Evidence for Catalytic Roles for *Plasmodium falciparum* Aminopeptidase P in the Food Vacuole and Cytosol*\(^{1,2}\)

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The metalloenzyme aminopeptidase P catalyzes the hydrolysis of amino acids from the amino termini of peptides with a prolyl residue in the second position. The human malaria parasite *Plasmodium falciparum* expresses a homolog of aminopeptidase P during its asexual intraerythrocytic cycle. *P. falciparum* aminopeptidase P (PIAPP) shares with mammalian cytosolic aminopeptidase P a three-domain, homodimeric organization and is most active with Mn(II) as the cofactor. A distinguishing feature of PfAPP is a 120-amino acid amino-terminal extension that appears to be removed from the mature protein. PfAPP is present in the food vacuole and cytosol of the parasite, a distribution that suggests roles in vacuolar hemoglobin catabolism and cytosolic peptide turnover. To evaluate the plausibility of these putative functions, the stability properties of recombinant PfAPP were evaluated at the acidic pH of the food vacuole and at the near-neutral pH of the cytosol. PfAPP exhibited high stability at 37 °C in the pH range 5.0–7.5. In contrast, recombinant human cytosolic APP1 was unstable and formed a high molecular weight aggregate at acidic pH. At both acidic and slightly basic pH values, PfAPP efficiently hydrolyzed the amino-terminal X-Pro bond of the nonapeptide bradykinin and of two globin pentapeptides that are potential *in vivo* substrates. These results provide support for roles for PfAPP in peptide catabolism in both the food vacuole and the cytosol and suggest that PfAPP has evolved a dual distribution in response to the metabolic needs of the intraerythrocytic parasite.

Malaria remains one of the most deadly global infectious diseases with an estimated 500 million clinical cases and 2 million deaths annually (1, 2). Clinical manifestations of the disease arise as the protozoan malaria parasite replicates asexually within human erythrocytes. Five species of the genus *Plasmodium* infect humans. The cytoadherent properties of red blood cells infected with *Plasmodium falciparum*, coupled with the ability of the parasite to reach high parasitemia, make it the most virulent species. The emergence of strains of *P. falciparum* that are resistant to affordable anti-malarial drugs such as chloroquine has complicated efforts to manage malaria, and new drugs are urgently needed.

Aminopeptidases catalyze the hydrolysis of amino acids from the amino termini of proteins and peptides. They participate in a wide range of biological processes, including peptide catabolism, protein maturation, antigen presentation on immune cells, and regulation of hormone activity. During the asexual erythrocytic replication cycle of the malaria parasite, aminopeptidases contribute to the catabolism of peptides generated by two major proteolytic pathways. One of these is initiated at the proteasome, a multifunctional protease that plays an important role in the turnover of ubiquitinated cellular proteins in the cytosol (3–5). In addition, the parasite transports host red blood cell cytosol (consisting primarily of hemoglobin) to an acidic degradative organelle, the food vacuole, where it is degraded in a proteasome-independent pathway (6, 7). As up to 75% of the host cell hemoglobin is catabolized during the intraerythrocytic cycle (8, 9), flux through the vacuolar pathway is substantial. Three aminopeptidases have been identified as key players in recycling amino acids from peptides generated by the proteasomal and vacuolar catabolic pathways: leucine aminopeptidase, aminopeptidase N (PfA-M1), and aminopeptidase P (10–14). The latter two enzymes have been found in the food vacuole and therefore may play a direct role in hemoglobin catabolism (11). An aspartyl aminopeptidase is also expressed in asexual stage parasites and hydrolyzes amino-terminal aspartyl and glutamyl substrates (15); however, disruption of its gene does not prevent efficient intraerythrocytic replication (11).

Aminopeptidase P (APP)\(^2\) homologs exhibit high specificity for proline in the second position of the substrate (the P1’ position in the nomenclature of Schechter and Berger (16)) and catalyze the hydrolysis of the X-Pro amide bond, where X is any aminoacyl residue (17). Because of the cyclic nature of the proline side chain, X-Pro-containing peptides are not easily accommodated in the active sites of broad specificity aminopeptidases (17). In mammals, three APP isozymes have been identified. APP1 is found in the cytosolic fraction of cell lysates and has been characterized from a variety of tissues (18–20). Although this enzyme has not, to our knowledge, been localized in intact cells, the apparent lack of specific targeting information is con-

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\(^{\text{2}}\) The abbreviations used are: APP, aminopeptidase P; BSA, bovine serum albumin; hAPP1, human aminopeptidase P1; IMAC, immobilized metal affinity chromatography; Lys(Abz)-Pro-Pro-NA, lysine(N'-2-aminobenzoyl)-Pro-Pro-4-nitroanilide; PfAPP, P. falciparum aminopeptidase P; TEV, tobacco etch virus; YFP, yellow fluorescent protein; HPLC, high pressure liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; rPfAPP, recombinant PfAPP; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
sistent with a role in cytosolic peptide turnover. Active cytosolic forms of APP have been reported in plants (21), fruit flies (22), the microsporidian parasite Encephalitozoon cuniculi (23), and in cellular tissues in Caenorhabditis elegans (24), where it is believed to play a role in the catabolism of peptides produced from ingested bacteria. Mammalian APP2 is a glycosylated ectoenzyme anchored into the membrane of endothelial and epithelial cells with a glycosylphosphatidylinositol attachment (25). The best characterized role of APP2 is the inactivation of the plasma hormone bradykinin, a nonapeptide, through cleavage of the Arg-Pro amino-terminal peptide bond (26, 27). inhibition of APP2 potentiates the vasodilatory and cardioprotective properties of bradykinin, and APP2 has been considered a target for the development of cardiovascular drugs (28–30). A third isoform, APP3, has been identified in the human genome and may be a mitochondrial enzyme but has not yet been characterized (31). Prokaryotic APP homologs contribute to intracellular peptide turnover (32).

*P. falciparum* aminopeptidase P (PfAPP) appears to be important for intraerythrocytic growth, as parasites with a disrupted PfAPP gene could not be isolated (11). We have previously localized a PfAPP-yellow fluorescent protein fusion to the food vacuole and the cytosol of the parasite (11). The cytosolic pool of PfAPP probably fulfills a role in peptide turnover and amino acid recycling that is orthologous to those of the cytosolic enzymes described above. In contrast, there is no report to our knowledge of an aminopeptidase P homolog functioning in an acidic environment akin to the malarial food vacuole. Moreover, characterization of mammalian aminopeptidase P homologs typically reveals a pH optimum of 7–8 with relatively little, if any, activity in the pH range 5.0–5.5 (18–20). Although we have previously detected PfAPP activity at acidic pH (11), the catalytic efficiency of the enzyme has not been characterized. Thus, at the outset of this study it was not clear whether PfAPP has a significant catalytic role in the food vacuole.

Here we have localized untagged, native PfAPP in the parasite and have confirmed the dual cytosolic/vacuolar distribution of the enzyme. The domain organization, quaternary structure, and metal requirement of PfAPP were characterized. To evaluate the plausibility of a catalytic role for PfAPP at acidic and near-neutral pH, its stability in the pH range 5.0–7.5 was assessed and compared with that of human cytosolic APP1, an enzyme that does not, to our knowledge, have a physiological role in an acidic environment. The catalytic efficiency of PfAPP at a range of pH values was characterized with three *X*-Pro-containing peptides, two of which are found in the sequences of human α- and β-globin and therefore represent potentially physiological substrates.

**EXPERIMENTAL PROCEDURES**

Parasite Culture and Isolation—*P. falciparum* clone 3D7 parasites were cultured in human O+ erythrocytes (Interstate Blood Bank) in RPMI 1640 medium supplemented with 27 mm sodium bicarbonate, 11 mm glucose, 0.37 mm hypoxanthine, 10 mg/ml gentamicin, and 5 g/liter Albumax I (Invitrogen). Cultures were synchronized by sorbitol treatment (33). Parasites used for immunoblotting and gel filtration chromatography were isolated from intact red blood cells by treatment with 1 mg/ml saponin (34).

Recombinant Protein Expression and Purification—DNA coding for residues 129–777 of *P. falciparum* aminopeptidase P (gene ID PF14_0517) was amplified by PCR from clone 3D7 genomic DNA using the forward primer 5’-GCA-CGGGATCCGAAAACCTGTGAATTTTCGAGGGAAT-ACTCTGCTGCTAGATTAG and the reverse primer 5’-GCACCAGAGCTTTATTTATGTTATGAAATCGCAATT-GGTT. PCR products were digested with BamHI and HindIII (underlined) and ligated into the same sites in the T7 expression vector pET45b (Novagen). The sequence encoding full-length human cytosolic aminopeptidase P1 (XP-PeP1) was amplified from a plasmid containing a cDNA from an ovarian adenocarcinoma cell line (Mammalian Genome Collection 10592, American Type Tissue Collection) with primers 5’-GCACCGGTACCGAAAACCTGTATTTTCGAGGATCCTGCTGCTAGATTAG and 3’-GCA-CAAAGCCTTTATGCTGTTTGGAGATGGGTTGC and was cloned into the KpnI and HindIII sites of pET45b. Both aminopeptidase sequences were in-frame with a vector-encoded amino-terminal hexahistidine tag and a primer-encoded tobacco etch virus (TEV) protease cleavage site ENLYFQS ([35]; italicized sequence in the 5’-primers). Coding sequences were verified by DNA sequencing.

*Escherichia coli* BL21(DE3) Rosetta 2 cells (Novagen) containing the PfAPP or hAPP1 expression plasmid were grown to an absorbance at 600 nm of 0.8, and protein expression was induced by adding 1 mm isopropyl β-D-thiogalactopyranoside for 3 h at 37 °C (PfAPP) or 25 °C (hAPP1). Cell pellets were resuspended in immobilized metal affinity chromatography (IMAC) buffer (20 mm NaH2PO4, pH 7.4, 500 mm NaCl, 30 mm imidazole) supplemented with 1 mm 4-(2-aminoethyl)benzenesulfonyl fluoride and 1 mg/ml hen egg white lysozyme and were incubated on ice for 30 min. After sonication, the lysate was clarified by centrifugation at 20,000 g for 15 min at 4 °C. Protease inhibitors were added to the supernatant at 16 mg/g of cell pellet wet weight. The mixture was stirred on ice for 15 min, and the precipitated material was removed by centrifugation as above. The clarified lysate was loaded onto a Ni2+-charged HisTrap column, and protein was incubated with His-tagged TEV protease (expressed and purified as described (36)) at an aminopeptidase/TEV protease molar ratio of 10:1 (PfAPP) or 30:1 (hAPP1) in 50 mm Tris–HCl, pH 8.0, overnight at 4 °C. The cleavage mixture was passed over a Ni2+-charged HisTrap column, and the TEV protease-cleaved protein eluting in the flow through was dialyzed into 50 mm Tris–HCl, pH 7.5, 200 mm NaCl at 4 °C and then concentrated in an Ultra-4 centrifugal device (Amicon). The protein was injected onto a Superdex 200 10/30 gel filtration column (GE Healthcare) equilibrated in 50 mm Tris–HCl, pH 7.5, 200 mm NaCl. For PfAPP, the major (dimer) peak was collected, concentrated, and repurified on the Superdex column. Purified protein was supplemented with 1 mm MnCl2.
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snap-frozen in liquid nitrogen, and stored at −80 °C. Protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 62,370 M⁻¹ cm⁻¹ (PfAPP) and 104,280 M⁻¹ cm⁻¹ (hAPP1), which were calculated from the amino acid sequences using the ProtParam tool on the ExPASy website.

Native and Recombinant PfAPP Quaternary Structure—Approximately 10⁰ trophozoite-stage, saponin-treated parasites were suspended in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl containing 1 μM N-(trans-epoxy succinyl)- l-leucine 4-guanidinobutylamide, 1 μM pepstatin, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonfyl fluoride. After sonication, the lysate was clarified by centrifugation at 100,000 g for 60 min at 4 °C. The supernatant was injected onto a Superdex 200 10/300 column equilibrated in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl. PfAPP activity in eluted fractions was determined using the fluorogenic substrate (N²-2-aminobenzyloxyl)-lysyl- prolyl-proline-4-nitroanilide (Lys(Abz)-Pro-Pro-NA; see under “Enzyme Assays and Kinetic Analysis”). 90 μg of purified recombinant PfAPP was injected onto the gel filtration column and assayed in the same fashion. The molecular mass of PfAPP was estimated from a calibration curve generated with ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (14 kDa). The void volume was estimated with blue dextran 2000.

Antibodies and Immunoblotting—Anti-PfAPP sera were produced in rabbits using IMAC-purified recombinant PfAPP as the immunogen (Cocalico Biologicals). Serum VP167 was used in all experiments presented here. The serum was diluted 1:10,000 in immunoblotting experiments. Signal was detected by chemiluminescence using a horseradish peroxidase-conjugated anti-rabbit secondary antibody and the ECL kit (GE Healthcare) and was detected on film.

Immunofluorescence and Cryo-immunoelectron Microscopy—For immunofluorescence assays, clone 3D7 parasites were fixed in suspension with 4% paraformaldehyde and 0.0075% glutaraldehyde, permeabilized, and blocked as described previously (37). Fixed parasitized erythrocytes were incubated with anti-PfAPP serum or preimmune serum (1:2700 dilution) for 1 h at room temperature, washed, and incubated with an Alexa 594-conjugated anti-rabbit secondary antibody (Invitrogen). Cells were allowed to settle onto polyethyleneimine-coated coverslips and were mounted with ProLong Gold (Invitrogen). Images were collected on a Zeiss AxioImager M1 equipped with a 4.6 mm; Alltech) equilibrated in 4% acetoacetic acid and were injected onto a Prevail C18 column (5 μm particle size, 250 × 4.6 mm; Alltech) equilibrated in 4% acetonitrile, 0.08% phosphoric acid. Substrate and product were resolved with a linear gradient from 20 to 45% acetonitrile in 0.08% phosphoric acid and were detected by absorbance at 214 nm. Product concentrations were calculated by relating peak areas to that of a known quantity of bradykinin(2–9) (Bachem) and was detected on film.

Enzyme Assays and Kinetic Analysis—Routine PfAPP or hAPP1 assays were carried out using the quenched fluorogenic substrate Lys(Abz)-Pro-Pro-NA (39) Bachem) at 100 μg) or PfAPP (15 μg) was diluted into 50 mM sodium succinate, pH 5.5, or 50 mM Tris-HCl, pH 7.5, supplemented with 200 mM NaCl and 1 mM MnCl₂ and then dialyzed against the same buffer for 3 h at 8 °C. Dialyzed samples were incubated at 37 °C for 30 min and transferred to ice. Quaternary structure was analyzed on a Superdex 200 column equilibrated in the same buffer as was used for dialysis.

Enzyme Assays and Kinetic Analysis—Routine PfAPP or hAPP1 assays were carried out using the quenched fluorogenic substrate Lys(Abz)-Pro-Pro-NA (39) Bachem) at 100 μg (unless otherwise stated) in 50 mM Tris-HCl, pH 7.5, 1 mM MnCl₂. Assays were read in microplates at 37 °C using a Victor3 fluorometer (PerkinElmer Life Sciences) with excitation and emission wavelengths of 340 and 430 nm, respectively.

PfAPP-catalyzed hydrolysis of the peptide substrates bradykinin (Bachem) and HbPep1 and HbPep2 (custom-synthesized at >95% purity by EZBiolab) was monitored using a high pressure liquid chromatography (HPLC)-based assay (40). PfAPP (4–73 ng) was incubated in 100 mM buffer (Tris-HCl, pH 7.5, BisTris-HCl, pH 6.5, sodium succinate, pH 5.5, or MES, pH 5.5), 1 mM MnCl₂, and a range of substrate concentrations at 37 °C for 30 min. Reactions were stopped by adding 10 μl of glacial acetic acid and were injected onto a Prevail C18 column (5 μm particle size, 250 × 4.6 mm; Alltech) equilibrated in 4% acetonitrile, 0.08% phosphoric acid. Substrate and product were resolved with a linear gradient from 20 to 45% acetonitrile in 0.08% phosphoric acid and were detected by absorbance at 214 nm. Product concentrations were calculated by relating peak area to that of a known quantity of bradykinin(2–9) (Bachem) or of HbPep1 or HbPep2 that had been quantitatively converted to product by incubation with PfAPP. The amount of enzyme used depended on the substrate and pH and was adjusted so that less than 10% of substrate was converted to product.
Kinetic parameters were obtained by nonlinear regression fit to the Michaelis-Menten Equation 1,

\[ v = \frac{[E]_0[S]k_{cat}}{K_m + [S]} \]  

(Eq. 1)

where \( v \) is the initial rate, \([E]_0\) is the enzyme concentration, and \([S]\) is the substrate concentration, using KaleidaGraph 4.0. Where substrate inhibition was observed, the data were fit by nonlinear regression to Equation 2 for uncompetitive substrate inhibition

\[ v = \frac{[E]_0[S]k_{cat}}{K_m + [S] + [S]/K_i} \]  

(Eq. 2)

where \( K_i \) is the inhibition constant (41).

Mass Spectrometry—PfAPP-catalyzed hydrolysis products were purified by HPLC using the conditions described under “Enzyme Assays and Kinetic Analysis” but with phosphoric acid in the mobile phase replaced with 0.1% formic acid. Samples were dried under vacuum and resuspended in 10 \( \mu \)l of 50% acetonitrile, 0.1% trifluoroacetic acid, 0.2% formic acid in a sonicating water bath for 5 min. Samples were mixed 1:1 with matrix solution (20 mg/ml 2,5-dihydroxybenzoic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 1% phosphoric acid), and 1 \( \mu \)l of the mix was spotted onto a MALDI target plate and allowed to air dry. Data were collected in positive reflectron mode using an Applied Biosystems 4800 MALDI TOF/TOF.

RESULTS

Distribution of Aminopeptidase P in Protozoa—A single-exon gene (PF14_0517) on chromosome 14 of \( P. falciparum \) encodes a 777-amino acid homolog of aminopeptidase P (termed PfAPP). Single putative orthologs of PfAPP were found in the genome sequences of mammalian and avian \textit{Plasmodium} species (\textit{Plasmodium vivax}, \textit{Plasmodium knowlesi}, and \textit{Plasmodium gallinaceum} (incomplete fragment)) as well as in other apicomplexans for which high quality genome sequence was available (\textit{Toxoplasma gondii}, \textit{Neospora caninum}, \textit{Cryptosporidium hominis}, and \textit{Theileria parva}; supplemental Table S1). A BLAST search of protozoan genomes using the catalytic domain of PfAPP as a query sequence revealed the presence of putative APP homologs in the genomes of the trypanosomatids \textit{Trypanosoma cruzi}, \textit{T. brucei}, and \textit{Leishmania major} as well as in \textit{Dictyostelium discoideum} and \textit{Tetrahymena thermophila}. APP homologs were not found in the genome sequences of \textit{Giardia lamblia} and \textit{Trichomonas vaginalis}; however, our search turned up multiple putative pro-lidase homologs (X-Pro dipeptidase; supplemental Table S1). Prolidase is related to APP and hydrolyzes an amino-terminal X-Pro bond; however, unlike APP, prolidase only accepts dipeptide substrates. These results indicate that APP is present in all apicomplexan species examined and is widely, but perhaps not universally, distributed among protozoa. It does appear, however, that the ability to catalyze the hydrolysis of an amino-terminal X-Pro bond is encoded in all protozoan genomes examined (either as APP or prolidase or both) and may therefore be an essential metabolic activity.

Localization of native PfAPP—We have previously shown that endogenous PfAPP tagged with yellow fluorescent protein (YFP) exhibits a bipartite distribution in the food vacuole and cytosol of the parasite (11). To confirm that this unusual distribution occurs in the absence of the YFP tag, native PfAPP was localized in aldehyde-fixed parasites using antibodies raised against recombinant PfAPP. In indirect immunofluorescence experiments, an anti-PfAPP serum strongly labeled the food vacuole with somewhat weaker labeling of the cytosol of the parasite (Fig. 1A). A similar vacuolar/cytosolic pattern of anti-PfAPP/immunogold labeling was observed by cryo-immunoelectron microscopy (Fig. 1B). The distribution of gold particles in the food vacuole (79%) and outside of the food vacuole but within the parasite (21%) in Fig. 1B agrees well in a qualitative sense with the relative vacuolar and cytosolic fluorescence intensities in Fig. 1A. In both experiments, no labeling was observed with preimmune serum. These results are in accord with the previously reported distribution of PfAPP-YFP (11) and confirm the presence of vacuolar and cytosolic pools of PfAPP in the parasite.

Characterization of Native PfAPP—Although mammalian APP1 and \textit{E. coli} APP catalyze the same reaction, they differ in...
the number of domains and in quaternary structure. *E. coli* APP possesses two domains: a carboxyl-terminal catalytic domain and a smaller amino-terminal domain that contributes to the formation of the tetrameric quaternary structure (42). In human APP1 (hAPP1), which forms a dimer, two domains (I and II) precede the carboxyl-terminal catalytic domain (III). Domains I and II share sequence similarity, and both are structurally similar to the amino-terminal domain of *E. coli* APP (43). Alignment of PfAPP with hAPP1 and with predicted sequences from other apicomplexan genomes reveals that the apicomplexan enzymes contain the three-domain organization of hAPP1 rather than the two-domain structure of prokaryotic APP (supplemental Fig. S1). The alignment also reveals in PfAPP an amino-terminal extension of 120 amino acids relative to the start of hAPP1 that is not present in the other apicomplexan homologs (supplemental Fig. S1). Analysis of the first 70 residues of this amino-terminal extension with the signal peptide prediction algorithm SignalP 3.0 (44) reveals a putative signal peptide from residues 5 to 16 (supplemental Table S1 and supplemental Fig. S1). No signal peptide was detected in any of the apicomplexan homologs (supplemental Table S1). Possible roles for the PfAPP amino-terminal extension are discussed under “Discussion.”

To characterize native PfAPP, polyclonal antibodies against recombinant enzyme (see under “Expression of Recombinant Proteins”) were raised in two rabbits. Both anti-PfAPP sera (but not the preimmune sera) recognized a single major band with a molecular mass of ∼73 kDa in an extract of trophozoite- and schizont-stage parasites (Fig. 2A and data not shown). Minor species at around 90 and 55 kDa were also apparent. To determine which of these bands corresponds to active PfAPP, an extract of soluble trophozoite-stage proteins was fractionated on a gel filtration column, and PfAPP activity was quantified (Fig. 2B). PfAPP eluted in a single peak with an apparent molecular mass of 157 kDa. Anti-PfAPP immunoblotting of active fractions revealed that both the major 73-kDa and the minor 55-kDa species co-migrated with activity (Fig. 2C). Native PfAPP is therefore a homodimer if a monomer molecular mass of 73 kDa is assumed. It appears from these data that full-length PfAPP, with a predicted molecular mass of 90 kDa, is processed into a 73-kDa mature form, likely through removal of the amino-terminal extension. The 55-kDa species, which migrates with active, dimeric PfAPP, probably represents a minor fraction of mature PfAPP that has been proteolytically clipped at an internal site. Consistent with this idea, we observe a 20-kDa band on overexposed immunoblots that likely represents the second proteolytic fragment.

The dimer interface of hAPP1 is dominated by hydrophobic interactions between the catalytic domains (domain III) of the monomers (43). Of the 11 residues identified in the hydrophobic interface, seven are conserved in hydrophobic character in PfAPP (supplemental Table S2). The four that are not conserved in PfAPP lie at the edge of the interface and are likely able to reposition their polar side chains to interact with bulk solvent. We propose that PfAPP, like hAPP1, forms a homodimer through interaction of two catalytic domains.

Expression of Recombinant Proteins—To characterize in detail the enzymatic properties of PfAPP, we expressed it in *E. coli*. As the major form of the enzyme in parasites appears to lack the amino-terminal extension, we expressed an amino-terminally truncated form of PfAPP (residues 129–777). The recombinant protein, termed rPfAPP, carried an amino-terminal hexahistidine tag with a TEV protease recognition sequence between the tag and rPfAPP. After purification and cleavage of the His6 tag with TEV protease (Fig. 3A), rPfAPP had a nearly identical mobility on an SDS-polyacrylamide gel to that of
native PfAPP (Fig. 3B). When analyzed by gel filtration chromatography, the major peak of rPfAPP activity co-eluted with that of dimeric native PfAPP (Fig. 2B). An active, minor peak (estimated ~20% of total activity) that might represent a dimer of dimers (4.9 polypeptides based on a monomer mass of 73 kDa) was observed. This species appeared to be in equilibrium with the dimeric species, as it could not be depleted by purifying the major (dimer) peak. Human APP1 (residues 1–623) was purified in a similar fashion (Fig. 3C) and migrated on a gel filtration column as expected for a dimeric species (data not shown).

Effect of Metal Ions on rPfAPP Activity—Metal ions in the active site of aminopeptidase P play a key role in catalysis by polarizing a bound water molecule and facilitating deprotonation to the nucleophilic hydroxide species (42). In the x-ray crystal structures of E. coli APP and hAPP1, two Mn²⁺ ions are bound in the active site by one histidine, two glutamate, and two aspartate ligands (43, 45). In contrast, pig kidney APP2 has been reported to contain 1 m eq of Zn²⁺ (46). To assess the metal requirement of PfAPP, the enzyme was dialyzed against 1 mM EDTA. After this treatment, activity decreased over 80% (Fig. 4A); residual activity was probably because of incomplete removal of active site metal ions. Following removal of EDTA, re-activation of the enzyme was attempted by addition of various bivalent cations. Addition of 1 mM Mn²⁺ resulted in a return to the control (undialyzed) level of activity (Fig. 4A). Co²⁺ partially activated the enzyme, whereas Mg²⁺ had no effect, and Zn²⁺, Ni²⁺, and Cu²⁺ were inhibitory at 1 mM. These data are consistent with a role for Mn²⁺ rather than Zn²⁺ in the active site of PfAPP.

To examine the relationship between Mn²⁺ concentration and activity against a peptide substrate, rates of PfAPP catalysis of bradykinin hydrolysis were determined at exogenous Mn²⁺ concentrations ranging from 0 to 4 mM (Fig. 4B). Low millimolar levels of Mn²⁺ resulted in a 3–4-fold increase in PfAPP activity. Similar levels of activation in the presence of millimolar concentrations of Mn²⁺ have been observed with mammalian cytosolic APP1 (18–20, 47, 48). Exogenous Mn²⁺ may stimulate activity by promoting occupation of the low affinity Mn²⁺ site in the active site of the enzyme (see under “Discussion”). For this reason, we routinely added 1 mM MnCl₂ to PfAPP assays.

Stability of rPfAPP and hAPP1—pH can be an important factor in the stability of enzyme activity over time. A pH of 7.2 has been reported for the P. falciparum cytosol (49), and published values for the food vacuole have ranged from 4.5 to 5.8 with most determinations falling within the range 5.0–5.5 (49–53). To assess the functional potential of PfAPP in both cellular compartments, we measured the stability of PfAPP activity at 37 °C over 1 h at pH values ranging from 5.0 to 7.5. As metalloenzymes are often stabilized in the presence of metal ligand, we included 1 mM MnCl₂ in the assay. In addition, bovine serum albumin was added at 100 μg/ml to prevent nonspecific adsorption of the enzyme to surfaces following dilution into the activity assay. At pH 7.5 and 6.5, no loss of activity was observed over the course of the 60-min incubation (Fig. 5A). At pH 5.5...
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and 5.0, PfAPP activity was only slightly less stable, with about 80% retention of activity after 60 min.

To determine whether stability in the pH range of 5.0–7.5 is a property intrinsic to aminopeptidase P enzymes, we evaluated the stability of recombinant hAPP1. Human APP1 was chosen for this comparison because it shares with PfAPP a three-domain organization, a dimeric quaternary structure, and high activity with Mn(II) in the active site; however, unlike PfAPP, hAPP1 is not known to function in an acidic environment. In marked contrast with PfAPP, hAPP1 rapidly lost activity at pH 5.5 with only 30% of initial activity present after a 10-min incubation (Fig. 5B). At all pH values examined, loss of activity appeared to be a biphasic process having an initial exponential phase (Fig. 5B). At pH 5.5, over 90% of initial activity was lost in the exponential phase, during which enzyme activity decayed with a half-time of 6 min. Stability of activity at pH 5.5 was not affected by the buffer in the assay; succinate, citrate, and MES yielded similar results. Likewise, addition of 10% glycerol to the assay or substitution of BSA with 0.1% Triton X-100 did not enhance hAPP1 stability at pH 5.5. It has been reported that hAPP1 purified from E. coli cells grown in standard Luria-Bertini broth has sub-stoichiometric levels of Mn²⁺ and, in addition, contains substantial amounts of the presumed nonphysiological metals Mg²⁺ and Fe³⁺ (43). To assess whether inappropriate or incomplete occupancy of the metal-binding sites of hAPP1 could explain its low stability at acidic pH, we dialyzed hAPP1 against 1 mM MnCl₂ overnight and purified hAPP1 from cells grown in medium containing 1 mM MnCl₂, a condition that was shown to yield nearly full Mn²⁺ occupancy of the divalent metal active site (43). In neither case was enzyme stability enhanced at pH 5.5.

Li et al. (43) have shown that mutation of a tryptophan residue in the hAPP1 dimer interface to glutamate (W477E) results in a monomeric protein. The W477E mutant exhibited less than 10% of wild-type activity, which suggests that monomeric hAPP1 has weak, if any, activity. We speculated that dimer instability of hAPP1 at acidic pH might be the reason for the inactivation observed in Fig. 5B. To test this idea, we dialyzed hAPP1 into pH 5.5 or pH 7.5 buffer (including in both cases 1 mM MnCl₂ and 200 mM NaCl), incubated the dialyzed enzyme hAPP1 into pH 5.5 or pH 7.5 buffer (including in both cases 1 mM MnCl₂ and 200 mM NaCl), and assessed the oligomerization state at pH 5.5 was only 8% that in the pH 7.5 control.

It appears that the aggregate consisted mainly of inactive void volume, with no apparent dimeric or monomeric protein. The W477E mutant exhibited less activity at pH 5.5 than in the pH 7.5 control (This study).

The dimeric species was the major form of the enzyme. At pH 5.5, a minor, broad peak appeared at lower elution volume (centered at 10 ml in the right panel of Fig. 5C), which suggested the formation of a small amount of aggregate at acidic pH. At both pH 7.5 and 5.5, a small fraction of rPAPP eluted in the void volume; however, the size of this peak did not vary with pH. Together, our data point to a substantial difference in the stabilities of PfAPP and hAPP1 at acidic pH.

| Enzyme                | pH  | Kₘ  | kₐₜ | kₐₜ/Kₘ | Kₘ   | Ref.   |
|-----------------------|-----|-----|-----|--------|------|-------|
| Substrate, bradykinin |     |     |     |        |      |       |
| PfAPP                  | 7.5 | 0.14| 16  | 1.1 × 10⁻² | –    | This study |
| PfAPP                  | 6.5 | 0.97| 88  | 9.1 × 10⁻⁴ | –    | This study |
| PfAPP                  | 5.5 | 6.7 | 1.6 × 10⁻² | 2.3 × 10⁻⁴ | –    | This study |
| hAPP1                  | 8.0 | 0.078| 3.8 | 4.9 × 10⁻⁴ | 43   |       |
| APP2                   | 6.8 | 0.021| 12  | 5.7 × 10⁻² | 67   |       |
| E. coli APP            | 8.0 | 0.36| 69  | 1.9 × 10⁻² | 66   |       |
| Substrate, HbPep1      |     |     |     |        |      |       |
| PfAPP                  | 7.5 | 0.51| 8.6 | 1.7 × 10⁴ | –    | This study |
| PfAPP                  | 5.5 | 0.86| 5.4 | 6.3 × 10⁻⁴ | –    | This study |
| Substrate, HbPep2      |     |     |     |        |      |       |
| PfAPP                  | 7.5 | 1.4 | 1.5 × 10⁻⁵ | 1.1 × 10⁻³ | 4.4  | This study |
| PfAPP                  | 5.5 | 1.8 | 12  | 6.5 × 10⁻⁵ | –    | This study |

**TABLE 1**

Kinetic parameters for the hydrolysis of X-Pro peptides by PfAPP and selected homologs

The sequence of each substrate is given in single-letter amino acid code. All values were determined at 37 °C except for those for E. coli APP, which were determined at 40 °C. A dash in the Kₘ column indicates that substrate inhibition was not observed (PfAPP) or reported previously (human APP1, rat APP2, and E. coli APP).

### pH Dependence of rPfAPP Catalysis

If the notion of catalytic roles for PfAPP in the food vacuole and cytosol is to be plausible, the enzyme must not only be stable but must also function as an efficient catalyst at acidic and near-neutral pH. We determined the pH dependence of the kinetic parameters for the PfAPP-catalyzed hydrolysis of three peptide substrates having an X-Pro amino-terminal sequence. The first peptide we analyzed was the nonapeptide hormone bradykinin (RPPGFSPFR), a physiological substrate of mammalian APP2. Although this peptide is almost certainly not a physiological substrate of PfAPP, it has been used to characterize the properties of both eukaryotic and prokaryotic APP homologs and therefore serves as a point of comparison between homologs. Plots of initial rate against substrate concentration for rPfAPP-catalyzed hydrolysis of bradykinin at pH 7.5, 6.5, and 5.5 fit well to the Michaelis-Menten equation (supplemental Fig. S2). Kinetic constants for hydrolysis of bradykinin by PfAPP and by mammalian APP1 and APP2 and E. coli APP are compiled in Table 1. At pH 7.5 and 6.5, the catalytic constants (Kₘ, kₐₜ, and kₐₜ/Kₘ) of rPfAPP are similar to those of eukaryotic and prokaryotic homologs. One difference that stands out is the substantially lower Kₘ value for hydrolysis of bradykinin by rat APP2; this difference is probably attributable to the fact that bradykinin is a physiological substrate of APP2 but not of PfAPP. As the pH of the reaction is lowered to 5.5 both the Kₘ and the kₐₜ values for rPfAPP hydrolysis increase significantly (48- and 10-fold increases from pH 7.5 to 5.5, respectively). The net result is that the catalytic efficiency (kₐₜ/Kₘ) at pH 5.5 is 5-fold lower than at pH 7.5.

We next evaluated the ability of rPfAPP to catalyze the hydrolysis of the X-Pro bond in two potentially physiological pentapeptide substrates found in the sequences of human α- and β-globin. Both globin chains contain seven proline residues, and catabolism of these polypeptides in the food vacuole is likely to give rise to peptides with a proline in the second position. We selected the sequences FPHFD (from α-globin; termed HbPep1) and YPWTQ (from β-globin; termed HbPep2). These sequences were chosen as they contain bulky residues preceding and following the prolyl residue and were...
tetrapeptides (supplemental Table S3). Products of HbPep1 and HbPep2 hydrolysis were the expected SPFR, also conforms to the 

...ions, as the octapeptide product of the first reaction, PPGF-

...sumably generated through two consecutive hydrolysis reac-

...product consisted of bradykinin-(3–9), which co-migrated with 

...at pH 7.5 and 5.5, the dominant product was bradykinin(2–9)

...MALDI-TOF mass spectrometry. For hydrolysis of bradykinin

...dropped around 10-fold, whereas the

...Km

...cat values for hydrolysis

...PfAPP activity was greatly reduced following dialysis against 

...spp.; thus, in this genus APP appears

...to be the only enzyme capable of catalyzing the hydrolysis of an 

...terminal X-Pro bond. All protozoan genomes examined here possess at least one (putative) enzyme that can catalyze the 

...hydrolysis of the X-Pro bond. These observations point to a 

...critical role for this activity in protozoan metabolism. The idea 

...that APP/prolidase provides an essential metabolic activity is 

...reinforced by a previous study of 34 genomes across the three 

...domains of life that identified APP/prolidase as one of 80 uni-

...versally conserved orthologous groups (55). It was also sug-

...gested that X-Pro hydrolytic activity was present in the last 

...common ancestor of life (55).

...In P. falciparum, aminopeptidase P (PFAPP) has an intriguing 

...bipartite distribution in the acidic food vacuole, the site of 

...hemoglobin catabolism, and in the cytosol. Although a cytos-

...olic role for APP in peptide turnover in eukaryotes as diverse as 

...mammals, plants, fruit flies, and nematodes is well established 

...(18–22, 24), this is the first example, to our knowledge, of an 

...APP homolog residing in an acidic intracellular compartment. 

...PFAPP has the three-domain, dimeric structure of hAPP1. 

...Conservation of the hydrophobic dimer interface of hAPP1 (43) 

...suggests that the quaternary structures are very similar in 

...both enzymes. Unlike hAPP1 and the other apicomplexan sequences, PFAPP possesses an amino-terminal extension of 120 amino acids that precedes the first conserved domain. Near the beginning of this extension lies a putative signal pep-

...tide for import into the endoplasmic reticulum. We speculate 

...that full-length PFAPP traffics to the food vacuole via the endo-

...plasmic reticulum as has been observed for several other vacu-

...olar peptidases (56–58). The apparent lack of a signal peptide 

...from the other apicomplexan APP sequences is consistent with 

...the absence of a food vacuole in these organisms; these APP 

...homologs likely reside in the cytosol. Based on the size of the 

...major PFAPP species in parasite extracts and the co-elution on 

...a gel filtration column of the native enzyme with recombinant 

...PFAPP lacking the amino-terminal extension, it appears likely 

...that the amino-terminal extension is absent from the mature 

...protein. We attempted to confirm the absence of the amino-

...terminal extension by amino-terminal sequencing of immuno-

...purified native PFAPP but were unable to obtain a sequence for 

...unknown reasons.

...rPFAPP activity was greatly reduced following dialysis against 

...EDTA-containing buffer and was nearly fully restored in the 

...presence of 1 mM Mn2+. Similar observations have been made 

...with mammalian cytosolic and E. coli APP homologs, which 

...have led to the conclusion that APP is an Mn(II)-dependent 

...enzyme (18, 19, 45). Structural studies of hAPP1 and E. coli APP 

...have revealed a di-Mn2+ cluster, with a water/hydroxide mole-

...cule bridging the two metal ions in a position to undertake 

...nucleophilic attack of the X-Pro bond (42, 43). However, uncer-
tainty regarding the architecture of the APP active site in *vivo* persists. Metal-enzyme stoichiometries of 1:1, rather than 2:1, have been reported for recombinant human APP1 and pig kidney APP2 (46, 47). Moreover, the metal found in APP2 was Zn(II) rather than Mn(II) (46). Although atomic structures of the homologous enzyme methionine aminopeptidase at first revealed a di-Co(II) active site very similar in structure to that of APP, more recent evidence suggests that the enzyme is active with a single metal ion in the active site (59). In our opinion, three lines of evidence favor the di-Mn(II) configuration as the best current model for the APP active site. First, recombinant human APP1 that was extensively dialyzed against metal-free buffer retained two Mn(II) ions in the active site (43). Second, a mutant (D260A) of *E. coli* APP that contains only the putative essential Mn(II) ion is inactive (60). Third, an inactive mutant (H243A) of *E. coli* APP that contains an intact di-Mn(II) active site complexed with a tripeptide substrate reveals an interaction between the substrate amino terminus and the putative nonessential Mn(II) ion, which suggests a role for this metal in substrate binding (61). We found that low millimolar levels of Mn(II) stimulated rPfAPP activity severalfold, as has been observed previously with mammalian APP1 (18–20, 47, 48). There is evidence that one of the two metal sites in the active site of methionine aminopeptidase, and possibly also in *E. coli* APP, has a low affinity for metal (45, 59). The stimulation of APP activity observed in the presence of millimolar levels of Mn(II) may result from occupancy of this low affinity metal-binding site. Whether the putative low affinity site of PfAPP is occupied in *vivo*, and if so by which metal ion, is an interesting question for further study. The inhibition of PfAPP by Zn(II) (and possibly also Ni(II) and Cu(II)) is likely due to stabilization of the enzyme-product complex as observed for *E. coli* APP (45).

Recombinant PfAPP activity was highly stable at 37 °C over 1 h in the pH range 5.0 to 7.5, which suggests that this enzyme is sufficiently robust to function in the food vacuole lumen. Contrasting with the high stability of rPfAPP, hAPP1 activity was lost rapidly at pH 5.5. Our observations are consistent with a literature report of low stability at acidic pH of native hAPP1 purified from human platelets (20); therefore, it seems unlikely that hAPP1 is post-translationally modified in a way that would enhance its stability at acidic pH. Loss of activity of hAPP1 appeared to be associated with the formation of a high molecular weight aggregate. These data suggest that the stability at acid pH found with PfAPP is not a universal feature of threedomain, dimeric aminopeptidase P.

Using three peptide substrates, we found that PfAPP has catalytic efficiencies at pH 7.5 of $10^4$ to $10^5$ M$^{-1}$ s$^{-1}$, which are similar to those of prokaryotic and eukaryotic APP homologs. At pH 5.5, catalytic efficiencies for each substrate were somewhat lower than the respective values at pH 7.5 but still ranged around $10^3$ M$^{-1}$ s$^{-1}$. We conclude from these data that PfAPP is an efficient catalyst at pH values expected for both the food vacuole and the parasite cytosol. There was no consistent trend in the pH dependence of $K_m$ or $k_{cat}$ values for the three PfAPP substrates examined, which suggests that the effect of pH on these parameters is complex and may be strongly affected by the particular sequence of the substrate. We attempted to determine the kinetic constants for hAPP1-catalyzed hydrolysis of bradykinin at pH 5.5 using a 1-min assay to minimize loss of activity. Although we were able to detect activity in this assay, the experiments were not sufficiently reproducible to allow confident determination of kinetic constants. Mass spectrometric identification of the products of PfAPP-mediated hydrolysis is consistent with strict X-Pro aminopeptidase activity. We found that PfAPP can slowly hydrolyze the Pro-Pro bond in bradykinin (2–9). This finding is significant as the sequence Pro-Pro is found in β-globin.

Together, the data presented here support our model for intracellular peptide catabolism in the malaria parasite (11) in which PfAPP has catalytic roles in both the cytosol and the food vacuole. By analogy with other organisms, the cytosolic pool of PfAPP likely has a housekeeping role in the turnover of peptides generated by the proteasome. We suggest that *Plasmodium* spp. have recruited PfAPP to the food vacuole to facilitate hemoglobin catabolism. Human α- and β-globin each contain seven proline residues. Food vacuole endopeptidases such as plasmscins and falcipains could directly generate peptides with an X-Pro amino terminus as they are able to cleave substrates with a P2′ proline residue (62–64). Peptide trimming by broad specificity aminopeptidases has been shown to generate aminopeptidase P substrates in the bacterial cytosol (32). We have localized *P. falciparum* aminopeptidase N to the food vacuole (11), where it could perform an analogous role. Finally, we note that the millimolar $K_m$ values observed at pH 5.5 do not rule out a catalytic role for PfAPP in the vacuole. Hemoglobin is the predominant soluble protein of the red blood cell cytosol at a concentration of 20 mM protomer (65). Even taking into account a dilution of 10-fold in the lumen of the food vacuole, oligopeptide concentrations could still reasonably be expected to exist at low millimolar concentrations, which would be sufficiently high for PfAPP to be a significant factor in peptide catabolism.

The available phylogenetic, biochemical, and genetic evidence suggests that PfAPP may present an interesting new target for the development of peptidase-directed anti-malarial drugs. Given the apparent universal conservation of X-Pro hydrolytic activity (55) and its catalytic roles in the intraerythrocytic parasite, we predict that inhibition of PfAPP activity would be detrimental to parasite growth and replication. Our inability to obtain parasites with a disrupted PfAPP coding sequence (11) is consistent with this idea. Although inhibition of host APP homologs would be a concern in a strategy targeting PfAPP, we note that PfAPP, unlike its mammalian APP2 homolog (66), can act on substrates with a bulky P2′ residue (HbPep2). Thus, specificity for PfAPP over APP2 could potentially be achieved by taking advantage of differences in the S2′ subsites.

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