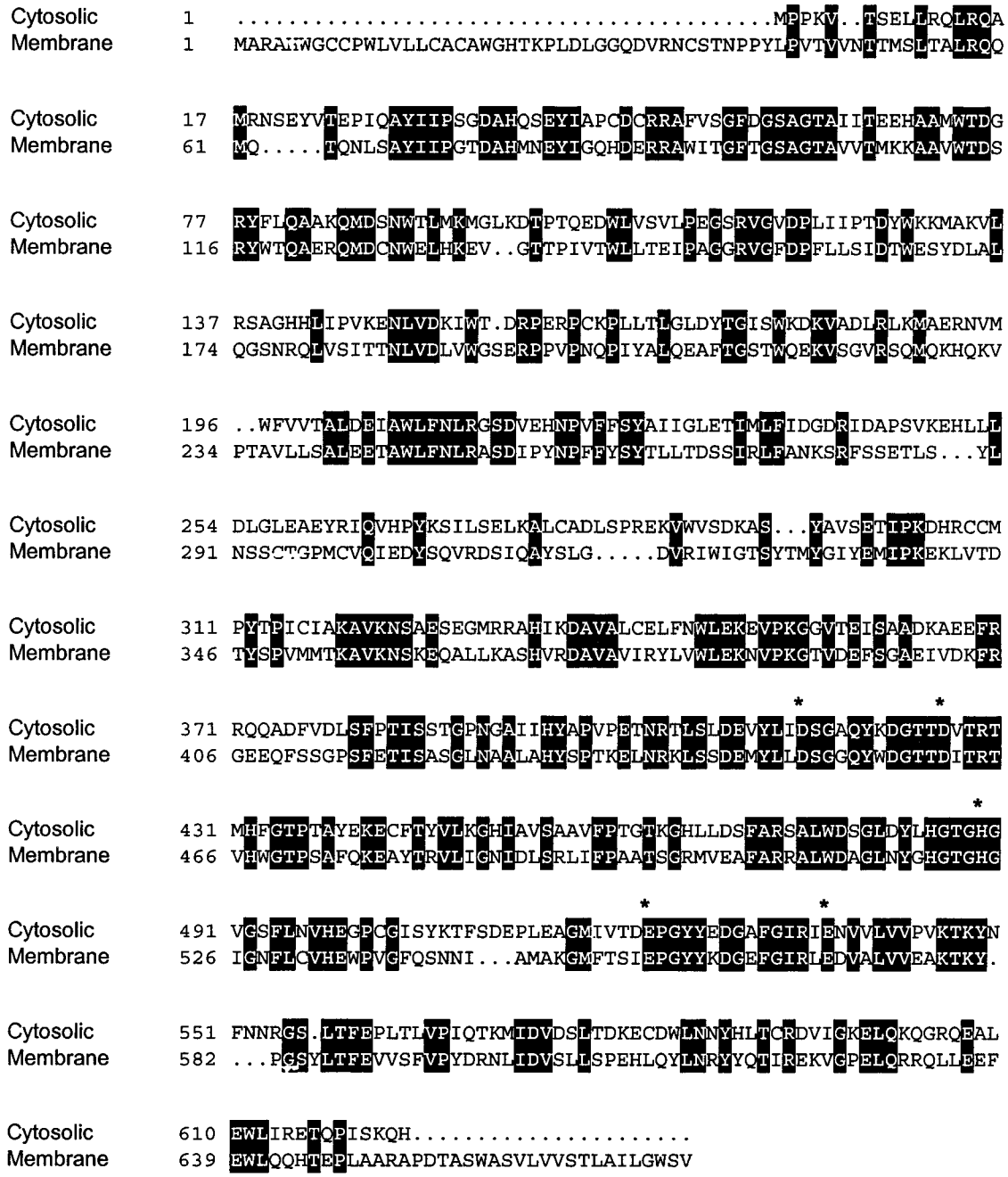

FIGURE 2: Amino acid sequence alignment of human cytosolic and membrane-bound aminopeptidase P. The sequences were aligned with the program PILEUP from the Wisconsin-GCG suite. On the basis of sequence alignments with *E. coli* methionyl aminopeptidase (*17*) and *E. coli* AP-P (*18*), residues predicted to be metal ligands are marked by asterisks. For more detail see Cottrell et al. (*27*).

shown). This is consistent with AP-P existing as a homodimer, in agreement with data on the protein purified from human leukocytes (*9*). The interaction between the subunits could not be disrupted by the addition of β-mercaptoethanol, 1 M NaCl, 1% Triton X-100, or 4 mM CHAPS (data not shown).

The purified human cytosolic AP-P expressed in *E. coli* hydrolyzed bradykinin with K_m of 100.6 μM and k_{cat} of 4.48 s^{-1}, comparing favorably with data previously reported for the enzyme purified from human platelets (K_m = 66 μM) (*10*). The enzyme was able to hydrolyze substance P in the expected way, removing the N-terminal arginine residue with a specific activity of 77.1 $\pm$ 6.3 μmol min^{-1} mg^{-1} and a k_{cat} of 90.01 s^{-1}. Assays with the tripeptide substrate Gly-Pro-hydroxyPro were conducted but no breakdown was observed. The optimum pH for the bacterially expressed enzyme was 8.2, very similar to that of the enzyme purified from human leukocytes (pH 8.0) (*9*).

Effect of Inhibitors on Human Cytosolic AP-P. The effect of various inhibitors on the hydrolysis of bradykinin by the human cytosolic AP-P purified from *E. coli* was determined (Table 1). The chelating agents, EDTA and 1,10-phenanthroline, both inhibited the enzyme, although even at 1 mM EDTA caused maximally 49% inhibition, while 1,10-phenanthroline completely inhibited AP-P at a concentration of 100 μM. Dithiothreitol partially inhibited the enzyme, as seen previously for the enzyme purified from human leukocytes (*9*). The specific AP-P inhibitor apstatin (*8*) completely inhibited the hydrolysis of bradykinin at 100 μM. The angiotensin-converting enzyme inhibitor enalaprilat and

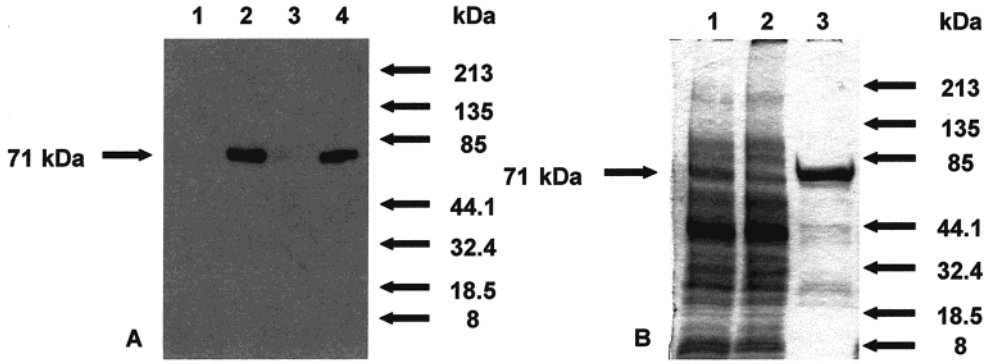

FIGURE 3: Analysis of expressed human cytosolic aminopeptidase P. (A) COS-1 cells were transiently transfected with pCIhcAP-P1 or the control vector, pCIneo. Whole-cell lysates and membrane and cytosolic fractions were prepared as described under Materials and Methods. The fractions were separated on a 3−17% polyacrylamide SDS gel and then subjected to immunoelectrophoretic blot analysis with an anti-His(C-terminal) horseradish peroxidase-conjugated antibody. Lane 1, mock-transfected cell lysate (10 μg); lane 2, transfected cell lysate (10 μg); lane 3, transfected membrane fraction (3 μg); lane 4, transfected cytosolic fraction (10 μg). (B) The product of pHDhcAP-P2 was purified from expressing *E. coli* (BL21 DE3) by Ni^{2+} affinity chromatography as described under Materials and Methods and analyzed on a 3−17% polyacrylamide SDS gel. The proteins were visualized by staining with Coomassie Blue. Lane 1, whole-cell extract before affinity chromatography (20 μg); lane 2, column flowthrough (15 μg); lane 3, eluted fraction (2 μg).

Table 1: Effects of Various Inhibitors on the Activity of *E. coli*-Expressed Aminopeptidase P[a]

inhibitor	concn (mM)	relative activity (%)
none		100 ± 2.7
EDTA	0.01	59.3 ± 5.4
	0.1	55.0 ± 3.0
	1	51.0 ± 1.6
1,10-phenanthroline	0.01	78.0 ± 7.7
	0.1	nd
dithiothreitol	0.01	94.4 ± 2.3
	0.1	66.9 ± 1.9
	1	35.1 ± 5.4
apstatin	0.001	78.2 ± 3.9
	0.01	68.9 ± 4.1
	0.1	nd
enalaprilat	1	93.3 ± 2.8
amastatin	0.1	95.2 ± 9.6

[a] AP-P (60 ng) purified from induced *E. coli* containing the vector pHDhcAP-P2 was incubated on ice with the indicated concentration of inhibitor for 30 min. On addition of 100 μM bradykinin, the samples were incubated at 37 °C for 30 min. Product and substrate were separated and quantified by HPLC as described under Materials and Methods. The results are the mean (± SEM) of triplicate assays. (nd) None detected.

the general aminopeptidase inhibitor amastatin did not significantly inhibit the enzyme.

Metal Ion Content of Human Cytosolic AP-P. Human cytosolic AP-P was purified from the *E. coli* (BL21 DE3) cells and subjected to ICP atomic emission spectroscopy analysis. The metal content of an aliquot containing 6.7 mg of purified protein was analyzed. The data indicated Mn in a molar ratio of metal:protein of 0.99:1, while negligible amounts of Co (0:1), Fe (0.07:1), and Zn (0.11:1) were present. Another aliquot (6.7 mg) of purified protein was incubated with 1,10-phenanthroline to remove any metal prior to analysis. After treatment with the chelating agent, the data indicated similar negligible quantities of Co (0:1), Fe (0.02:1), and Zn (0.09:1), while the Mn content was reduced by a factor of 10 (0.09:1).

Effect of Divalent Cations on the Activity of AP-P. The effect of increasing concentrations of Mn^{2+} on the hydrolysis of bradykinin by the human cytosolic AP-P purified from *E. coli* was examined (Figure 4). Mn^{2+} was seen to promote

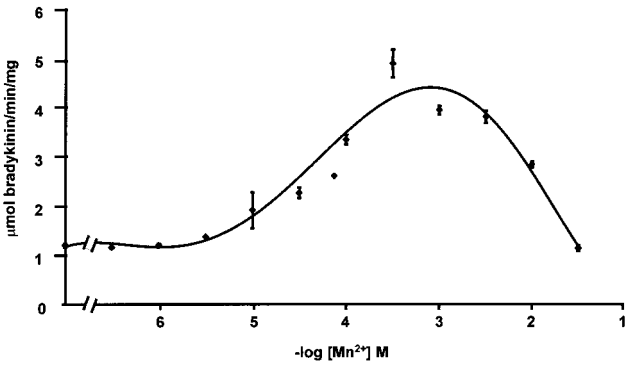

FIGURE 4: Effect of manganese ions on the activity of cytosolic aminopeptidase P toward bradykinin. Human cytosolic AP-P was purified from *E. coli* expressing pHDhcAP-P2. AP-P (60 ng) was preincubated on ice with the indicated concentration of Mn^{2+} for 30 min before the addition of 100 μM bradykinin. The reaction was allowed to proceed for 30 min at 37 °C. Product and substrate were separated and quantified by HPLC as described under Materials and Methods.

activity of the enzyme, with maximal activity observed at a metal ion concentration of approximately 300 μM. The effect of other divalent cations on the activity of the *E. coli*-expressed human cytosolic AP-P was also investigated (Table 2). Ca^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} were all inhibitory, with Zn^{2+} showing the greatest inhibition. Mg^{2+} was also inhibitory but to a much lesser extent. Assays containing Co^{2+} ions showed slight activation at 10 μM and 100 μM concentrations, but at 1 mM Co^{2+} was inhibitory. The effect of glutathione on the activity of the *E. coli*-expressed AP-P was assessed in order to mimic the reducing conditions in the cytosol of a mammalian cell (Table 3). Glutathione alone at 1 mM slightly inhibited (10%) the activity of the human cytosolic AP-P toward bradykinin. In the presence of Mn^{2+} and Co^{2+} glutathione caused a substantial increase in enzyme activity, while with Zn^{2+} there was a slight rise in enzyme activity. The hydrolysis of substance P by the *E. coli*-expressed cytosolic AP-P was significantly activated by 1 mM Mn^{2+} (271% of the activity relative to that in the absence of Mn^{2+}), with only slight additional activation observed in the presence of 1 mM Mn^{2+} and 1 mM glutathione (310% of the activity relative to that in the absence of Mn^{2+} and

Table 2: Effect of Divalent Cations on the Activity of *E. coli*-Expressed Aminopeptidase P

divalent ion	concn (mM)	relative activity (%)	divalent ion	concn (mM)	relative activity (%)
none		100 ± 11.4	Mn^{2+}	0.01	160 ± 5.9
Ca^{2+}	0.01	51.9 ± 2. 5		0.1	279.8 ± 9.3
	0.1	49.7 ± 2.7		1	240.2 ± 7.6
	1	16.9 ± 0.6	Ni^{2+}	0.01	68.4 ± 7.2
Co^{2+}	0.01	103.0 ± 5.2		0.1	70.5 ± 10.2
	0.1	109.5 ± 4.1		1	6.5 ± 2.1
	1	71.0 ± 1.3	Zn^{2+}	0.01	33.9 ± 8.7
Cu^{2+}	0.01	58.8 ± 4.9		0.1	nd
	0.1	33.8 ± 0.8		1	nd
	1	nd			
Mg^{2+}	0.01	73.3 ± 3.4			
	0.1	70.4 ± 4.6			
	1	61.8 ± 3.5			

[a] AP-P (60 ng) purified from induced E. coli (BL21 DE3) containing the vector pHDhcAP-P2 was incubated on ice with the indicated concentration of divalent cation for 30 min. On addition of 100 μM bradykinin, the samples were incubated at 37 °C for 30 min. The product and substrate were separated and quantified by HPLC as described under Materials and Methods. The results are the mean (± SEM) of triplicate assays. (nd) None detected.

Table 3: Effect of Divalent Ions and Glutathione on the Activity of *E. coli*-Expressed Aminopeptidase P[a]

divalent ion	glutathione (1 mM)	relative activity (%)
none	−	100.0 ± 5.2
	+	89.8 ± 6.7
Co^{2+}	−	109.5 ± 4.1
	+	280.1 ± 9.0
Mn^{2+}	−	279.8 ± 9.3
	+	459.7 ± 16
Zn^{2+}	−	nd
	+	25.1 ± 1.4

[a] AP-P (60 ng) purified from induced *E. coli* was preincubated on ice with 100 μM indicated divalent ion in the absence or presence of 1 mM glutathione for 30 min. On addition of bradykinin (100 μM), the samples were incubated at 37 °C for 30 min. The results are the mean (± SEM) of triplicate assays. (nd) None detected.

glutathione). AP-P expressed in COS-1 cells was also activated by Mn^{2+} and Co^{2+} and inhibited by Zn^{2+} (data not shown).

Reactivation of the Metal-Free Enzyme. The human cytosolic AP-P purified from *E. coli* was incubated overnight with 1,10-phenanthroline to remove any metal ions from the protein (see Materials and Methods). This metal-free enzyme failed to hydrolyze bradykinin. The enzyme was then incubated with various divalent cations in the absence and presence of glutathione in an attempt to reactivate it. In the absence of glutathione, enzyme activity was recovered by the addition of 1 mM Mn^{2+} but not by the addition of 100 μM Mn^{2+} (Table 4). Glutathione, at either 1 or 10 mM, resulted in enzyme activity being recovered at either a 10 000-fold lower or a 1000-fold lower concentration of Mn^{2+}, respectively. At 1 mM glutathione, enzyme activity was recovered at a 10-fold lower Mn^{2+} concentration than with 10 mM glutathione, and higher levels of activity were observed. The enzyme was also reactivated in the presence of 1 mM Fe^{2+} and 10 mM glutathione, giving an activity of approximately 57% when compared with manganese (100 μM) and glutathione (1 mM). Neither Co^{2+} (10 nM−1 mM) nor Zn^{2+} (10 nM−1 mM) was able to reactivate the metal-free enzyme in either the presence or absence of glutathione (1 mM). AP-P expressed in COS-1 cells was also purified and treated with 1,10-phenanthroline to remove the metal ion. The purified metal-free AP-P expressed in COS-1 cells could also be reactivated by Mn^{2+} in the presence or absence of glutathione (data not shown).

Table 4: Effect of Manganese and Glutathione on the Activity of *E. coli*-Expressed Aminopeptidase P[a]

Mn^{2+} concn (M)	glutathione (mM)	activity (nmol of bradykinin/30 min)
10^{-4}		nd
10^{-3}		0.70
10^{-8}	1	nd
10^{-7}	1	0.33
10^{-6}	1	0.79
10^{-5}	1	1.07
10^{-4}	1	1.38
10^{-3}	1	1.37
10^{-8}	10	nd
10^{-7}	10	nd
10^{-6}	10	0.85
10^{-5}	10	0.98
10^{-4}	10	1.13
10^{-3}	10	0.95

[a] Approximately 35 mg of purified E. *coli*-expressed aminopeptidase P was incubated overnight on ice in a solution of 0.1 M Tris-HCl, pH 8.2, and 20 mM 1,10-phenanthroline. The buffer was exchanged for 0.1 M Tris-HCl, pH 8.2. Protein (60 ng) was preincubated with Mn^{2+} and/or glutathione at the appropriate concentration for 30 min on ice. Bradykinin was added to a final concentration of 100 μM, and the assays were incubated at 37 °C for 30 min. The results are the means of duplicate assays with differed by less than 10%. (nd) None detected.

DISCUSSION

In the present study we have, for the first time, expressed a functional form of the human cytosolic AP-P in both mammalian and bacterial cells. The cDNA isolated clearly encodes a cytosolic form of human AP-P, as it lacks the sequences encoding the N-terminal signal peptide and C-terminal GPI anchor addition signal present on the cDNA encoding the membrane-bound form (*3*, *6*). Upon expression in COS-1 cells, the encoded protein was located exclusively in the soluble fraction. The enzyme produced from both the mammalian and the bacterial expression systems exhibited identical characteristics, and as more protein could be isolated from the *E. coli* cells, this form of the protein was extensively studied. The expressed form of human AP-P was able to hydrolyze the bioactive peptides bradykinin and substance P but had no activity toward Gly-Pro-hydroxyPro, a substrate commonly used to assay the mammalian membrane-bound AP-P (*1*, *2*). The molecular weight of the expressed protein, its oligomeric state, and its pH optimum for enzyme activity are essentially identical to those previously reported for the cytosolic form of AP-P purified from human sources (*9*, *10*).

The metalloenzyme nature of the expressed human cytosolic AP-P was initially confirmed by its inhibition by the chelating agents EDTA and 1,10-phenanthroline. AP-P purified from human leukocytes was inhibited similarly by EDTA and 1,10-phenanthroline (*9*). Like the membrane-bound form of AP-P (*2*), the cytosolic form was not inhibited by the general aminopeptidase inhibitor amastatin. A cytosolic form of AP-P purified from guinea pig brain was also insensitive to amastatin at the same concentration (*12*). The

selective inhibitor, apstatin, that was developed to inhibit the membrane-bound form of AP-P (*8*) also inhibited the cytosolic form. Thus, apart from the different ability to hydrolyze Gly-Pro-hydroxyPro, the cytosolic and membrane-bound forms of mammalian AP-P appear remarkably similar in terms of their catalytic activity and sensitivity to inhibitors.

During the course of the present study the cDNA encoding a putative cytosolic form of rat AP-P was cloned from a liver cDNA library (*28*). The open reading frame of this clone encoded a protein of 623 residues with a predicted molecular mass of 69,657 Da that has 96% identity at the amino acid level with the human cytosolic AP-P reported here. The expressed rat liver AP-P was shown to be active only toward a synthetic 13 amino acid substrate, with no indication of whether it could hydrolyze bradykinin or substance P.

ICP atomic emission spectroscopy analysis revealed that the human cytosolic AP-P contained 1 mol of Mn per 71 kDa subunit and no detectable levels of Co, Fe, or Zn. This contrasts markedly with the mammalian membrane-bound form of AP-P, which contains 1 mol of Zn and no Co (*7*, *22*). The soluble *E. coli* AP-P also contains Mn (*18*). This difference in metal ion content may reflect an intracellular cytosolic location for the Mn-containing AP-Ps versus an extracellular location for the Zn-containing membrane-bound AP-P. The membrane-bound form of AP-P has its active site facing the extracellular fluid where the concentration of Zn can be as high as 100 μM. In contrast, the concentration of free Zn in the cytoplasm is less than 1 nM due to protein binding and efficient Zn efflux mechanisms (*29*). In comparison, manganese is relatively abundant (10 μM) in the cytoplasm (*30*).

The activity of the human cytosolic AP-P toward bradykinin was stimulated by the addition of Mn^{2+} ions to the assay. This activation profile is similar to the effect of Mn^{2+} on the activity of the porcine membrane-bound AP-P toward Gly-Pro-hydroxyPro as substrate (*2*). With the latter enzyme, when bradykinin was used as substrate, Mn^{2+} had an inhibitory effect (*7*). Following removal of the metal ion, the membrane-bound AP-P was reactivated with Co^{2+} and to some extent with Zn^{2+} in addition to Mn^{2+} (*7*), again contrasting with the cytosolic form, which could only be reactivated with Mn^{2+}. These differences may reflect the metal ion content of these two forms of mammalian AP-P in vivo.

Physiological concentrations of the reductant glutathione, as found in the cytosol of yeast, have been shown to affect the metal ion activation of yeast type I methionyl aminopeptidase (*31*). Although previously classified as a Co^{2+}-containing enzyme, treatment of recombinant apomethionyl aminopeptidase with 12.5 μM Zn^{2+} produced an enzyme that was as active as that reconstituted with 200 μM Co^{2+}. In the presence of a physiological concentration (5 mM) of glutathione, the Co^{2+}-reactivated methionyl aminopeptidase was inactive, whereas the Zn^{2+}-reactivated enzyme was 1.7-fold more active than the enzyme assayed in the absence of glutathione. These data led those authors to propose that Zn^{2+} rather than Co^{2+} is likely to be the true cofactor in vivo. In the present study, glutathione significantly enhanced the reactivation of apo-AP-P by Mn^{2+}. However, even in the presence of glutathione no reactivation of the enzyme was observed with either Zn^{2+} or Co^{2+}, and only partial activity was obtained with Fe^{2+}. Thus, our data strongly suggest that the metal ion cofactor present in human cytosolic AP-P is indeed Mn^{2+} and not Zn^{2+}, Co^{2+}, or Fe^{2+}.

From comparison of the metal binding residues with those present in methionyl aminopeptidase and *E. coli* AP-P, the other members of clan MG, bacterial X−Pro dipeptidase and mammalian AP-P, would be predicted to have two metal ions at their active sites. On purification, bacterial X−Pro dipeptidase was found to contain 1 mol of Co^{2+} but was enzymically inactive, requiring the addition of further Co^{2+} for full activity or Mn^{2+} for partial activity (*32*). In contrast, both human cytosolic AP-P and porcine membrane-bound AP-P (*22*) were active with only one metal ion present, although addition of further metal ions increased the enzyme activity. It should be noted that although human cytosolic AP-P was activated by the addition of Co^{2+} or Mn^{2+}, in contrast to X−Pro dipeptidase, the greater activation was observed with Mn^{2+}.

The metal binding properties of *E. coli* methionyl aminopeptidase as a function of its activity have recently been investigated (*20*). Maximal enzymatic activity was observed after the addition of only 1 equiv of divalent metal ion, either Co^{2+} or Fe^{2+}. Binding of excess metal ions resulted in loss of activity. The data obtained indicated that there were two metal binding sites having different affinities for divalent ions. The high-affinity site had a K_d in the micromolar range and contained the only active-site histidine residue, whereas the low-affinity site had a K_d in the millimolar range. The enzyme appeared to work optimally when only the high-affinity metal binding site was occupied. The ligands that form the low-affinity metal binding site in *E. coli* methionyl aminopeptidase were predicted to bind the cationic N-terminal amino group of the substrate (*20*), and thus binding of metal ions to this low-affinity site may have a regulatory role similar to that in carboxypeptidase A (*33*). Inhibition by millimolar Mn^{2+} was also observed with the human cytosolic AP-P and with the porcine membrane-bound enzyme (*2*). The second metal ion binding site in mammalian AP-P may therefore also be involved in regulation and/or positioning of the substrate. Although molecular modeling and mutagenesis have shown that mammalian AP-P has a very similar active-site configuration to that of other members of clan MG (*27*), elucidation of the crystal structure of a mammalian AP-P will help to confirm the predicted metal ligands, other residues involved in catalysis, and the identity of the metal ion within the active site.

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