The M18 Aspartyl Aminopeptidase of the Human Malaria Parasite Plasmodium falciparum

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A member of the M18 family of aspartyl aminopeptidases is expressed by all intra-erythrocytic stages of the human malaria parasite Plasmodium falciparum (PfM18AAP), with highest expression levels in rings. Functionally active recombinant enzyme, rPfM18AAP, and native enzyme in cytosolic extracts of malaria parasites are 560-kDa octomers that exhibit optimal activity at neutral pH and require the presence of metal ions to maintain enzymatic activity and stability. Like the human aspartyl aminopeptidase, the exopeptidase activity of PfM18AAP is exclusive to N-terminal acidic amino acids, glutamate and aspartate, making this enzyme of particular interest and suggesting that it may function alongside the malaria cytosolic neutral aminopeptidases in the release of amino acids from host hemoglobin-derived peptides. Whereas immunocytochemical studies using transgenic P. falciparum parasites show that PfM18AAP is expressed in the cytosol, immunoblotting experiments revealed that the enzyme is also trafficked out of the parasite into the surrounding parasitophorous vacuole. Antisense-mediated knockdown of PfM18AAP results in a lethal phenotype as a result of significant intracellular damage and validates this enzyme as a target at which novel antimalarial drugs could be directed. Novel phosphinic derivatives of aspartate and glutamate showed modest inhibition of rPfM18AAP but did not inhibit malaria growth in culture. However, we were able to draw valuable observations concerning the structure-activity relationship of these inhibitors that can be employed in future inhibitor optimization studies.

It is estimated that 3.2 billion people currently live in areas where there is a risk of malaria transmission. Three to five hundred million of these individuals become infected each year and over two million die. The groups most affected by malaria are children under five years of age and pregnant women in sub-Saharan Africa. Parasite resistance to most of the currently used antimalarial drugs is now widespread and resistance to new drugs is developing. With an effective vaccine at least 15 years away there is an urgent need for new malaria treatments.

During the intraerythrocytic phase of development the parasite digests 65–75% of the host cell hemoglobin. A proportion of this digested hemoglobin (16%) is used for protein synthesis. Hemoglobin degradation is also important in reducing the colloid-osmotic pressure within the infected erythrocyte, which prevents premature cell lysis during parasite growth and establishes a concentration gradient by which rare amino acids enter the malaria-infected erythrocyte from host serum. The degradation of hemoglobin to peptide fragments occurs within a specialized acidic digestive vacuole (DV) by the action of aspartic, cysteine, and metalloendoproteases and by dipeptidases. However, small peptide fragments are transported from...
the DV to the parasite cytosol where they are degraded into free amino acids by amino- and carboxypeptidases (5).

There are eight aminopeptidases within the genome of the most clinically significant malaria species, *Plasmodium falciparum* (www.plasmodb.org): four methionine aminopeptidases, two neutral aminopeptidases (leucine aminopeptidase (M17LAP) and membrane alanine aminopeptidase (M1MAA)), a prolyl aminopeptidase (PAP), and an aspartyl aminopeptidase (M18AAP). Using specific enzyme inhibitors, one of the methionine aminopeptidases has been validated in *vitro* and *in vivo* as a potential drug target (7) and inhibitors of the M1MAA and M17LAP have been shown to prevent malaria growth in culture (8). Therefore, other aminopeptidases may also prove to be good targets for new antimalarial agents, particularly as part of drug combinations.

Aspartyl aminopeptidases are members of the M18 family of metalloproteases (www.merops.sanger.ac.uk). Unlike the methionine and neutral aminopeptidases, few aspartyl aminopeptidases (M18AAP) have been characterized; at present, there is only limited information reported for the aspartyl aminopeptidase of mammals (9), yeast (10), and bacteria.10 The lack of available substrate and inhibitor reagents has contributed to our poor understanding of the function of these enzymes. However, because of their restricted specificities for the N-terminal acidic amino acids, aspartic and glutamic acid, which cannot be cleaved by any other aminopeptidases, they are thought to act in concert with other aminopeptidases to facilitate protein turnover. In humans, a more specific function in the conversion of angiotensin II to the vasoactive angiotensin III within the brain has been implicated (12).

Here, we report for the first time the physicochemical properties, cellular expression, and distribution of the *P. falciparum* aspartyl aminopeptidase (PfM18AAP). We have produced a functionally active recombinant form of the enzyme that exhibits comparable properties to the native form measured in malaria cytosolic extracts. Our studies show that the PfM18AAP is expressed in the parasite cytosol and exported to the parasitophorous vacuole of the parasite indicating that whereas the enzyme may function in the final stages of hemoglobin digestion it may also have an additional function outside the parasite. Antisense-mediated inhibition of the PfM18AAP results in a lethal phenotype as a result of significant morphological changes to the parasite and, therefore, pinpoints the enzyme as a promising target for new anti-malarial drug development. However, novel inhibitors of aspartyl aminopeptidases that exhibit modest activity against the native and recombinant PfM18AAP do not prevent the growth of the parasites in culture.

**EXPERIMENTAL PROCEDURES**

*Parasites and Preparation of Parasite Extracts—* *P. falciparum* clone D10 was cultured as described (13). For experiments investigating the stage-specific expression of PfM18AAP, parasites were synchronized using two rounds of sorbitol treatment (14), and parasites harvested at ring, trophozoite, and schizont stages.

After washing infected red blood cells in PBS, parasites were released by incubation with either (1) 0.03% saponin/PBS on ice or (2) with 600 units/ml streptolydin O/PBS at 37 °C. Resulting parasite pellets were washed three times with PBS, re-suspended in 100 μl of PBS, and extracted by two cycles of freeze-thaw at −80 °C followed by centrifugation at 14,000 × g. Supernatants were stored at −20 °C.

Membrane preparations of infected red blood cells were produced by hypotonic lysis followed by centrifugation at 5,000 × g (15). The crude membrane pellets were treated with 0.5 ml of 0.1 M sodium carbonate or 1 ml of 1% Triton X-100 and separated into pellet and supernatant by centrifugation at 16,000 × g. Proteins in the supernatant were concentrated by precipitation with trichloroacetic acid (10% final concentration).

**Production, Purification, and Characterization of Functionally Active Recombinant Aspartyl Aminopeptidase—**The Plasmodb annotated gene sequence PfI1570c encoding the putative PfM18AAP was chemically synthesized by GENEART GmbH (GeneArt, Germany) using codons for optimized gene expression in the yeast *Pichia pastoris*. As reported by Stack et al. (16) for the M17 leucine aminopeptidase, the malaria PfM18AAP gene with the codons optimized for *P. pastoris* was successfully expressed in a functional form in insect cells. Potential N-linked glycosylation sites were removed in gene synthesis by replacing the asparagine of all Asn-X-Thr/Ser with Gln. This construct was recombined with BaculoDirect™ C-terminal linear DNA (Invitrogen) and transfected into *Sf9* (*Spodoptera frugiperda*) cells (16). Like the M17 leucine aminopeptidase, the malaria PfM18AAP gene while synthesized in the codon style of *P. pastoris*, was not able to be expressed successfully in yeast (16).

For protein expression, *Sf9* insect cells were infected at the cell density 3 × 10⁶ cells/ml with PfM18AAP recombinant *Baculovirus* at a multiplicity of infection of 2–5 plaque forming units/cell. rPfM18AAP, which was expressed bearing a His₆ tag, was isolated from insect cells by affinity chromatography on a Ni-NTA-agarose column as previously described (16). The purity and molecular size of isolated rPfM18AAP was analyzed using 12% reducing SDS-PAGE and gel filtration HPLC on a Superdex-200 column using a Pharmacia Biotech Smart System. The mobile phase was PBS, the column was run at a flow rate of 40 μl per min, and 40-μl fractions were collected.

The activity and substrate specificity of purified rPfM18AAP was determined by measuring initial rates of hydrolysis of the fluorogenic peptide substrates H-Asp-NHMec and H-Glu-NHMec at an excitation wavelength of 370 nm and an emission wavelength of 460 nm using a Bio-Tek KC4 microfluorimeter (16). rPfAAP (60 nm) was incubated in 50 mM Tris-HCl, pH 7.5, for 30 min before addition to the substrate. The pH profile for rPfM18AAP activity was determined from the initial rates of H-Asp-NHMec hydrolysis carried out in constant ionic strength (I = 0.1) with acetate/Mes/Tris buffers, pH 4–11 (17). The pH stability was determined by incubating rPfM18AAP in these buffers for 1 h at 37 °C before assaying for residual activity at pH 7.5.

To investigate the effect of metal ions on enzymatic kinetic parameters, rPfM18AAP was incubated with various metal ions for 30 min prior to initiation of the enzymatic reaction. Initial rates were obtained at 37 °C over a range of H-Asp-NHMec
substrate concentrations spanning $K_m$ (0.2–500 μM) and at fixed enzyme concentrations. The effect of bestatin, metal chelators, and diithiothreitol on PfM18AAP activity was investigated by measuring the initial rate of hydrolysis of 25 μM H-Asp-NHMec at pH 7.5 in the presence of each compound. Each rate was compared with the control rate containing only enzyme and substrate.

M18AAP activity in parasite extracts were determined by first incubating aliquots of the extract in 50 mM Tris- HCl, pH 7.5, containing 1 mM CoCl$_2$ for 20 min before addition to 25 μM H-Asp-NHMec. Leucyl aminopeptidase activity in the extracts was measured using 10 μM H-Leu-NHMec as described (16).

Phosphorus Containing Inhibitors—A series of α-phosphonic (compounds 1, 4, 7, and 8) and α-phosphinic (2 and 5) analogues of acidic amino acids (18), as well as phosphate dipeptides (3 and 6) (19) were tested for their inhibitory activity toward PfM18AAP using the fluorogenic peptide assay described above.

Polyclonal Antibody Production and Immunoblotting Analysis—Polyclonal antiserum was prepared against a 15-mer peptide, C*FSHKENSQNKRDDQ, corresponding to amino acid residues 211–224 of the putative P. falciparum aspartyl aminopeptidase (PFI1570c) as described previously (20). Proteins of saponin-lysed parasite extracts were resolved on reducing 10% SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with the anti-PfM18AAP antisera (1:250 dilution) followed by a horseradish peroxidase-labeled antimouse IgG antibody (1:5000 dilution, Chemicon International Inc.) (21). The membrane was stripped and re-probed with an anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) rabbit antibody (1:5000 dilution) to demonstrate equal loading and transfer of malaria proteins (22).

Fluorescence Microscopy—Fluorescence and phase-contrast images were collected with an Axioscope 2 Mot+ (Zeiss) equipped with a Zeiss ×63/1.4 Plan Apochromat lens. Live parasites were mounted in PBS and observed at ambient temperature. Parasite DNA was visualized by adding Hoechst dye (0.5 μg/ml) and incubating at 37 °C for 10 min prior to mounting. For indirect fluorescence, concanavalin A (0.5 mg/ml) was added to each well of a multwell slide and incubated for 30 min at 37 °C after which infected red blood cells were added, incubated at room temperature for 15 min, and unbound cells were removed by washing with PBS. The cells were fixed in 4% formaldehyde, 0.005% glutaraldehyde and probed with anti-PfM18AAP antiserum or with a mouse monoclonal antibody to GFP or c-myc (all diluted 1:500). Bound antibody was visualized with goat anti-mouse Ig-Cy2 (10 μg/ml).

Transmission Electron Microscopy—Infected red blood cells were fixed with 3% glutaraldehyde in cacodylate buffer, pH 7.2, and processed according to standard methods (23). After embedding in Spurr low viscosity resin, ultrathin sections (~50–60 nm thick) were prepared and stained with uranyl acetate and lead citrate and examined with a JEOL 1200EX transmission electron microscope operating at 80 kV.

Northern Blotting—Northern blotting was performed with total RNA extracts prepared using TRIzol (Invitrogen) (21). Blots were probed with a purified 1713-bp PCR fragment corresponding to the full-length CDS of the aspartyl aminopeptidase (PFI1570c) amplified from genomic P. falciparum DNA using primers PFI1570ASF (ctgcagatggataagaaagctaggga) and PFI1570ASR (agatctttgtggacacatggga). Probes were labeled with [α-32P]dATP by random priming (DECAprime II, Ambion Inc.). The probe was hybridized overnight at 40 °C in a hybridization buffer containing formamide (Northern Max; Ambion). The filter was washed once at low stringency and twice at high stringency (Northern Max; Ambion), then exposed overnight.

For specific sense and antisense probes, 26 ng of the above purified PCR product was primed with either a 3’ primer (actctgtaagacctatt) to generate a probe that specifically binds to the endogenous mRNA or a 5’ primer (atgtgtcataaggttagg) to produce a probe that binds specifically to the transgenic antisense RNA. Geometric amplification of the PCR product was performed using Taq polymerase under the following cycling conditions: denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, followed by extension at 68 °C for 2 min, for 30 cycles. These probes were hybridized to blots containing RNA extracted from both D10 wild-type parasites and transgenic cultures.

Construction of the Transgenic Expression Plasmids—PFI1570c was amplified from P. falciparum clone D10 genomic DNA. The forward primer for PFI1570c was PFII1570F (agatcttggataagaaagctaggga) and contained a BglII restriction site (in bold). The reverse primer was PFII1570R (ctgcagatggataagaaagctaggga) and contained a PstI site (in bold). Antisense primers were designed with restriction sites in the reverse orientation (PstI forward and BglII reverse). The PCR products were cloned into pGEM using a TA cloning system (Promega) and sequenced to confirm that no Taq-associated errors had occurred. In the case of GFP and c-myc constructs full-length fragments were digested out of the pGEM vector using BglII and PstI and subcloned into the Gateway™ compatible entry vectors pHGFpB and pHcmycB (Gateway, Invitrogen) that had previously been digested using BglII and PstI. Either a GFP tag or a c-myc tag were ligated in-frame at the 3’ end of the introduced gene sequence, respectively (24). These introduced genes were under the control of the HSP86 promoter. The antisense sequence was cloned into the pHcmycB vector. These entry vectors were designated pH-PFI1570c-GFP and pH-PFI1570c-cmyc, respectively, whereas the antisense plasmid was designated pHB-PFI1570-AS. Using those entry vectors and Gateway™ compatible destination vectors with a destination cassette and a second cassette containing the human dihydrolorate reductase synthase gene under the control of the P. falciparum calmodulin promoter as a selectable marker, cloning reactions were then performed. These final plasmids were designated pHH1-PFI1570c-GFPB (GFP tag), pHH1-PFI1570c-cmycB (c-myc tag), and pHH1-PFI1570c-AS (antisense). For transfection, ring stage parasites were subjected to electroporation in the presence of 50 μg of plasmid DNA as described (25). Parasites resistant to WR99210 were obtained up to 25 days later.

Sequence Analysis—Aspartyl aminopeptidase proteases from various species were retrieved from the NCBI data base. BLAST hits were aligned using CLUSTAL W at PBIL and alignments drawn using ESPriT 2.0.
**P. falciparum M18AAP**

**RESULTS**

Interrogation of PlasmoDB Discovered a Putative Aspartyl Aminopeptidase—PFI1570c, the putative aspartyl aminopeptidase of *P. falciparum* is located on chromosome 9 and as annotated by PlasmoDB consists of a single open reading frame of 1713 bp that translates into a protein of 570 amino acids. PFI1570c contains two signature domains, an N-terminal aspartyl aminopeptidase domain (LAP4: residues 1-186, e value 5e-34), and a C-terminal aminopeptidase 1 zinc metalloprotease M18 domain (Peptidase_18: residues 246-556, e value 2e-47). The overall sequence identity between the aspartyl aminopeptidases (M18AAP) of the various rodent *Plasmodium* sp. is 61–65% and would be in the region of 83–88% only for the fact that *PfM18AAP* possesses two unique sequence insertions. These insertions, which are predicted to form loops, are present in the central portions of the enzymes and clearly do not prevent the enzyme from forming an octomeric structure (see below). Whereas low levels of identity exists between the *PfM18AAP* and the human (31%) and yeast M18 aspartyl aminopeptidases (27%) the three histidine residues (His-94, His-170, and His-440) that are predicted from site-directed mutagenesis studies to be critical for enzymatic activity and another (His-352) essential for stabilization of quaternary structure of human M18AAP are conserved and are indicated in the alignment presented in supplementary materials Fig. S1.

Biochemical Characterization of Functionally Active Recombinant *PfM18AAP*—Recombinant *PfM18AAP* was purified from Baculovirus-transformed insect cells by affinity chromatography on a Ni-NTA resin and resolved as a single protein by SDS-PAGE (Fig. 1A). Purified *PfM18AAP* efficiently cleaved the simple fluorogenic substrates H-Asp-NHMec and H-Glu-NHMec with *k*_**cat**/*K**_m** values of 129.9 and 82.3 s** − 1, respectively (Table 1). The purified enzyme exhibited aminopeptidase activity against the fluorogenic substrate H-Asp-NHMec between pH 6.0 and 9.0 with optimal activity at pH 7.5 (Fig. 1B). The enzyme was stable when stored for 1 h at 37 °C over the pH range 6 to 11. No hydrolysis was observed against the fluorogenic substrates H-Leu-NHMec, H-Phe-NHMec, H-Ala-NHMec, H-Pro-NHMec, H-Gly-NHMec, H-Val-NHMec, H-Arg-NHMec, and H-Ile-NHMec when the substrate concentration was 100 µM.

*PfM18AAP* activity was reduced to 14 and 8.3% after incubating with 10 mM EDTA and 20 mM a-phenanthroline, respectively, demonstrating that metal ions are necessary for enzyme activity (data not shown). A study of the effect of various metal ions on the activity of the enzyme showed that it was enhanced by Co(II), but not by Ca(II), Fe(II), Mg(II), Mn(II), and Ni(II), whereas Zn(II) at a concentration of 1 mM abolished enzyme activity (data not shown). When the enzyme was incubated for 30 min in 50 mM Tris-Cl, pH 7.5, and containing 1 mM Co(II) the *k*_**cat**/*K**_m** values of the enzyme for substrates H-Asp-NHMec and H-Glu-NHMec increased ~8- and 30-fold, respectively (Table 1). These enzyme kinetics studies showed that *k*_**cat** values increased while the *K**_m** values remained unchanged, which indicates that the metal ion does not affect binding of the substrate but does increase the catalytic efficiency of the enzyme.

**PfM18AAP Activity in Soluble Extracts of Malaria Parasites**—Activity against the M18AAP-specific substrates H-Asp-NHMec and H-Glu-NHMec was detected in soluble extracts of malaria parasites and had similar characteristics to the activity of purified *PfM18AAP*. The *K**_m** values with parasite extracts of 216 and 251 µM for H-Asp-NHMec and H-Glu-NHMec, respectively, are similar to the *K**_m** values of 327 and 136 µM obtained with *PfM18AAP* for the same substrates (Table 1). The specific activity (µmol of NHMec released/min/mg of protein) for the enzyme in these extracts was 662.5 and 607 for
H-Asp-NHMec and H-Glu-NHMec, respectively. Furthermore, the activity against these substrates was enhanced almost 10-fold by Co(II) but was not enhanced by other metal ions studied (data not shown).

The molecular size of the PfM18AAP and native enzyme in parasite extracts were determined by HPLC size chromatography by assaying fractions for activity toward H-Asp-NHMec. H-Asp-NHMec-cleaving activity in parasite extracts eluted from the HPLC molecular size column at the same retention time as the activity of purified PfM18AAP corresponding to a molecular size of 560 kDa (Fig. 1C). The data suggest that both native and recombinant PfM18AAP possesses an octomeric structure consistent with that observed for human M18AAP (26).

PfM18AAP Is Transcribed and Translated in Intra-erythrocytic Stage Parasites—Northern blot analysis showed that wild type D10 parasites transcribed a single species of mRNA with an apparent size of ~3 kb when hybridized using a 1713-bp fragment comprising the complete CDS of the PfM18AAP (Fig. 2A). PfM18AAP appeared to be most abundantly expressed in ring stage parasites (Fig. 2A). Western blot analysis showed that when saponin-lysed parasite extracts of the same cultures were probed with anti-PfM18AAP antiserum a single protein species with an apparent molecular mass of 65 kDa was observed, which corresponded closely with the theoretical molecular mass of 65.6 kDa (Fig. 2B).

Immunoblot analysis of soluble, peripheral membrane-bound and integral membrane-bound fractions of isolated malaria-infected erythrocytes showed that PfM18AAP was exclusively associated with the soluble fraction (Fig. 3A). However, immunoblot analysis of saponin-lysed D10 parasite extracts showed that the 65-kDa species was present in both the parasite pellet (P1) and soluble fractions (S1) (Fig. 3B, panel i). As saponin treatment permeabilizes both the erythrocyte and the parasitophorous vacuole membrane (PVM) that surrounds the parasite but not the parasite membrane itself these data indicate that PfM18AAP is expressed in the parasite cytosol and is also exported out of the parasite. ImmunobLOTS of streptolysin O-lysed malaria-infected erythrocytes, which lyse the erythrocyte membrane but not the PVM, showed that the enzyme is not transported into the erythrocyte cytosol but is retained between the PVM and the parasite membrane (Fig. 3B, panel i, S2 and P2). The data were further substantiated by probing the same extracts with anti-GAPDH (Fig. 3B, panel ii), which is known to be a cytosolic enzyme that is also secreted into the space between the parasite and PVM (22).

Immunocytochemical Localization of PfM18AAP—Immunofluorescence analysis of parasite-infected erythrocytes using anti-PfM18AAP antibody located the PfM18AAP enzyme to the parasite cytosol (Fig. 4A). The same staining pattern was also seen with transgenic D10 parasites expressing a GFP-tagged PfM18AAP probed with anti-GFP antibody confirming that the enzyme is routed to the cytosol (Fig. 4B). In addition, direct live fluorescence microscopy on these transgenic parasites also revealed a diffuse staining pattern characteristic of localization to the parasite cytosol (Fig. 4C; immunoblots using anti-GFP confirmed that parasites expressed a GFP-tagged protein, data not shown). Staining with specific antisera was
only seen in parasitized red blood cells as confirmed by nuclear staining with Hoechst dye (Fig. 4, A–C) and no staining was observed in wild-type D10 parasites (data not shown).

Transfection Experiments Confirm That PFI1570c Is the Source of M18AAP Activity in Malaria Parasites—The plasmid pHHI-PFI1570c-cmycB was transfected into *P. falciparum* clone D10 ring-stage parasites to establish transgenic parasites with increased expression of *Pf*M18AAP enzyme. Western blots confirmed that tagged *Pf*M18AAP was expressed in this transgenic line (Fig. 5A). In other experiments, parasites were transfected with pHHI-PFI1570c-AS to knockdown *Pf*M18AAP enzyme expression. Northern blot analysis showed that the antisense parasites transcribed a single species of mRNA with an apparent size of ~3 kb when hybridized with either a double-stranded probe or a single-stranded sense probe. This was slightly larger than in the parental line D10. When probed with a single-stranded antisense probe the transfected parasites transcribed a single species of mRNA with an apparent size of ~3 kb (Fig. 5B).

Soluble extracts of cultured wild-type and transgenic parasites were assayed for *Pf*M18AAP activity using the substrate H-Asp-NHMec. Extracts derived from the pH1-PFI1570c-cmycB-transfected parasites exhibited a 4-fold higher *Pf*M18AAP activity than D10 control extract. In contrast, pHHI-PFI1570c-AS-transfected parasites showed ~80-fold lower activity than D10 control extracts (Fig. 5C). As a control, we measured the activity of a previously characterized aminopeptidase, a M17 leucyl aminopeptidase (M17LAP), with the substrate H-Leu-NHMec (16) and demonstrated that this was expressed at similar levels in the wild-type and the two transgenic D10 parasite lines (Fig. 5C). No aspartyl activity was detected in extracts of uninfected erythrocytes.

Transfection with Antisense Plasmid pHHI-1570c-AS Induces Cellular Damage—To examine whether inhibition of *Pf*M18AAP enzyme expression in the parasite transfected with the plasmid pHHI-1570c-AS had an effect on parasite development these were selected with 5 nM WR99210 and maintained in culture. To increase the copy number of the plasmid the parasites were then further selected with 10 nM (2 times) WR99210. Significant cellular alterations were observed by electron microscopy of the transgenic parasite cultures selected on 2X WR compared with wild-type D10 parasites. Of partic-
Inhibition of PfM18AAP by Phosphorus Containing Inhibitors—Several α-phosphonic (compounds 1, 4, 7, and 8, Fig. 7), α-phosphinic (2 and 5), and phosphinate dipeptides (3 and 6) were tested for their inhibitory activity toward PfM18AAP. Their structures are shown in Fig. 7 and the inhibitory activity presented in Table 2. The compounds are best described as moderate inhibitors of the enzyme; however, valuable observations concerning the structure-activity relationship of these inhibitors could be drawn. First, a strong preference for the glutamate side chain is clearly visible. The most active, phosphonic analogue of glutamate (compound 4, tested in the form of the pure enantiomer R, corresponding to the relative configuration L, exhibited a $K_i$ of 0.34 μM) appeared between 2 and 3 orders of magnitude more potent than the homologues shorter and longer by a single methylene group (compounds 1 and 8), respectively. This observation is also supported by the quite unexpected high activity of compound 7, which represents an analogue of glutamic acid with two carboxylic groups being replaced by the phosphonic moieties. Second, although the difference in inhibitory activity for AspP[CH$_2$]Ala and GluP[CH$_2$]Ala dipeptide analogues (see 3 versus 6) was less significant, the latter substrate was 3-fold more potent, which agrees with our studies using fluorogenic substrates that showed Glu is accepted in the P1 site of PfM18AAP more readily than Asp (Table 1). Third, a meaningful, albeit somewhat surprising, observation was the decrease of inhibitor potency in any attempts of inhibitor elongation by the introduction of a P1’ substituent. Thus, neither the P-modified phosphonic amino acid analogues nor the phosphinic dipeptides retained the activity exhibited by the simplest phosphonic amino acid derivatives (compare 1 versus 3 and 4 versus 6, for example).

Activity of Phosphorus Containing Inhibitors Against Malaria Parasite in Vitro—The inhibitors described above did not exhibit any significant inhibition of the growth of P. falciparum D10 parasites in culture (even at 100 μM final concentrations) using methods described previously (16).

**DISCUSSION**

The number of gene sequences in the public data bases that encode members of the M18 aspartyl aminopeptidase family are mounting due to genomic sequencing projects, but previous to the present study only two members have been biochemically characterized, those derived from human (9) and yeast (10). In fact, because the yeast enzyme, referred to as yeast aminopeptidase I, exhibits a preference for leucine over acidic amino acid residues and lacks a well conserved His-352, Wilk...
et al. (26) suggested that it was not a true M18 aspartyl aminopeptidase; a second sequence Yhr113wp, which does possess the conserved His-352, is now considered the genuine yeast M18AAP (10) (see supplementary materials Fig. S1). The \textit{P. falciparum} PF11570c gene is located on chromosome 9 and encodes an enzyme with conserved N- and C-terminal domains typical of the M18AAP family, retains the conserved His-352 and possesses three other histidine residues (His-94, His-170, and His-440) that were shown by Wilks et al. (26) using site-directed mutagenesis to be essential for enzymatic activity of the human M18AAP. Because the M18AAP peptidases lack the typical HEXXH zinc-binding motifs found in other metalloaminopeptidases it was postulated that His-94, His-170, and His-440 coordinate the active site zinc metal ions in this family of enzymes (26). Our biochemical investigations of native and recombinant malarial aspartyl aminopeptidase demonstrated that the enzyme requires metal ions for its activity and has a strict preference for N-terminal acidic amino acids, glutamate and aspartate (the enzyme failed to cleave hydrophobic, basic or neutral amino acids) and, thus, we can confidently place it among the M18AAP family. Uniquely, the \textit{Pf}M18AAP possesses two large sequence insertions in the central portion of the molecule, but given the fact that we could produce a functionally active recombinant enzyme that retains these inserts they clearly do not affect the formation of the multimeric quaternary structure of the enzyme or the structure of its active site. In the absence of a three-dimensional structure for the M18AAPs we cannot determine how these insertions are arranged in space but because they are predicted to be loops they presumably emanate freely from the core structure of the molecule. Furthermore, because they are absent from the homologous enzymes expressed in another human malaria, \textit{P. vivax}, and in several rodent malarias, they are not suspected to play a critical role in enzyme function.

Our cytochemical studies with wild-type and transgenic malaria parasites revealed that the \textit{Pf}M18AAP is routed to and expressed in the cytosol of the parasite. We recently described another malaria aminopeptidase, a M17 leucine aminopeptidase (16) (M17LAP), with exopeptidase specificity for N-terminal hydrophobic residues, Leu and Phe, that was also localized in this compartment. Accordingly, as has been suggested to take place in mammalian cells (9, 26), the two enzymes may function in concert in protein catabolism. The catabolic processes they are likely involved in, include the breakdown and turnover of malaria cellular proteins and the release of amino acids from peptides that are generated from the digestion of host hemoglobin within the malaria DV. The latter peptides are transported from the DV to the cytosol for final processing to free amino acids that are used in the anabolism of proteins required for the rapidly growing intracellular parasite. \textit{Pf}M18AAP would be necessary for tackling the acidic amino acids, Glu and Asp residues, which comprise \~8% of the total amino acid content of hemoglobin and cannot be removed by \textit{Pf}M17LAP (16). Earlier studies by us (27) implied the presence of these two aminopeptidase classes in malaria because cytosolic extracts could effectively cleave hemoglobin-derived peptides containing hydrophobic and acidic amino acids in their sequence. However, these studies also inferred the presence of additional aminopeptidases that could remove amino acids such as alanine, lysine, valine, and proline that are not cleaved by the \textit{Pf}M18AAP or \textit{Pf}M17LAP. Two other aminopeptidases identified in the malaria genome, an M1 membrane alanine aminopeptidase (M1AAA) and a prolyl aminopeptidase, can putatively cleave these amino acids and, therefore, complete the line-up of exopeptidases required for the total degradation of hemoglobin to free amino acids in the parasite cytosol.

More specific functions for human M18AAP are emerging in the literature and include processing of biologically active peptides with N-terminal Asp or Glu such as \(\beta\)-amyloid peptide, angiotensin II, cholecystokinin, and neuregulin (see Ref. 26). The \textit{Pf}M18AAP is also secreted outside the parasite and into the space between the parasite plasma membrane and the surrounding parasitophorous vacuole membrane, \textit{i.e.} into the parasitophorous vacuole, but not to the erythrocyte cytosol. Molecules passing into the parasite from the exterior, and vice versa, must pass though this space in which the parasite sits. Accordingly, we could speculate that the \textit{Pf}M18AAP has some role in processing proteins or peptides in transit in either direction, but presently we have no candidate substrates. Additionally, or alternatively, the enzyme may have a role to play in cell rupture and the release of merozoites by the processing of other molecules; again, we have not identified any particular host or parasite protein or peptide with an N-terminal Glu or Asp putative function in cell rupture. Using \textit{P. falciparum} phage display libraries Lauterback et al. (28) identified 7 proteins, including \textit{Pf}M18AAP, that bind to the erythrocyte cytoskeletal proteins spectrin and band 4.1 and may be involved in structurally re-organizing these during either the invasion or exit of the parasites. There are a number of malaria proteins that exist in the PV for which functions are unclear, however, others such as GAPDH are known housekeeping enzymes (22). The putative serine protease ABRA (29) and the SERA/SERP family of serine protease-like molecules have been shown to localize to the parasitophorous vacuole (30) and have been postulated to play a part in parasite egress but their exact role remains to be determined (31).

To investigate the essential nature of the \textit{Pf}M18AAP in \textit{P. falciparum} we created a knockdown-transgenic parasite line by transfecting parasites with an antisense plasmid. This antisense inhibition technique was recently developed for \textit{P. falciparum} in our laboratory (32) given that knockdown by RNA interference is generally considered ineffective in this organism (33). As antisense inhibition results in a knockdown of protein expression it also offers an alternative to knock-out of gene function and, therefore, the method can be employed to evaluate the importance of a gene product in parasite function and survival. In the present study the technique was demonstrated to successfully knockdown the expression of \textit{Pf}M18AAP activity because the transgenic parasites exhibited 80-fold less enzyme activity compared with wild-type parasites. The method was also highly target-selective as the relative activity of the malaria cytosolic M17LAP was shown to be unaffected by knocking down the \textit{Pf}M18AAP. Together with the data showing that overexpression of the \textit{Pf}M18AAP by transfecting parasites with the plasmid pH11-PFI1570c-cmycB resulted in an increase of M18AAP activity, these knockdown results prove that the
Pf\textsubscript{M17LAP} resulted in a reduction rather than an increase in their inhibitory constants ($K_i$). To this end, inhibitors with potency on par with our anti-MetAP1b, Pf\textsubscript{M1MAA}, and the Pf\textsubscript{M18AAP} possess an active site scaffold that binds metal ions (which activate the H\textsubscript{2}O to form a hydroxide nucleophile that attacks the peptide bond) it may be judicious to exploit this in future multiple-target anti-malaria drug design. Furthermore, there is a precedent for this approach because drugs that target the metal center of metalloproteases such as matrix metalloproteases, tumor necrosis factor-\textgreek{a} converting enzyme, and histone deacetylase have been successfully used in treating cancer and arthritis (11, 36).

**Acknowledgments**—We thank Dr. Deborah Stenzel at the Analytical Electron Microscopy facility, Queensland University of Technology in Brisbane, Australia, for performing the transmission electron microscopy. We thank Prof. Leann Tilley of the La Trobe University Melbourne for the generous donation of anti-GAPDH antibody.

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*P. falciparum* M18AAP