Characterization of the *Plasmodium falciparum* M17 Leucyl Aminopeptidase A PROTEASE INVOLVED IN AMINO ACID REGULATION WITH POTENTIAL FOR ANTIMALARIAL DRUG DEVELOPMENT

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Amino acids generated from the catabolism of hemoglobin by intra-erythrocytic malaria parasites are not only essential for protein synthesis but also function in maintaining an osmotically stable environment, and creating a gradient by which amino acids that are rare or not present in hemoglobin are drawn into the parasite from host serum. We have proposed that a *Plasmodium falciparum* M17 leucyl aminopeptidase (PfLAP) generates and regulates the internal pool of free amino acids and therefore represents a target for novel antimalarial drugs. This enzyme has been expressed in insect cells as a functional 320-kDa homo-hexamer that is optimally active at neutral or alkaline pH, is dependent on metal ions for activity, and exhibits a substrate preference for N-terminally exposed hydrophobic amino acids, particularly leucine. PfLAP is produced by all stages in the intra-erythrocytic developmental cycle of malaria but was most highly expressed by trophozoites, a stage at which hemoglobin degradation and parasite protein synthesis are elevated. The enzyme was located by immunohistochemical methods and by transfecting malaria cells with a PfLAP-green fluorescent protein construct, to the cytosolic compartment of the cell at all developmental stages, including segregated merozoites. Amino acid dipeptide analogs, such as bestatin and its derivatives, are potent inhibitors of the protease and also block the growth of *P. falciparum* malaria parasites in culture. This study provides a biochemical basis for the antimalarial activity of aminopeptidase inhibitors. Availability of functionally active recombinant PfLAP, coupled with a simple enzymatic readout, will aid medicinal chemistry and/or high throughput approaches for the future design/discovery of new antimalarial drugs.

The intra-erythrocytic stage of the malarial parasite *Plasmodium falciparum* is responsible for many of the clinical symptoms attributable to a disease that kills 2–3 million per year (1). It also represents a stage during which many metabolic pathways, unique to the parasite, are switched on and as a result has been the focus of the majority of antimalarial drug development strategies (2, 3). One essential pathway that has been a particular target for antimalarial drug discovery is the catabolism of erythrocyte hemoglobin; between 65 and 75% of the host cell hemoglobin is degraded in a process that results in the liberation of free amino acids (4–6). These free amino acids are utilized in a variety of critical processes including the following: (a) parasite protein synthesis and development (7), (b) the maintenance of osmotic pressure within the infected red blood cell that prevents premature cell lysis during the highly metabolic maturation and replication phases (8), and (c) the provision of a pool of free amino acids that serve as a concentration gradient against which an influx of amino acids that are rare or not present in hemoglobin enter the malaria-infected erythrocyte from host serum (9, 10). Although malaria parasites can acquire amino acids from the external environment, studies by Liu et al. (11) recently demonstrated that hemoglobin digestion is necessary for parasite survival and that this process alone can supply the parasite with most of its amino acid requirements.
Malarial M17 Leucyl Aminopeptidases

The initial steps in the catabolism of host hemoglobin take place within the acidic digestive vacuole (DV)\(^5\) of the parasite and involve a number of endopeptidases of various mechanistic classes, including aspartic proteases (plasmepsins I, II, and IV), cysteine proteinases (falcipains 2, 2', and 3), and a metalloprotease (falcilysin) (7, 12). An exopeptidase, dipeptidyl-aminopeptidase I, or cathepsin C, also exists in the DV and, as its name implies, can reduce peptides generated by endopeptidase activity to dipeptides (13). Studies using \textit{P. falciparum} demonstrated that soluble lysates of DVs could degrade human hemoglobin to small peptide fragments but were incapable of liberating free amino acids (14). In contrast, when human hemoglobin was incubated with cytosolic extracts, the main products generated were free amino acids suggesting the presence of an exopeptidase activity (mono-, di-, tri-, and carboxypeptidases) in this cellular compartment. These observations also imply that small peptide fragments derived from hemoglobin degradation are transported from the DV to the parasite cytosol for processing into free amino acids by these enzymes (14–16).

A number of reports have characterized aminopeptidase activity in cytosolic extracts of several \textit{Plasmodium} species (15–22). The activity was optimum at neutral pH, inactive below pH 6.0, and had a preference for synthetic substrates containing leucine and alanine at the N terminus (15, 16). These biochemical properties are consistent with an enzymatic function outside the DV and a proposed role in the terminal stages of hemoglobin catabolism because leucine and alanine constitute ~24% of the amino acids in this protein. Furthermore, a partially purified \textit{P. falciparum} aminopeptidase was capable of freeing amino acids from synthetic peptides representative of the products of endopeptidase-degraded hemoglobin, and was inactivated by the specific aminopeptidase inhibitors bestatin and nitrobestatin (16). These same inhibitors arrested the growth of \textit{Plasmodium chabaudi} \textit{chabaudi} and \textit{P. falciparum} parasites \textit{in vitro}, most particularly at the intra-erythrocytic trophozoite stages that express the highest aminopeptidase activity (16, 21).

Although a total of eight aminopeptidases have been identified among the annotated sequences of the 26-Mb \textit{P. falciparum} genome (23), only two belong to families of neutral aminopeptidases as follows: a 67.8-kDa M17-family leucyl aminopeptidase (PF14_0439), and a 122-kDa M1-family alanyl aminopeptidase (MAL13P1.56). The M17 aminopeptidases are classically described as cytosolic enzymes, whereas the M1 aminopeptidase transgenic parasites produced the products of endopeptidase-degraded hemoglobin, and was inactivated by the specific aminopeptidase inhibitors bestatin and nitrobestatin (16). These same inhibitors arrested the growth of \textit{Plasmodium chabaudi} \textit{chabaudi} and \textit{P. falciparum} parasites \textit{in vitro}, most particularly at the intra-erythrocytic trophozoite stages that express the highest aminopeptidase activity (16, 21).

In this study we have successfully produced a functionally active recombinant form of the malaria M17 leucyl aminopeptidase, r\textit{Pf}LAP, and report for the first time its physico-biochemical properties, cellular expression, and distribution. We interpret our findings in the light of previously published reports of aminopeptidase activities in extracts of malaria parasites. We also show that the aminopeptidase inhibitor, bestatin, and other dipeptide analogs are potent inhibitors of r\textit{Pf}LAP and prevent the growth of \textit{P. falciparum} parasites \textit{in vitro}. Our data provide further support for designating the M17 leucyl aminopeptidase as a promising target for new antimalarials.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture**—The asexual intra-erythrocytic stages of \textit{P. falciparum} parasites were cultured in RPMI 1640 medium containing 10% human serum (24). The parasites used were D10, a noncystodherent clone of FC27 lacking the right end of chromosome 9 (25), and were obtained from the Walter and Eliza Hall Institute, Melbourne, Australia. The parasites were synchronized using two rounds of sorbitol treatment (26), and stage-specific parasites were harvested at ring stage, early trophozoite stage, late trophozoite stage, and schizont stage.

**The \textit{P. falciparum} M17 Leucyl Aminopeptidase Gene, Codon Optimization, and Gene Synthesis**—The M17 leucyl aminopeptidase gene sequence (PF14_0439), as annotated by PlasmoDB, is located on chromosome 14 of \textit{P. falciparum}. It consists of a single exon of 1818 bp encoding a 605-amino acid protein. The gene was amplified from genomic DNA using primers described below but was also chemically synthesized by GENEART GmbH (GeneArt, Germany) using codons for optimized gene expression in the yeast \textit{Pichia pastoris}. Potential N-linked glycosylation sites were removed in gene synthesis by replacing the asparaginyl of all Asn-X-(Thr/Ser) motifs with Glu. These genes were ligated into pCR-Script cloning vector (Strategene, CA).

**Plasmid Construction and Transfection into Parasites**—For the transgenic expression of PF14_0439-GFP under the control of the \textit{hsp86} promoter, the complete M17 aminopeptidase gene PF14_0439 was amplified from D10 DNA using primers M17F (AGATCTAGTATTTTTTCTTCTTATGT), which contained a BglII restriction site (in boldface), and M17R (CTGAGTACCGGCTATTTTCATACAA), which contained a PstI site (in boldface), but not the putative stop codon of the gene. The gene was cloned into pHGB Gateway Entry vector containing the \textit{hsp86} promoter region and modified to contain BglII/PstI sites between the promoter and the GFP (gift from Dr. Chris Tonkin). This entry vector containing the PF14_0439-GFP sequence was used in a Clonase™ reaction (Invitrogen) with a pHHC*/DR0.28 vector containing a desti-
nation cassette to obtain vectors mediating expression of PF14_0439-GFP fusion proteins under the hsp86 promoter. Inserts of all constructs were confirmed by sequencing. Ring stage parasites were subjected to electroporation in the presence of 150 μg of plasmid as described (27, 28). Parasites resistant to WR99210 were obtained 15–22 days later.

Expression of Recombinant Malarial M17 Leucyl Aminopeptidase in Insect Cells—Successful functional expression of the M17 leucyl aminopeptidase (PfLAP) was achieved using a truncated form of the enzyme (Fig. 1A). This truncated form was prepared from the synthesized codon-biased leucyl aminopeptidase gene and was amplified by PCR using 5′ primers containing the additional bases CACC to allow directional cloning of the gene into the Gateway entry vector pENTR/D-TOPO (Invitrogen). The primers used were as follows: LAP forward primers contain-5′-CACCATGCTCTGAGGTTCCAC-3′, and LAP reverse 5′-GGCCTGAAAATACAGGTTTCACAC-3′. The pENTR construct housing the truncated leucyl aminopeptidase, pENTR-LAP, was verified by DNA sequencing. The verified construct was recombined into BaculoDirect™ C-Term linear DNA (Invitrogen) and transfected into Sf9 (Spodoptera frugiperda) cells according to the manufacturer’s protocol to generate the recombinant baculovirus carrying the aminopeptidase gene. Sf9 insect cells were maintained in serum-free SF-900II medium (Invitrogen) suspension cultures at 28 °C and orbiting at 120 rpm on a shaker platform. For protein expression, Sf9 insect cells were infected at the cell density 3 × 10⁶ cells/ml with rPfLAP recombinant baculovirus at an multiplicity of infection of 2–5 plaque-forming units/cell. The infections were allowed to proceed for 48 h at 28 °C before the cell pellets were being harvested by centrifugation at 8,000 × g for 15 min at 4 °C.

Purification and Molecular Size Analysis of rPfLAP—Baculovirus cells (~3.5 × 10⁶ cells/ml) containing rPfLAP were stored as ~15-mL pellets at ~80 °C. The pellet was thawed on ice and resuspended in cold PBS to a total volume of 100 ml. The suspension was sonicated three times for 10 s at 2-min intervals using a soni-probe. The suspension was frozen, thawed and sonicated an additional two times. The suspension was then centrifuged at 14,000 × g in a Sorvall RC-5 centrifuge for 30 min at 4 °C. The supernatant or soluble extract was filtered on ice through 0.45-μm HA Millipore membrane. The 85-ml filtrate was diluted to a total volume of 425 ml with 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM sodium chloride and 10 mM imidazole. The resulting protein solution was loaded on a 1-mL nickel-agarose column equilibrated with the same buffer. A sample of the eluate or flow-through was stored for SDS-PAGE analysis. The column was then washed with 10 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM sodium chloride and 20 mM imidazole. Leucyl aminopeptidase was eluted from the column in a total volume of 10 ml by addition of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM sodium chloride and 250 mM imidazole to the column. The eluate was dialyzed against PBS containing 10 mM ZnCl₂ for 16 h. Protein concentration of purified enzyme was measured using the Bio-Rad DC protein assay based on the Lowry method (29). Bovine serum albumin was the protein standard.

Purified rPfLAP and soluble extracts of malaria parasites (>80% trophozoites; prepared as described for extraction of insect cells above) were analyzed using a Smart System (Amerham Biosciences) HPLC equipped with a Superdex-200 gel filtration column. The samples (40 μl containing 10–20 μg of protein) were passed through the column at a flow rate of 40 μl per min and collected each minute into tubes pre-loaded with 40 μl of 1 mM CoCl₂ in 50 mM Tris-HCl, pH 8.0. Fractions were analyzed for aminopeptidolytic activity toward H-Leu-NHMe and Ala-NHMe as described below. Separation of the molecular size standards of apoferritin (440 kDa), β-amylase (232 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (29 kDa) was monitored at A₂₅₀. The molecular size of the PflAP and enzymes in the parasite soluble extracts was calculated from a plot of log M, versus elution time for these standards.

Enzymatic Analysis—Aminopeptidase activity was determined by measuring the release of the fluorogenic leaving group, NHMe, from a range of fluorogenic peptide substrates representative of the various amino acid groupings. Reactions were carried out in 96-well microtiter plates (200 μl total volume, 30 min, 37 °C) using a spectrofluorimeter (Bio-Tek KC4) with excitation at 370 nm and emission at 460 nm. Enzyme was incubated in 50 mM Tris-HCl, pH 8.0, and containing 1 mM CoCl₂ for 20 min before the addition of 10 μM H-Leu-NHMe. Initial rates were obtained at 37 °C over a range of substrate concentrations spanning Km values (0.2–500 μM) and at fixed enzyme concentrations in 50 mM Tris-HCl, pH 8.0. Inhibition experiments were carried out in the presence of substrate. Because bestatin and its analogs are time-dependent inhibitors of PflAP, progress curves were monitored until a final steady-state velocity, vₙ, was reached. K values were determined from Dixon plots of 1/vₙ versus inhibitor concentration when [S] ≪ Kₘ.

The metal ion dependence of PflAP was determined by assaying activity after preincubation of the enzyme (10 min, 37 °C) in 50 mM Tris-Cl, pH 8.0, containing a given metal chloride, before addition of H-Leu-NHMe substrate (10 μM). The PflAP apoenzyme was prepared by incubation of the enzyme with 10 mM o-phenanthroline in PBS for 15 h, followed by dialysis against PBS overnight. The ability of metal cations to reactivate the PflAP apoenzyme was determined by assaying its activity following preincubation (10 min, 37 °C) with 0.1 mM metal cations. Inhibition of PflAP activity by peptidase inhibitors was investigated by preincubating the enzyme (10 min, 37 °C) with EDTA and o-phenanthroline in 50 mM Tris-Cl, with and without 0.5 mM CoCl₂.

Northern Blotting—Northern blotting was carried out using total RNA extracts from stage-specific parasites (30, 31). The blot was probed with a DNA fragment corresponding to the full-length M17 leucyl aminopeptidase (PF14_0435) DNA coding sequence labeled with [α-³²P]dATP by random priming (DECAprime II Ambion). The blots were then stripped and re-probed with the P. falciparum S28 RNA probe to ensure equal loading in the lanes.

Polyclonal Antibody Production, Immunofluorescence Assays, and Immunoblotting Analyses—A 15-mer peptide CAGVSWNFKARKPKG corresponding to the amino acids residues 577–590...
Malarial M17 Leucyl Aminopeptidases

of the M17 leucyl aminopeptidase (PF14_0439) was synthesized (Sigma) and conjugated to diphtheria toxin through the N-terminally added cysteine residue. Mice were then immunized three times at 3-week intervals with 30–50 μg of peptide-diphtheria toxin conjugate per injection formulated in Freund’s Complete and Incomplete Adjuvant (10). Antibodies were also prepared in a similar manner to a recombinant PfLAP expressed in Escherichia coli BL21 (DE3).

Immunofluorescence assays were carried out as described before (32) using air-dried P. falciparum-infected red blood cells fixed with acetone. Cells were probed with mouse anti-M17 peptide (1/250) and then Cy2-conjugated goat anti-mouse AffiniPure antibodies (Jackson ImmunoResearch). The para-
M17 peptide (1/250) and then Cy2-conjugated goat anti-mouse

Phylogenetic Analysis of P. falciparum M17 Leucyl Aminopeptidase—The C-terminal catalytic region sequence of P. falciparum leucyl aminopeptidase (PF14_0439) (see Fig. 1A) was used to search the BLASTP data base. Selected BLAST hits were aligned with the aid of ClustalW within MEGA version 3.1 suite of programs (35). Regions of sequences that were difficult to align because of gaps were removed using Gblocks. Sequences were then realigned using ClustalW, and this align-
ment was then used to generate a phylogenetic tree. Phylo-
genetic analyses were conducted using MEGA version 3.1 (option: Neighbor-Joining, 1000 pseudo-replicates, and gaps were handled by pairwise deletion and distances calculated using Jones-Thornton-Taylor). To determine the strength of the groupings, bootstrap values for nodes were calculated by analyzing 1,000 bootstrap replicate data sets.

RESULTS

The P. falciparum M17 Leucyl Aminopeptidase Is Related to Other Apicomplexan Aminopeptidases—The general structure of the P. falciparum M17 leucyl aminopeptidase (PfLAP) is presented as a schematic in Fig. 1A, and its primary sequence is aligned with the LAPs of the various rodent (Plasmodium berghei, P. chabaudi chabaudi, and Plasmodium yoelii) malaria parasites in supplemental Fig. 1. The malaria LAPs are longer than LAPs from most other species because of an N-terminal extension containing an asparagine-rich low complexity region (LCR) (residues 31–79). The N-terminal extensions are found in each malaria leucyl aminopeptidase, but their sequences are highly variable and of various lengths (supplemental Fig. 1). Low complexity regions are found in many malarial proteins and are linked to the high A + T richness of the genome and high recombination rate (36).

Leucyl aminopeptidases of prokaryotes, plants, and animals consist of two domains, a less conserved N-terminal domain and a more conserved catalytic C-terminal domain. Evolutionary constraints are more stringent in the C-terminal domain because it contains the active site motifs that generate a scaffold capable of binding two zinc ions essential in substrate recognition (Fig. 1A). We assessed the phylogenetic relationship between the P. falciparum M17 leucyl aminopeptidase and that of its closest homologs by comparing their C-terminal catalytic domains only (residues 280–598 in the P. falciparum sequence, Fig. 1A). Fig. 1B shows that the P. falciparum leucyl aminopeptidase forms a distinct clade with various other Plasmodium species.6 The overall sequence identity between the various malaria LAPs is high (65–69%), and within the C-terminal domain it is 80–85%. The residues that bind the metal ion are thus highly conserved in malaria and most other LAPs (see supplemental Fig. 1). Residues Asp-379, Asp-459, and Glu-461 bind zinc 1, whereas Lys-374, Asp-399, and Glu-461 bind zinc 2. The residues Lys-386 and Arg-463 that act as electrophilic and proton donors, respectively, during the catalytic process are also conserved.

The plasmodial M17 leucyl aminopeptidases form a distinct group with other members of the Apicomplexa, including Cryptosporidium hominis and Cryptosporidium parvum as well

6 Sequences used in this study are as follows: P. falciparum LAP (accession number PF14_0439), P. yoelii LAP (accession number MALPY00521), P. berghei LAP (accession number PB_RP3746, www.plasmodb.org), and P. chabaudi chabaudi LAP (accession number PC_RP1908); C. hominis (accession number XP_667960), C. parum (accession number XP_826197), T. annulata (accession number CA176586), T. gondii (accession number 80.m00088), Nostoc BAB77761; Synechococcus elongatus CAA73771, Solana tumus tuberosum CAA48038, Lycopersicon esculentum AA015916, Medicago truncatula ABE92845; Arabidopsis thaliana NP_194821; Aquifex aeolicus NP_211437; Clostridium tetani NP_782447; Bacillus cereus AAP11794; Caenorhabditis elegans LAP-1 P34629; L. major AAL16097; S. mansoni AA502093; Rickettsia typhi AAU03616; E. coli PeP-A AP_004756; Anopheles gambiae EA06020; Drosophila melanogaster AAF50390; Ciona intestinalis 149954 (orthomCL DB internal accession); Fugu rubripes CA845956; Bovine lens AAB28170; Homo sapiens LAP AAD17527; Mus musculus LAP AAK13495; Rattus norvegicus AAH79381.

2072 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282•NUMBER 3•JANUARY 19, 2007
as *Theileria annulata* and *Toxoplasma gondii*. The apicomplexan group in turn forms a larger clade with various M17 LAP members of the flowering plants, which is not surprising given their close association and common ancestry (37). The M17 leucyl aminopeptidase of the only other parasite in this phylogram that uses hemoglobin as a source of nutrient, the human blood fluke *Schistosoma mansoni*, resides in a more distant clade, surprisingly grouping together with the M17 aminopeptidase of the protozoan *Leishmania major*.

**FIGURE 1. The *P. falciparum* M17 leucyl aminopeptidase.** A, schematic showing the structure of the *P. falciparum* M17 leucyl aminopeptidase, highlighting the asparagine-rich region unique to malaria parasites, the less conserved N-terminal domain, and the highly conserved C-terminal domain that contains all the residues that make up the catalytic site. The arrow points to residue 83 where the functionally active recombinantly expressed rPfLAP begins. B, the phylogenetic relationship of the *P. falciparum* M17 leucyl aminopeptidase with other members of the family. The *P. falciparum* LAP (accession number PF14_0439) forms a separate clade with other M17 LAPs from *P. yoelii*, *P. berghei*, and *P. chabaudi chabaudi*. The malaria LAPs are most closely related to enzymes from other apicomplexans such *Cryptosporidium hominis*, *C. parvum*, *T. annulata*, and *T. gondii*.

A truncated form of the enzyme (residues 83–598; see Fig. 1A) that lacked the asparagine-rich region was produced, but it too was not expressed in these systems. A similarly truncated gene was then synthesized using the codon bias of *P. pastoris*, and with all potential glycosylation sites removed, for the purpose of expression in this yeast system. Although this construct did not produce a recombinant product in *P. pastoris*, a recombinant protein was finally obtained by transforming insect cells with a baculovirus vector carrying this modified gene. The rPfLAP was easily extracted and solubilized from the insect cells by three cycles of freeze-thaw and sonication and was purified by affinity chromatography on Ni-NTA-agarose (Fig. 2A). The rPfLAP resolved as a single protein of expected approximate size, 60 kDa, in reducing SDS-PAGE, and 15–25 mg of protein was obtained from a 1-liter fermentation. This purified enzyme exhibited aminopeptidase activity against the fluorogenic peptide H-Leu-NMHec (\(K_m = 12.12 \mu M\)) over the pH range 7–11, with optimal activity at pH 8.5 and was most stable when stored at alkaline pH (Fig. 2, B and C).

rPfLAP Activity Is Dependent on Metal Ions—Members of the M17 leucyl aminopeptidase family are metalloproteases and therefore require the presence of metal cations to maintain enzymatic activity and stability (38–40). An analysis of the metal requirement for rPfLAP using the fluorogenic peptide H-Leu-NMHec as a substrate revealed that addition of Co(II) or Mn(II) ions to rPfLAP prior to mixing with substrate increased its activity up to 24-fold (Table 1). The divalent metal ions Ni(II), Mg(II), and Zn(II) increased activity up to 10-, 8-, and 4-fold, respectively, whereas various other ions enhanced activity no more than 2-fold. The only divalent ion to have an inhibitory effect on enzymatic activity was Cu(II) (1.0 mM).

Metal ions are clearly essential for the activity of rPfLAP, as the metal chelator o-phenanthroline was capable of abolishing enzyme activity (10 mM) in the presence or absence of Co(II) ions (Table 2). EDTA was less effective at removing metal ions from rPfLAP as 80% of enzymatic activity was retained even at concentrations of 10 mM; however, this metal chelator was capable of preventing activation of the enzyme by Co(II).
Malarial M17 Leucyl Aminopeptidases

A. purification of recombinant leucyl aminopeptidase was monitored by 12% reducing SDS-PAGE. **MW**, molecular size markers; lane 1, total freeze-thaw extract of insect cells infected with baculovirus carrying the PfLAP gene; lane 2, insoluble pellet of recombinant insect cells; lane 3, soluble supernatant extract of insect cells; lane 4, column run-through of Ni-NTA-agarose column; lane 5, sample from wash of Ni-NTA-agarose column; lane 6, insoluble pellet of recombinant insect cells; lane 7, column run-through of Ni-NTA-agarose column. Purified protein migrates at 60 kDa, which is expected for the truncated form of rPfLAP. rPfLAP exhibits activity in the neutral to alkaline pH range, 6.5 to 11.0 (Fig. 2, A), with a pH optimum at 8.2. The enzyme was most stable in alkaline pH (Fig. 2, B), and could be stored for 4 weeks at 4 °C and at pH 7.3 with- out significant loss of activity.

A preparation consisting largely of apoenzyme was prepared by incubating the rPfLAP enzyme with o-phenanthroline followed by dialysis to remove the metal ions and chelator complex. The apoenzyme activity was reduced to 8% of the holoenzyme, but this was capable of re-activation by the addition of metal cations; Co(II), Zn(II), and Mn(II) were most efficient at reactivating the enzyme (Table 1). Reactivated enzyme, however, never returned to the value of the holoenzyme, which suggests that removal of the metal ions can cause denaturation of the protease.

**rPfLAP and Native PfLAP Are Homohexameric Enzymes with a High Specificity for N-terminal Leucines—Enzyme kinetics studies using fluorogenic synthetic substrates revealed that, consistent with its classification as a member of the M17 leucyl aminopeptidase family, rPfLAP could efficiently cleave the hydrophobic amino acid leucine from the N terminus of synthetic peptides; its high affinity for this substrate is reflected in the enzyme kinetics (Table 2).**

**FIGURE 2. Purification of functionally active P. falciparum M17 leucyl aminopeptidase.** A, purification of recombinant leucyl aminopeptidase was monitored by 12% reducing SDS-PAGE. MW, molecular size markers; lane 1, total freeze-thaw extract of insect cells infected with baculovirus carrying the PfLAP gene; lane 2, insoluble pellet of recombinant insect cells; lane 3, soluble supernatant extract of insect cells; lane 4, column run-through of Ni-NTA-agarose column; lane 5, sample from wash of Ni-NTA-agarose column; lane 6, insoluble pellet of recombinant insect cells; lane 7, column run-through of Ni-NTA-agarose column. Purified protein migrates at 60 kDa, which is expected for the truncated form of rPfLAP. rPfLAP exhibits activity in the neutral to alkaline pH range, 6.5 to 11.0 (Fig. 2, A), with a pH optimum at 8.2. The enzyme was most stable in alkaline pH (Fig. 2, B), and could be stored for 4 weeks at 4 °C and at pH 7.3 without significant loss of activity.

**TABLE 1**

| Metal ion | Concentration (mM) | Relative activity (Holoenzyme) | Relative activity (Apoenzyme) |
|-----------|--------------------|-------------------------------|-------------------------------|
| None      | 100%               | 100%                          | 8%                            |
| Ca(II)    | 0.01               | 153 ± 5                       | 9%                            |
| 0.1       | 140 ± 1            |                               |                               |
| 1         | 127 ± 3            |                               |                               |
| Co(II)    | 0.01               | 1175 ± 6                      | 322                           |
| 0.1       | 2399 ± 70          |                               |                               |
| 1         | 2081 ± 46          |                               |                               |
| Cu(II)    | 0.01               | 227 ± 11                      | 15                            |
| 0.1       | 173 ± 5            |                               |                               |
| 1         | 52 ± 2             |                               |                               |
| Fe(II)    | 0.01               | 140 ± 11                      | 13                            |
| 0.1       | 211 ± 5            |                               |                               |
| 1         | 190 ± 17           |                               |                               |
| Mg(II)    | 0.01               | 180 ± 10                      | 17                            |
| 0.1       | 422 ± 14           |                               |                               |
| 1         | 788 ± 11           |                               |                               |
| Mn(II)    | 0.01               | 587 ± 23                      | 57                            |
| 0.1       | 1653 ± 26          |                               |                               |
| Ni(II)    | 0.01               | 2310 ± 24                     | 46                            |
| 0.1       | 334 ± 2            |                               |                               |
| 1         | 594 ± 26           |                               |                               |
| Zn(II)    | 0.01               | 374 ± 37                      | 128                           |
| 0.1       | 418 ± 6            |                               |                               |
| 1         | 420 ± 15           |                               |                               |

*100% activity is the rate of 73 mFu/s, and data reflect the mean relative activity ± S.D. (n = 3).

**TABLE 2**

| Reagent         | Concentration (mM) | Without Co(II) % | With Co(II) % |
|-----------------|--------------------|------------------|---------------|
| None            | 100                | 100              | 100           |
| o-Phenanthroline| 10                 | 0.6 ± 1          | 0.3 ± 0.04    |
|                 | 1                  | 44 ± 0.9         | 52 ± 0.6      |
|                 | 0.1                | 96 ± 5           | 80 ± 3        |
|                 | 0.01               | 107 ± 5          | 81 ± 6        |
| EDTA            | 10                 | 80 ± 5           | 6 ± 0.1       |
|                 | 1                  | 90 ± 4           | 88 ± 1        |
|                 | 0.1                | 112 ± 6          | 104 ± 15      |
|                 | 0.01               | 91 ± 4           | 93 ± 2        |

*Data reflect the mean relative activity of three assays ± S.D. (n = 3).**

**rPfLAP not preincubated with Co(II), 100% activity = rate of 51 mFu/s.**

**rPfLAP preincubated with 1 mM Co(II), 100% activity = rate of 1710 mFu/s.**
and isoleucine were not cleaved by rPfLAP (even at substrate concentrations of 200 μM). Additionally, the enzyme did not cleave substrates with small nonpolar (Gly), acid (Asp, Glu), or basic (Arg) amino acids.

We found that the substrate H-Ala-NHMec was poorly cleaved by rPfLAP with $k_{cat}/K_m = 3.78 \text{ M}^{-1} \text{s}^{-1}$. Because we (15, 16) and others (22, 41) have shown that this substrate is efficiently cleaved by aminopeptidases in soluble extracts of malaria parasites, we compared the elution profiles of these extracts to that of rPfLAP subjected to HPLC size chromatography. Each eluted fraction was analyzed for H-Leu-NHMec- and H-Ala-MHMec-cleaving activity in the presence and absence of Co(II) (Fig. 3). The rPfLAP resolved as a single enzymatic peak which, when compared with the molecular size standards, was calculated to be 320 kDa (Fig. 3, top panel and inset). This elution profile confirms that the rPfLAP exists as a homohexamer, which is common for M17 leucyl aminopeptidases (38, 39). In contrast, two peaks of H-Leu-NHMec-cleaving activity were detected in the soluble extracts of malaria, one at 320 kDa (with an identical retention time to the rPfLAP peak) and another at 82 kDa (Fig. 3, bottom panel). The 320-kDa enzymatic peak eluted from the rPfLAP and malaria extracts did not cleave H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate. The enzyme eluting in the 82-kDa peak, however, was capable of cleaving both H-Leu-NHMec and H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate. The enzyme eluting in the 82-kDa peak, however, was capable of cleaving both H-Leu-NHMec and H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate. The enzyme eluting in the 82-kDa peak, however, was capable of cleaving both H-Leu-NHMec and H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate. The enzyme eluting in the 82-kDa peak, however, was capable of cleaving both H-Leu-NHMec and H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate. The enzyme eluting in the 82-kDa peak, however, was capable of cleaving both H-Leu-NHMec and H-Ala-NHMec, and both activities were reduced by >95% if the fraction were not preactivated with Co(II) before addition of substrate.

### Table 3

| Substrate       | $k_{cat} \times 10^3$ | $K_m$ | $k_{cat}/K_m$ |
|-----------------|----------------------|------|--------------|
| H-Leu-NHMec     | 39.0 ± 1.0           | 12.12 ± 1.04 | 3.218        |
| H-Phe-NHMec     | 1.45 ± 0.09          | 7.92 ± 0.51 | 183.1        |
| H-Pro-NHMec     | 0.59 ± 0.02          | 234.91 ± 17.76 | 2.51        |
| H-Ala-NHMec     | 0.2 ± 0.003          | 52.90 ± 2.61 | 3.78         |

**Malarial M17 Leucyl Aminopeptidases**

**FIGURE 3.** HPLC gel filtration of recombinant and native *P. falciparum* leucyl aminopeptidase. The rPfLAP (3 μg) (upper panel) and soluble extracts of *P. falciparum* parasites (15 μg) (lower panel) were analyzed on a Superdex-200 HPLC column. Fractions were activated by addition of 1 mM Co(II) for 20 min before assessing their activity against the fluorogenic peptide substrates H-Leu-NHMec (small dashed line) and H-Ala-NHMec (large dashed line) as outlined under "Experimental Procedures." The solid line represents the elution of the mix of four proteins used as molecular size standards, apoferritin (440 kDa), β-amylase (232 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (29 kDa), which were monitored at $A_{280}$. A plot of log $(M_r)$ versus elution time for these standards was linear (inset).

**TABLE 3**

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| H-Ala-NHMec | 0.2 ± 0.003          | 52.90 ± 2.61 | 3.78         |
catabolism provides free amino acids to the cell and is performed by various aminopeptidases; M17 leucyl aminopeptidases are among the most predominant within the cytosol (42). The presence of an M17 leucyl aminopeptidase in malaria is thus not surprising, and these proteases likely perform the same housekeeping function as in any other cell; however, in malaria parasites the enzyme must take on the additional chore of freeing amino acids from peptides sent to the cytosol from the DV where the rapid turnover of host hemoglobin takes place. Regulating the cellular pool of amino acids is not only essential for protein synthesis in malaria but also for maintaining the correct osmotic balance within the host erythrocyte (8) and for setting up a gradient by which amino acids that are not provided by hemoglobin in sufficient quantities can be drawn from the external environment (9, 10). Thus, malaria aminopeptidases are critical to the survival of the parasite within the host erythrocyte.

Because of their unusually high A/T richness (81% in P. falciparum, 36), functional expression of malarial antigens in heterologous systems has proven difficult; mRNA transcripts are often unstable or prematurely terminated (43). Mehlin et al. (44) assessed the expression of 1000 P. falciparum genes (lacking transmembrane regions) in E. coli, but only 337 genes gave rise to recombinant proteins, and of these, only 63 were soluble in aqueous buffers (6.3% soluble expression rate). To overcome this problem, we synthesized the M17 leucyl aminopeptidase with a codon usage that was expected to favor expression in the yeast P. pastoris (and removed potential glycosylation sites as this organism tends to hyper-glycosylate), but neither the full-length gene nor a truncated form lacking the N-terminal extension containing the asparagine-rich LCR was expressed in this system. The truncated form of the same gene was accommodated by insect cells transformed by baculovirus, and functionally active enzyme was expressed in soluble form that was isolated by affinity chromatography on Ni-NTA-agarose. We have since obtained functionally active rPfLAP in E. coli BL21 (DE3) by transforming these with the P. pastoris-optimized truncated gene in the expression vector pTrcHisB (Invitrogen). However, the purified recombinant protein obtained using this system rapidly lost enzymatic activity (within 2 days) at 4°C. In contrast, the insect cell-produced enzyme retained activity for greater than 4 weeks when stored in neutral to slightly alkaline buffers (pH 7.3 to 8.2) at 4°C.

The expressed rPfLAP resolved at 60 kDa on reducing SDS-PAGE but HPLC size chromatography showed that the soluble protein exhibits a molecule size in the region of 320 kDa consistent with the enzyme forming a homohexamer, which has been described for several other leucyl aminopeptidases such as that of Leishmania mexicana (45), E. coli (46), pig kidney (47), and bovine lens (39). Removal of the N-terminal extension containing the asparagine-rich LCR clearly did not prevent correct folding of the protein nor the ability of the monomers to combine into hexamers. The N-terminal extension containing the asparagine-rich LCR varies in length and amino acid composition between the various human and rodent malarials, but the N- and C-terminal domains, particularly the latter that houses the catalytic apparatus, are highly conserved (80–85%). All the residues involved in substrate binding are absolutely conserved.
between the malaria species; and therefore, the enzymes derived from the rodent malaria would be expected to have identical substrate specificities to that of the \( P.falciparum \) characterized in this study. These comparisons are relevant because Ramjee et al. (48) demonstrated that the cysteine protease of rodent malaria \( P.berghei \) (bergherain-2) displayed significant differences in substrate specificity to its ortholog from the human malaria \( P.falciparum \) (falcipain 2, FP-2), and thus questioned the appropriateness of rodent malarial models for testing the \textit{in vivo} antimalarial activity of inhibitors directed at FP-2.

Also consistent with its membership of the M17 leucyl aminopeptidase family was the substrate preference of \( PfPfLAP \) for N-terminally exposed hydrophobic amino acids, most markedly leucine. The enzyme did not cleave aliphatic (Val and Ile), small nonpolar (Gly), acid (Asp and Glu), or basic (Arg) amino acids. Of particular interest, however, was our enzyme kinetic data showing the relative inability of \( PfPfLAP \) to cleave \( N \)-terminal alanine (~850-fold lower than leucine). This differential preference has been described for other M17 leucyl aminopeptidase, for example those isolated from \textit{Leishmania} species (45).

However, we have demonstrated previously that soluble extracts of \( P.falciparum \) malaria contained neutral aminopeptidase activity capable of effectively cleaving both H-Leu-NHMec and H-Ala-NHMec (16, 21), and similar activities have been reported by Florent et al. (22) using the same substrates. HPLC size separation chromatography revealed that the specific H-Leu-NHMec-cleaving activity in malaria extract resides in a 320-kDa peak with an identical retention time to the \( rPfLAP \) and that its activity is similarly enhanced by the metal ion Co(II). On the other hand, a separate aminopeptidase elutes in an 82-kDa peak that cleaves both H-Leu-NHMec and H-Ala-NHMec and does not exhibit activation in the presence of metal ions. Accordingly, we suggest that the 320 kDa in malaria extracts represents the native form of the hexameric M17 leucyl aminopeptidase. Considering its substrate specificity and molecular size, we suggest that the 82-kDa peak represents the M1 alanyl aminopeptidase described by Florent et al. (22) and that the peak may contain a combination of the soluble forms of 92 and 68 kDa that are processed from the 122-kDa membrane-bound parent molecule. Definitive proof of this suggestion, however, awaits further studies, including production of a functional recombinant M1 enzyme for comparative studies.

Both Northern blotting and immunoblotting studies show that \( PfPfLAP \) is expressed by all \textit{intra-erythrocytic} developmental stages of \( P.falciparum \). Our Northern blot data support gene expression profiling studies using microarrays that showed \( PfPfLAP \) mRNA levels are highest in the early trophozoite stages and decrease during the latter half of the cycle (49–51). Le Roch et al. (49) found that genes with similar expression profiles during the \textit{intra-erythrocytic} development of the parasite could be assigned to one of 15 clusters that correlated with the function of the gene products in the malarial cell. Thus, genes that were maximally expressed in the ring and trophozoite stages and declined in the latter stages of the life cycle were assigned to clusters 5–7 and included proteins involved in protein synthesis. \( PfPfLAP \) was assigned to cluster 6, and its expression was closely aligned with that of the cysteine protease falcipain-2 (PF11_0165) and plasmspepsins (PF14_0174; PF14_0075), which is consistent with its involvement with these enzymes in the turnover of host hemoglobin. But whether \( PfPfLAP \) plays additional functions in the latter developmental stages where expression is lower but still present within schizont and segregated merozoites is unknown. A role for an aminopeptidase in red cell invasion was suggested by studies by Olaya and Wasserman (52), and our laboratory has shown that the number of ring stage parasites in cultures 24 h after addition of infected erythrocytes to uninfected cells was reduced by the presence of bestatin (16). However, because the IC\(_{50}\) value of this effect was 25 \( \mu M \) (two times higher than that which prevents trophozoite and schizont growth, see this study and Ref. 16), we suspected that this observation was because of inhibition of schizont growth rather than cell invasion.

Quantitative analysis of mRNA levels during the \textit{intra-erythrocytic} stages of the parasite indicate that levels of \( M17 \) aminopeptidase mRNA transcripts are ~18-fold greater than those of the M1 aminopeptidase (49). Our analysis of malaria parasite-soluble extracts (~80% trophozoites) by HPLC size chromatography as described above (Fig. 3) do not suggest such a
large difference between the levels of expression of these two aminopeptidases; the total fluorogenic units calculated for the 320-kDa peak containing the M17 PfLAP is almost three times lower than that detected in the 82-kDa peak, which we believe represents the M1 alanyl aminopeptidase. However, the transcriptome and proteome data did not always correlate in the study of Le Roch et al. (49), who suggested that transcription and translation may not be tightly associated. Perhaps, differences in the rate of mRNA turnover or stability may also account for the observed discrepancies between the mRNA and enzymatic data.

We have shown previously that the aminopeptidase inhibitor bestatin can block the growth in culture of both human, P. falciparum, and rodent, P. chabaudi chabaudi, malaria (21). Bestatin exerts its greatest inhibitory effects on trophozoites (16), a time when cellular metabolism is at its peak, hemoglobin degradation is at its highest, and expression of PfLAP is elevated. In this study we show that bestatin is a potent inhibitor of rPfLAP with an inhibitory constant (Ki) of 25 nM; however, its derivative, nitrobestatin, exhibits almost a 10-fold greater inhibitory activity (Ki = 2.7 nM). Correlating with its greater anti-rPfLAP inhibitory activity, nitrobestatin was also a more potent inhibitor of P. falciparum growth in culture, displaying an IC50 of 8 μM, although this was less than 2-fold better than bestatin, with an IC50 of 14.87 μM. Studies have demonstrated that the uptake of bestatin by erythrocytes in vitro is very slow, 0.3% per min, as entry is entirely dependent on passive diffusion across the red blood cell plasma membrane (53). Because nitrobestatin possesses an additional electronegative group, passive transfer of this derivative would be expected to be slower compared with bestatin and may account for its low antimalarial activity relative to its anti-rPfLAP activity. Interestingly, although bestatin methyl ester (Ki = 138 nM) was 6-fold less inhibitory of rPfLAP compared with bestatin and 50-fold less compared with nitrobestatin, it showed a surprisingly high relative antimalarial killing activity in culture (IC50 of 20.5 μM). Replacement of the C-terminal carboxyl group of bestatin (COOH) with a methyl ester (COOCH3) greatly increases its hydrophobicity and therefore its ability to permeate cellular membranes (54). Thus, despite its lower anti-rPfLAP effects, bestatin methyl ester would penetrate both erythrocyte and parasite membranes more easily than the hydrophilic bestatin and nitrobestatin. Another important factor that may influence the rate of transport of these dipeptide analogs into the infected cell is their possible interaction with specialized transport channels and receptors that are inserted into the erythrocyte and parasitophorous membranes by the malaria parasites for the two-way trafficking of amino acids and other solutes (55). Overall, however, it must be pointed out that the inhibition constants for inhibition of PfLAP, Ki, for each of these compounds are much lower than their IC50 values, which may indicate that they do not gain access to the target enzyme easily.

Our data support the idea that the M17 rPfLAP is a target of the bestatin-mediated killing of malaria parasites. However, they do not exclude the possibility that this inhibitor kills malaria parasites by targeting other enzymes. Bestatin, bestatin methyl ester, and nitrobestatin can inhibit the aminopeptidase activities in both the 320- and 82-kDa peaks obtained in our HPLC gel filtration analysis of malaria extracts (not shown), which clearly demonstrates that these aminopeptidase inhibitors have an additional target enzyme in the parasite. The most obvious other candidate is the M1 alanyl aminopeptidase that has also been suggested to be involved in the liberation of free amino acids from the fragments of digested hemoglobin and probably works in concert with the M17 PfLAP. It is not surprising that blocking these enzymes would have such a dramatic effect on the growth in culture of malaria given that leucine and alanine constitute 24% of the amino acids of the hemoglobin (15) (without considering other hydrophobic amino acids, for example phenylalanine, methionine, and cysteine, that these enzymes may be responsible for releasing from hemoglobin-derived peptides). Two other malaria aminopeptidases, a prolyl aminopeptidase and aspartyl aminopeptidase, can be detected in soluble extracts of P. falciparum parasites using specific fluorogenic substrates and may be responsible for cleaving the bulky hydrophobic amino acid proline and the acidic amino acids (aspatic acid and glutamic acid) within hemoglobin, respectively, that are not cleaved by M17 or M1 aminopeptidases. However, the activity of these more specialized aminopeptidases is not affected by bestatin or its derivatives, thereby excluding them as targets of the antimalarial activity of these inhibitors (data not shown).

Much of the evidence supporting the hemoglobin digestive pathway as a therapeutic target comes from studies involving the major DV endopeptidases, the aspartic proteases (plasmepsins I, II, and IV), and cysteine proteinases (falcipains 2, 2', and 3) (7, 12). Indeed, inhibitors against the malarial aspartic proteases prevent parasite growth in culture (56) and cure malaria in a P. chabaudi chabaudi mouse model (57). Similarly, the cysteine protease inhibitor E64 prevents the development of cultured parasites by blocking the hydrolysis of hemoglobin and also cures Plasmodium vinckei-infected mice (58, 59). These inhibitors induce the characteristic swelling of the DV because of the accumulation of undegraded hemoglobin (60, 61). More recent gene disruption studies of both the aspartic and cysteine protease gene families have revealed the synergistic nature of these enzymes (62–64). However, disruption studies by Omara-Opyene et al. (62) demonstrated that the loss of any single DV plasmepsin or in combination (plasmepsin IV-I double knock out) is in itself insufficient to produce a lethal phenotype. Inactivated genes cannot only be compensated by other DV plasmepsins but also by members of the falcipain protease gene family. Similarly, disruption of any of the DV falcipains known to be involved in hemoglobin digestion can be compensated by increased expression of other members of the family (63, 65). Liu et al. (11) demonstrated that a knock out involving a combination of genes from both protease families (disrupted falcipain-2 on a plasmepsin IV-I double knock-out back round) had a negative but lethal effect on parasite growth in medium when parasites were cultured in medium containing isoleucine as the sole exogenously added amino acid. Collectively, these results suggest significant redundancy in the proteolytic systems within the DV and, more importantly, indicate that in order for therapeutic strategies to be
successful inhibitors should be capable of targeting both families of proteases in combination (11).

Importantly, malaria parasites express only one class of the M17 and M1 aminopeptidases and therefore redundancy of function is somewhat restricted. At present, specific inhibitors of each of these aminopeptidases are not available to assess the extent to which they may compensate for each other within the parasite cytosol. However, studies are underway to examine this issue using transgenic and antisense techniques to knock out or knock down the production of each enzyme. It must also be mentioned that various other exopeptidases, including dipeptidases, tripeptidases, and carboxypeptidases, are expressed by malaria parasites and presumably work in concert with the aminopeptidases to release amino acids from hemoglobin (13).

Bestatin is a dipeptide analog first discovered as an antibiotic with aminopeptidase inhibitory activity in culture filtrates of the bacterium Streptomyces olivoreticuli (66). A major target of bestatin-mediated inhibition in mammals is aminopeptidase N, an M1 alanyl membrane aminopeptidase localized to the intestinal brush border and on the surface of many immune cells where it is better known as CD13 (67). Bestatin induces immunomodulatory effects on certain immune effector cells but has very low toxicity in experimental animals and humans (68–71) and, consequently, has been formulated for the safe therapeutic treatment of certain cancers such as squamous cell carcinoma in humans (53, 72).

The data presented in this study provides a meaningful biochemical explanation for the antimalarial activity of broad spectrum aminopeptidase inhibitors, such as bestatin. These dipeptide analogs will be useful scaffolds on which novel small molecule inhibitors could be designed to potently and selectively inhibit either or both the P. falciparum M17 leucyl and M1 alanyl aminopeptidases (10). An additional challenge now exists for medicinal chemists to design related compounds that can readily cross the erythrocyte and parasite membranes by either simple or facilitated diffusion to gain access to their targets. Availability of the functionally active rPfJLAP described in this study, coupled with the simple and rapid fluorogenic enzymatic readout, will be invaluable for screening these derivatives or for directing high throughput screening of inhibitors in small-molecule banks. Acquiring functionally active recombinant M1 alanyl aminopeptidase is a priority in our laboratory so that this screening can be carried out with both enzymes simultaneously.

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Malarial M17 Leucyl Aminopeptidases
Malarial M17 Leucyl Aminopeptidases

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