THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 3, pp. 2069–2080, January 19, 2007

Characterization of the *Plasmodium falciparum* M17 Leucyl Aminopeptidase

A PROTEASE INVOLVED IN AMINO ACID REGULATION WITH POTENTIAL FOR ANTIMALARIAL DRUG DEVELOPMENT[*][S]

Received for publication, September 29, 2006, and in revised form, November 8, 2006 Published, JBC Papers in Press, November 15, 2006, DOI 10.1074/jbc.M609251200

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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 (*Pf*LAP) 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. *Pf*LAP 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 *Pf*LAP-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 *Pf*LAP, 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.

* This work was supported in part by an Australian Research Council Discovery Project Grant DP0666128 and by an Australian Government Endeavour Scholarship (to E. C. and J. P. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] Supported by Basic Research Grant SC/2003/111 from Enterprise Ireland.

[2] Supported by National Health and Medical Research Council Program Grant 290208 and by a generous donation from Mark Nicholson, Alice Hill, and the Tudor Foundation.

[3] Recipient of a University of Queensland postdoctoral fellowship and a Ramaciotti development grant.

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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 *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 *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 *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 *Plasmodium chabaudi chabaudi* and *P. falciparum* parasites *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 *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 aminopeptidases are typically membrane-bound; however, Florent and co-workers (22) detected M1 alanyl aminopeptidase activity in soluble extracts of *P. falciparum* and used antipeptide antibodies to identify 98- and 68-kDa immunoreactive processed forms of the parent 122-kDa membrane-bound aminopeptidase. In an attempt to identify whether the M17 or M1 aminopeptidase, or both, was the target of the bestatin-mediated killing of *P. falciparum,* we generated transgenic parasites that overexpressed these aminopeptidases (10). Only the parasites overexpressing the M17 leucyl aminopeptidase gene produced a functional enzyme, whereas the M1 aminopeptidase transgenic parasites produced an inactive protein of 120 kDa that did not process to smaller fragments. The transgenic *P. falciparum* parasites overexpressing the M17 aminopeptidase were less susceptible to killing by bestatin when compared with wild-type parasites pinpointing this enzyme as a target for this antimalarial inhibitor (10).

In this study we have successfully produced a functionally active recombinant form of the malaria M17 leucyl aminopeptidase, r*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*Pf*LAP and prevent the growth of *P. falciparum* parasites *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 *P. falciparum* parasites were cultured in RPMI 1640 medium containing 10% human serum (24). The parasites used were D10, a noncytoadherent 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 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 *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 *Pichia pastoris*. Potential *N*-linked glycosylation sites were removed in gene synthesis by replacing the asparagine of all Asn-*X*-(Thr/Ser) motifs with Glu. These genes were ligated into pCR-Script cloning vector (Stratagene, CA).

Plasmid Construction and Transfection into Parasites—For the transgenic expression of PF14_0439-GFP under the control of the *hsp86* promoter, the complete M17 aminopeptidase gene PF14_0439 was amplified from D10 DNA using primers M17F (**AGATCT**ATGTATTTTTCTTCCTTATGT), which contained a BglII restriction site (in boldface), and M17R (**CTGCAG**TAGAGCGTCATTGAGTACAAA), 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 *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-

[5] The abbreviations used are: DV, digestive vacuole; H-Leu-NHMec, L-leucine-4-methylcoumarinyl-7-amide; GFP, green fluorescent protein; mFu, millifluorescent units; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; *Pf*LAP, *P. falciparum* M17 leucyl aminopeptidase; LAP, leucyl aminopeptidase; r*Pf*LAP, recombinant *Pf*LAP; Ni-NTA, nickel-nitrilotriacetic acid; LCR, low complexity region.

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 (*Pf*LAP) was achieved using a truncated form of the enzyme (Fig. 1*A*). 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 5′-CACCATGGCTTCTGAGGTTCCAC-3′, and LAP reverse 5′-GCCCTGAAAATACAGGTTTTCCAAAGCGTCGTTC-AAAAC-3′. The pENTR construct housing the truncated leucyl aminopeptidase, pENTR-LAP, was verified by DNA sequencing. The verified construct was recombined with 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 3×10^6 cells/ml with r*Pf*LAP 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 $\times$ *g* for 15 min at 4 °C.

Purification and Molecular Size Analysis of rPfLAP—Baculovirus cells ($\sim 3.5 \times 10^6$ cells/ml) containing r*Pf*LAP 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 $\times$ *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 μM $ZnCl_2$ 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 r*Pf*LAP and soluble extracts of malaria parasites (>80% trophozoites; prepared as for described for extraction of insect cells above) were analyzed using a Smart System (Amersham 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 $CoCl_2$ in 50 mM Tris-HCl, pH 8.0. Fractions were analyzed for aminopeptidolytic activity toward H-Leu-NHMec and Ala-NHMec 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 A_{280}. The molecular size of the *Pf*LAP and enzymes in the parasite soluble extracts was calculated from a plot of log M_r *versus* elution time for these standards.

Enzymatic Analysis—Aminopeptidase activity was determined by measuring the release of the fluorogenic leaving group, NHMec, 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 $CoCl_2$ for 20 min before the addition of 10 μM H-Leu-NHMec. Initial rates were obtained at 37 °C over a range of substrate concentrations spanning K_m 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 *Pf*LAP, progress curves were monitored until a final steady-state velocity, v_s, was reached. K_i values were determined from Dixon plots of $1/v_s$ *versus* inhibitor concentration when $[S] \ll K_m$.

The metal ion dependence of *Pf*LAP 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-NHMec substrate (10 μM). The *Pf*LAP 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 *Pf*LAP apoenzyme was determined by assaying its activity following preincubation (10 min, 37 °C) with 0.1 mM metals. Inhibition of *Pf*LAP 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 $CoCl_2$.

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-^{32}P]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 C*AGVSWN-FKARKPKG corresponding to the amino acids residues 577–590

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 *Pf*LAP 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 parasite nuclei were visualized with Hoechst dye (0.5 μg/ml). Samples were viewed on an Axioscope 2 Mot + (Zeiss) equipped with a Zeiss 63x/1.4 Plan Apochromat lens. Images were captured with an Axiocam MRm camera (Zeiss) using Axiovision AC software (Zeiss). PF14_0435-GPF-tagged transgenic parasites were visualized in a live fluorescence assay.

Protein extracts were resolved on reducing SDS-polyacrylamide gels (10%) and transferred to 0.2-μm polyvinylidene difluoride membranes (Macherey-Nagel). Membranes were probed with mouse anti-M17 peptide (1/250), stripped, and reprobed with anti-*P. falciparum* glyceraldehyde-3-phosphate dehydrogenase monoclonal antibodies (a kind gift of Dr. C. Daubenberger, Swiss Tropical Institute, Basel, Switzerland) diluted at 1/2000 to demonstrate equal loading (31). Sheep horseradish peroxidase-conjugated anti-mouse secondary antibodies (Chemicon) were used at 1/5000, and detection was carried out using ECL (Amersham Biosciences).

In Vitro Sensitivity of Parasites to Aminopeptidase Inhibitors—*P. falciparum* D10 parasites were cultured as described (24). Stock solutions of inhibitors were prepared in 100% Me_2SO. The *in vitro* sensitivity of each parasite population to the aminopeptidase inhibitors bestatin {(−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine}, bestatin methyl ester, and nitrobestatin was determined using [^{3}H]hypoxanthine incorporation (33). Briefly, serial dilutions of each inhibitor were prepared in culture media (0.05–50 μM) and added with [^{3}H]hypoxanthine (0.5 μCi/well) to asynchronous cultures at 0.5% parasitemia and 2% hematocrit. After a 48-h incubation, the amount of [^{3}H]hypoxanthine incorporation was measured, and the concentrations of inhibitor required to prevent incorporation by 50% (IC_{50}) were determined by linear interpolation of inhibition curves (34). Each assay was performed in triplicate on three separate occasions.

Phlyogenetic Analysis of P. falciparum M17 Leucyl Aminopeptidase—The C-terminal catalytic region sequence of *P. falciparum* leucyl aminopeptidase (PF14_0439) (see Fig. 1*A*) 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 alignment was then used to generate a phylogenetic tree. Phylogenetic 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 Apicoplexan Aminopeptidases—The general structure of the *P. falciparum* M17 leucyl aminopeptidase (*Pf*LAP) is presented as a schematic in Fig. 1*A*, 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. 1*A*). 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. 1*A*). Fig. 1*B* 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-379, 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 homnis* and *Cryptosporidium parvum* as well

[6] Sequences used in this study were 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. parvum* (accession number XP_626197), *T. annulata* (accession number CAI76586), *T. gondii* (accession number 80.m00088), Nostoc BAB77761; *Synechococcus elongatus* CAA73771, *Solanum 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* PepA 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.

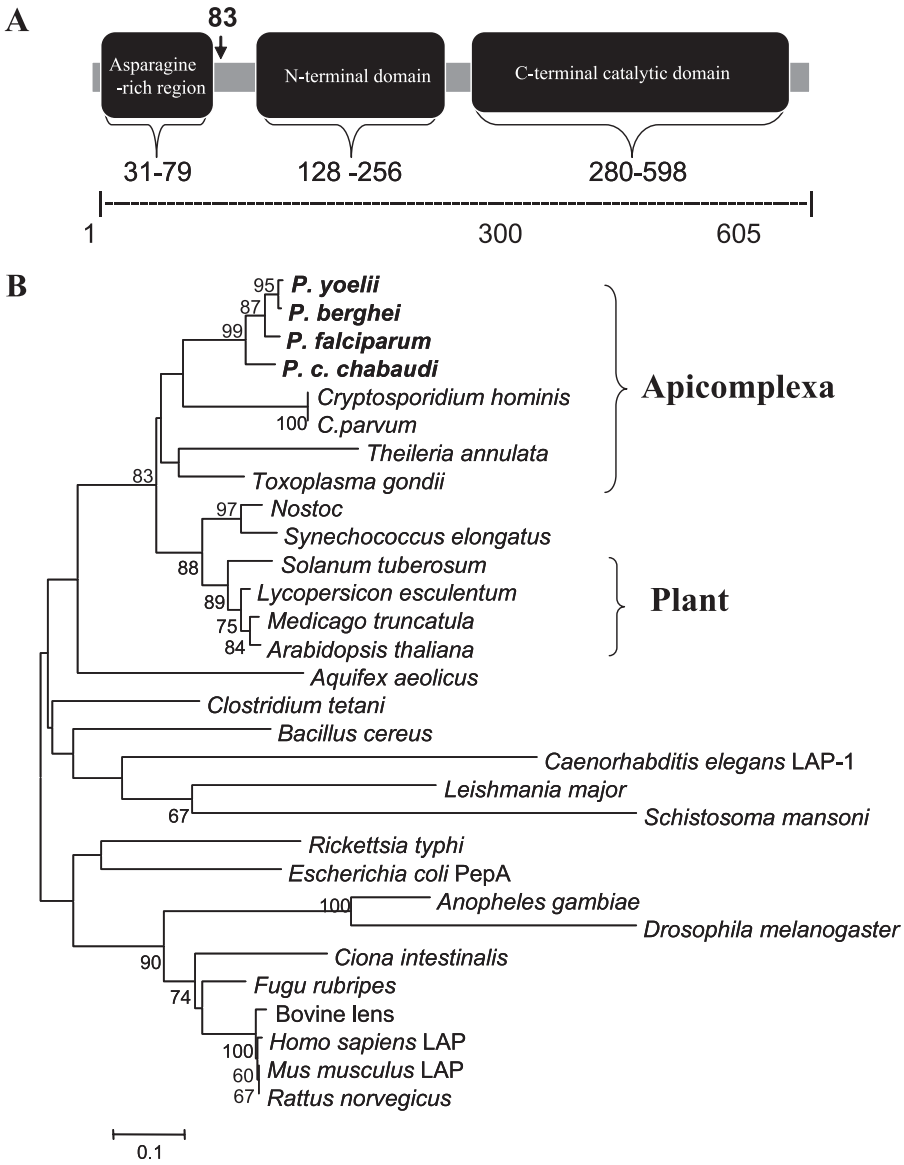

FIGURE 1. **The *P. falciparum* M17 leucyl aminopeptidase.** *A*, *schematic* showing the structure of the *P. falciparum* M17 leucyl aminopeptidase, highlighting the asparagine-rich region unique to malaria parasites, the less conserved N-terminal domain, and the highly conserved C-terminal domain that contains all the residues that make up the catalytic site. The *arrow* points to residue 83 where the functionally active recombinantly expressed r*Pf*LAP 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 *Crytosporidium hominis*, *C. parvum*, *T. annulata,* and *T. gondii*.

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*.

Functionally Active Recombinant P. falciparum M17 Leucyl Aminopeptidase Can Be Expressed in Insect Cells—Functional expression of the full-length *P. falciparum* leucyl aminopeptidase was unsuccessful in prokaryotic (*E. coli*) and eukaryotic (*P. pastoris*) expression systems (data not shown). A gene encoding a truncated form of the enzyme (residues 83–598; see Fig. 1*A*) 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 r*Pf*LAP 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. 2*A*). The r*Pf*LAP 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 μM) 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 r*Pf*LAP using the fluorogenic peptide H-Leu-NHMec as a substrate revealed that addition of Co(II) or Mn(II) ions to r*Pf*LAP 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 r*Pf*LAP, 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 r*Pf*LAP 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).

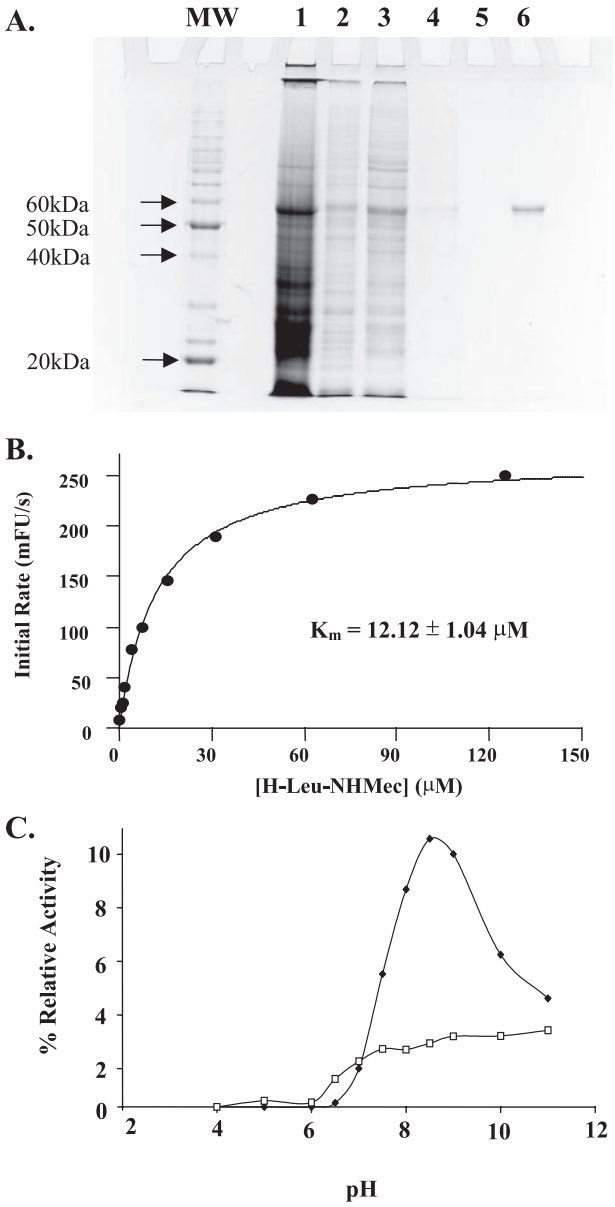

FIGURE 2. **Purification of functionally active *P. falciparum* M17 leucyl aminopeptidase.** *A*, purification of recombinant leucyl aminopeptidase was monitored by 12% reducing SDS-PAGE. *MW*, molecular size markers; *lane 1*, total freeze-thaw extract of insect cells infected with baculovirus carrying the *Pf*LAP 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*, eluted purified r*Pf*LAP from Ni-NTA-agarose column. Purified protein migrates at 60 kDa, which is expected for the truncated form of r*Pf*LAP indicated in Fig. 1*A*. *B*, enzyme assays with the fluorogenic peptide substrate H-Leu-NHMec demonstrate that the r*Pf*LAP exhibits typical Michaelis-Menten enzymatic kinetics with a K_m constant of 12.12 μM for this substrate. *C*, the r*Pf*LAP exhibits activity in the neutral to alkaline pH range, 6.5 to 11.0 (*solid diamonds*), with a pH optimum at 8.2. The enzyme was most stable in alkaline pH (*open squares*) and could be stored for 4 weeks at 4 °C and at pH 7.3 without significant loss of activity.

A preparation consisting largely of apoenzyme was prepared by incubating the r*Pf*LAP 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.

TABLE 1
Effect of divalent metal ions on recombinant *P. falciparum* M17 leucyl aminopeptidase (holoenzyme) and its metal-depleted apoenzyme

Metal ion	Concentration	Relative activity	
		Holoenzyme[a]	Apoenzyme[b]
	mM	%	
None		100	8
Ca(II)	0.01	153 ± 5	
	0.1	140 ± 1	9
	1	127 ± 3	
Co(II)	0.01	1175 ± 6	
	0.1	2399 ± 70	322
	1	2081 ± 46	
Cu(II)	0.01	227 ± 11	
	0.1	173 ± 5	15
	1	52 ± 2	
Fe(II)	0.01	140 ± 11	
	0.1	211 ± 5	13
	1	190 ± 17	
Mg(II)	0.01	180 ± 10	
	0.1	422 ± 14	17
	1	788 ± 11	
Mn(II)	0.01	587 ± 23	
	0.1	1653 ± 26	57
	1	2310 ± 24	
Ni(II)	0.01	334 ± 2	
	0.1	594 ± 26	46
	1	1002 ± 59	
Zn(II)	0.01	374 ± 37	
	0.1	418 ± 6	128
	1	420 ± 15	

[a] 100% activity is the rate of 73 mFu/s, and data reflect the mean relative activity ± S.D. (n = 3).
[b] Data reflect the relative activity of *Pf*LAP following treatment with *o*-phenanthroline (10 mM); 100% activity is the rate of 6 mFu/s.

TABLE 2
Effect of metal chelators on *P. falciparum* M17 leucyl aminopeptidase activity

Reagent	Concentration (mM)	Relative activity[a]	
		Without Co(II)[b]	With Co(II)[c]
		%	
None		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 ± 0.6
EDTA	10	80 ± 5	6 ± 0.1
	1	90 ± 4	88 ± 1
	0.1	112 ± 6	104 ± 15
	0.01	91 ± 4	93 ± 2

[a] Data reflect the mean relative activity of three assays ± S.D. (n = 3).
[b] *Pf*LAP not preincubated with Co(II), 100% activity = rate of 51 mFu/s.
[c] *Pf*LAP preincubated with 1 mM Co(II), 100% activity = rate of 1710 mFu/s.

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, r*Pf*LAP could efficiently cleave the hydrophobic amino acid leucine from the N terminus of synthetic peptides; its high affinity for this substrate is reflected in both a low K_m and a high k_{cat} value resulting is a very high overall catalytic efficiency, k_{cat}/K_m, of 3218 M^{-1} s^{-1} (Table 3). The enzyme exhibited a marked preference for this amino acid compared with the hydrophobic amino acid phenylalanine (k_{cat}/K_m = 183 M^{-1} s^{-1}), and even more so to proline (k_{cat}/K_m = 2.51 M^{-1} s^{-1}). Aliphatic hydrophobic amino acids such as valine

TABLE 3

Kinetic parameters for the hydrolysis of fluorogenic peptide substrates by the recombinant *P. falciparum* M17 leucyl aminopeptidase

Substrate	$k_{cat} \times 10^3$	K_m	k_{cat}/K_m
	s^{-1}	μM	$M^{-1} s^{-1}$
H-Leu-NHMec	39.0 ± 1.0	12.12 ± 1.04	3218
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

and isoleucine were not cleaved by r*Pf*LAP (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 r*Pf*LAP with $k_{cat}/K_m = 3.78\ M^{-1}\ s^{-1}$. 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 r*Pf*LAP subjected to HPLC size chromatography. Each eluted fraction was analyzed for H-Leu-NHMec- and H-Ala-MH-Mec-cleaving activity in the presence and absence of Co(II) (Fig. 3). The r*Pf*LAP 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 r*Pf*LAP 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 r*Pf*LAP peak) and another at 82 kDa (Fig. 3, *bottom panel*). The 320-kDa enzymatic peak eluted from the r*Pf*LAP 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 its activity was not significantly affected by a preincubation period with Co(II) (Fig. 3).

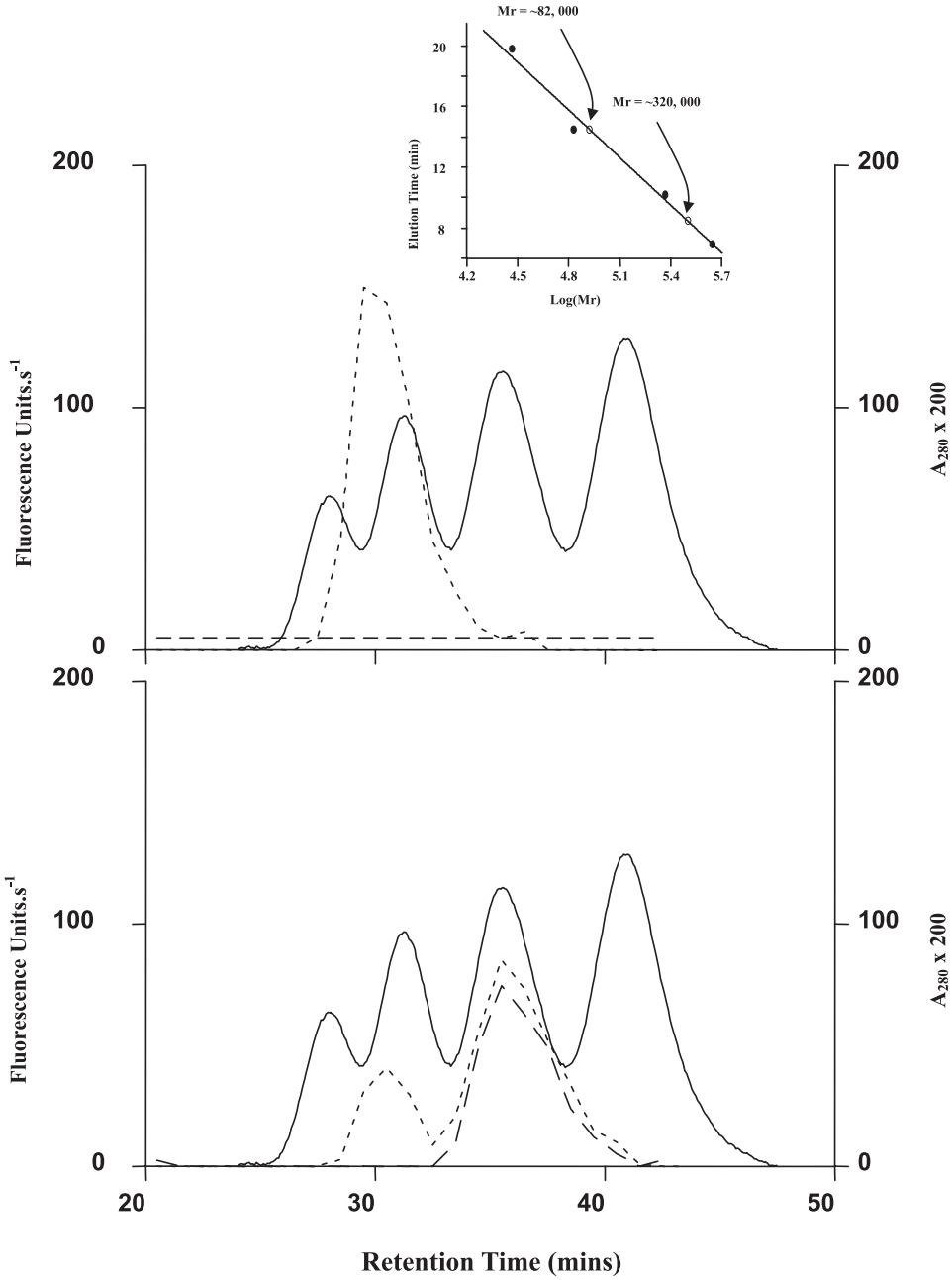

FIGURE 3. **HPLC gel filtration of recombinant and native *P. falciparum* leucyl aminopeptidase.** The r*Pf*LAP (3 μg) (*upper panel*) and soluble extracts of *P. falciparum* parasites (15 μg) (*lower panel*) were analyzed on a Superdex-200 HPLC column. Fractions were activated by addition of 1 mM Co(II) 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*).

PfLAP Is Expressed in the Cytosol throughout Intra-erythrocytic Development—Intra-erythrocytic *Pf*LAP mRNA expression levels, shown in Fig. 4*A*, are highest in the ring stage parasites followed by the trophozoite stages, with levels dropping during the latter half of the developmental cycle. Immunoblotting studies using sera prepared against a synthetic *Pf*LAP peptide (anti-M17) detected a single protein of the expected 68 kDa and showed that it is expressed during all stages of intra-erythrocytic development, including schizonts (Fig. 4*B*).

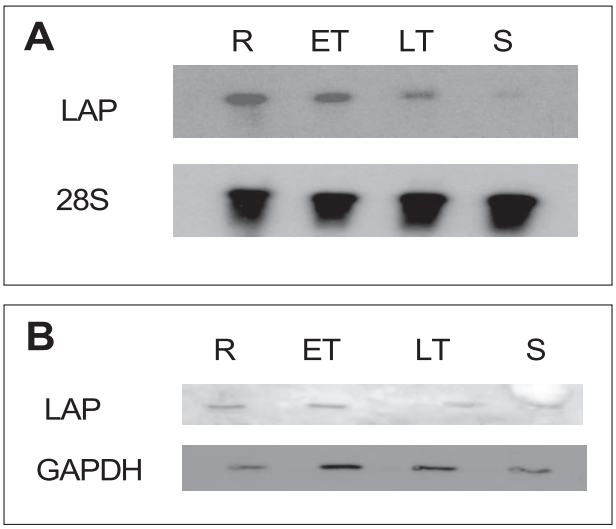

FIGURE 4. **Expression of the *P. falciparum* M17 leucyl aminopeptidase in the intra-erythrocytic stages of development.** *A*, total RNA isolated from stage-specific parasites was probed with a DNA fragment corresponding to the full-length M17 leucyl aminopeptidase DNA (*LAP*) before being stripped and re-probed with the *P. falciparum* S28 RNA probe (*28S*) to ensure equal sample loading in the lanes. *B*, protein extracts from stage-specific parasites were resolved on reducing SDS-polyacrylamide gels (10%), transferred to 0.2-μm polyvinylidene difluoride membranes, and probed with mouse anti-M17 peptide (*LAP*), stripped and re-probed with anti-*P. falciparum* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) monoclonal antibodies. Parasites were synchronized using two rounds of sorbitol treatment, and stage-specific parasites were harvested at early ring stage (*R*), early trophozoite stage (*ET*), late trophozoite (*LT*) ,and schizont stage (*S*).

To determine the cellular location of the *Pf*LAP protein, we probed parasite-infected erythrocytes with antiserum prepared against the recombinant enzyme and found immunoreactivity localized to the parasite cytoplasm (Fig. 5*A*). Confirmation of this localization for the *Pf*LAP was obtained using a live fluorescence assay to visualize the presence of *Pf*LAP-GFP fusion protein in *P. falciparum* erythrocytic stages transfected with the PF14_0439-GFP construct (Fig. 5*B*). All intra-erythrocytic stages, including segregating merozoites, displayed diffuse staining indicative of a cytoplasmic localization; however, the intensity of the staining was greatest in the early and late trophozoite stages.

Inhibitors of rPfLAP Are Also Inhibitors of P. falciparum Growth in Vitro—The ability of bestatin and two derivatives, nitrobestatin and bestatin methyl ester, to inactivate r*Pf*LAP was compared with their antimalarial activity against *P. falciparum* clone D10 in culture (Table 4). Bestatin was a potent inhibitor of r*Pf*LAP with a K_i = 25 nM and, as demonstrated previously (10, 16), was active against the intra-erythrocytic development of malaria parasites, displaying an IC_{50} value of 14.9 μM. Nitrobestatin exhibited an almost 10-fold greater inhibitory activity against r*Pf*LAP than bestatin, but its antimalarial activity was only slightly increased (IC_{50} = 8 μM). The bestatin methyl ester derivative, on the other hand, exhibited a 6-fold lower inhibitor value for r*Pf*LAP (K_i = 138 nM), but its killing activity against the parasites in culture was only slightly less than bestatin (IC_{50} = 20.5 μM).

DISCUSSION

All living cells maintain a closely regulated balance between protein catabolism and anabolism. The final step in protein 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 r*Pf*LAP 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 r*Pf*LAP 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 malarias, 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

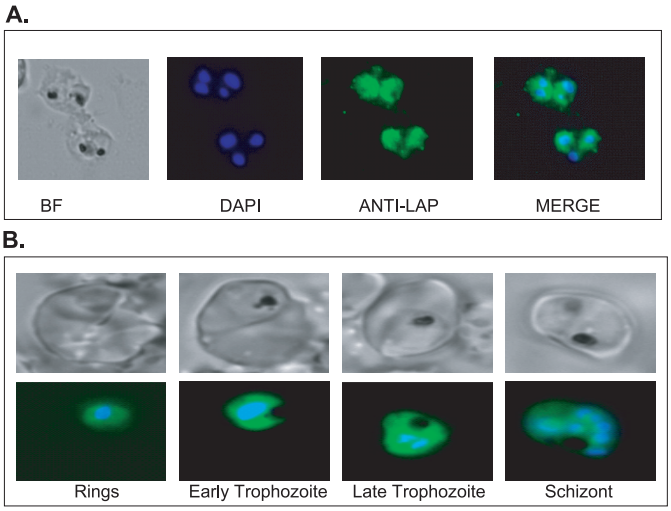

FIGURE 5. **Localization of the *P. falciparum* M17 leucyl aminopeptidase in the intra-erythrocytic parasites.** *A*, immunofluorescence assays were carried out using air-dried *P. falciparum*-infected red blood cells fixed with acetone. Cells were probed with mouse anti-M17 peptide (1/250) and then Cy2-conjugated goat anti-mouse antibodies. *B*, transgenic *P. falciparum* strain D10 parasites expressing the complete M17 leucyl aminopeptidase linked to green fluorescent protein were visualized in a live fluorescence assay. The parasite nuclei were visualized with Hoechst dye (0.5 μg/ml). Samples were viewed on an Axioscope 2 Mot + (Zeiss) equipped with a Zeiss 63x/1.4 Plan Apochromat lens. Images were captured with an Axiocam MRm camera (Zeiss) using Axiovision AC software (Zeiss). *BF*, bright field; *DAPI*, 4,6-diamidino-2-phenylindole.

TABLE 4

Inhibition of recombinant *P. falciparum* M17 leucyl aminopeptidase activity and blocking of the *in vitro* growth of the parasite (DC10 strain) by bestatin and its derivatives

Inhibitor	Inhibitory activity against recombinant *P. falciparum* LAPK_i (nM)	Inhibition of the *in vitro* growth of *P. falciparum* IC_{50} (μM)
Bestatin	25	14.87
Nitrobestatin	2.7	8
Bestatin methyl ester	138	20.5

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 r*Pf*LAP characterized in this study. These comparisons are relevant because Ramjee *et al.* (48) demonstrated that the cysteine protease of rodent malaria *P. berghei* (berghepain-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 *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 r*Pf*LAP 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 r*Pf*LAP 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 *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 r*Pf*LAP 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 *Pf*LAP is expressed by all intra-erythrocytic developmental stages of *P. falciparum*. Our Northern blot data support gene expression profiling studies using microarrays that showed *Pf*LAP 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 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. *Pf*LAP was assigned to cluster 6, and its expression was closely aligned with that of the cysteine protease falcipain-2 (PF11_0165) and plasmepsins (PF14_0174; PF14-_0075), which is consistent with its involvement with these enzymes in the turnover of host hemoglobin. But whether the *Pf*LAP plays additional functions in the latter developmental stages where expression is lower but still present within schizonts 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 schizont-infected erythrocytes to uninfected cells was reduced by the presence of bestatin (16). However, because the IC_{50} value of this effect was 25 μM (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 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 *Pf*LAP 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 *Pf*LAP is elevated. In this study we show that bestatin is a potent inhibitor of r*Pf*LAP with an inhibitory constant (K_i) of 25 nM; however, its derivative, nitrobestatin, exhibits almost a 10-fold greater inhibitory activity (K_i= 2.7 nM). Correlating with its greater anti-r*Pf*LAP inhibitory activity, nitrobestatin was also a more potent inhibitor of *P. falciparum* growth in culture, displaying an IC_{50} of 8 μM, although this was less than 2-fold better than bestatin, with an IC_{50} 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-r*Pf*LAP activity. Interestingly, although bestatin methyl ester (K_i = 138 nM) was 6-fold less inhibitory of r*Pf*LAP compared with bestatin and 50-fold less compared with nitrobestatin, it showed a surprisingly high relative antimalarial killing activity in culture (IC_{50} of 20.5 μM). Replacement of the C-terminal carboxyl group of bestatin (COOH) with a methyl ester ($COOCH_3$) greatly increases its hydrophobicity and therefore its ability to permeate cellular membranes (54). Thus, despite its lower anti-r*Pf*LAP 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 parasitophorus 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 *Pf*LAP, K_i, for each of these compounds are much lower than their IC_{50} values, which may indicate that they do not gain access to the target enzyme easily.

Our data support the idea that the M17 r*Pf*LAP 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 *Pf*LAP. 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 (aspartic 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 falcipian-2 on a plasmepsin IV/I double knock-out back round) had a negative but nonlethal 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 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 r*Pf*LAP 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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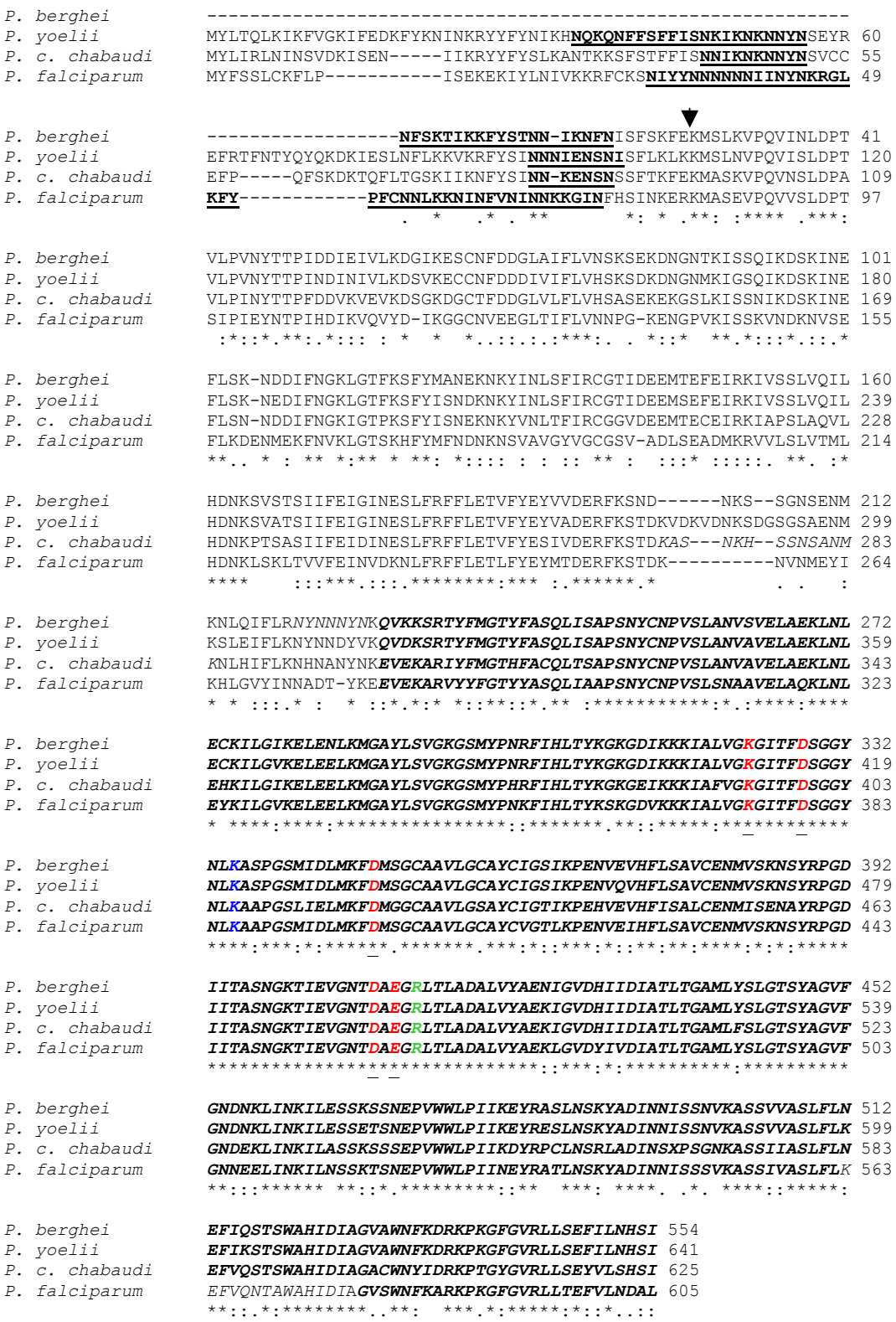
Supp. Figure 1. Comparison of the primary sequences of the human and rodent malaria M17 leucyl aminopeptidases. The primary sequence of the P. falciparum M17 leucyl aminopeptidase (PF14_0439) was aligned with its ortholog from P. yoelii (MALPY00521), P. berghei (PB_RP3746) and P. c. chabaudi (PC_RP1908). Conservation between amino acids is indicated by asterisk and dots in the alignment. The arrow indicates the beginning of the truncated rPfLAP. The asparagine-rich LCRs in the N-terminal extension of the malaria sequences are indicated each in bold and underlined. The less-conserved N-terminal domain is in normal font, while the more conserved C-terminal region is presented in bold and italics. LAPs bind two zinc ions that are pentahedrally-coordinated within the active site. The residues binding the metal ions are conserved; zinc 1 is bound by Asp 379, Asp 459 and Glu 461 while zinc 2 is bound by Lys 374, Asp 379, Asp 399 and Glu 461 (all are red and underlined). The conserved Lys 386 (blue) and Arg 463 (green) act as an electrophile and proton donor, respectively.

Supplementary Figure 1.



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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J. Biol. Chem. 2007, 282:2069-2080.
doi: 10.1074/jbc.M609251200 originally published online November 15, 2006
