Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*

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Running Title: *Falcipain-2, a P. falciparum trophozoite hemoglobinase*
SUMMARY

Trophozoites of the malaria parasite *Plasmodium falciparum* hydrolyze erythrocyte hemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis. Cysteine protease inhibitors block hemoglobin degradation, indicating that a cysteine protease plays a key role in this process. A principal trophozoite cysteine protease was purified by affinity chromatography. Sequence analysis indicated that the protease is encoded by a previously unidentified gene, falcipain-2. Falcipain-2 was predominantly expressed in trophozoites, was concentrated in food vacuoles, and was responsible for at least 93% of trophozoite soluble cysteine protease activity. A construct encoding mature falcipain-2 and a small portion of the prodomain was expressed in *Escherichia coli* and refolded to active enzyme. Specificity for the hydrolysis of peptide substrates by native and recombinant falcipain-2 was very similar, and optimal at acid pH in a reducing environment. Under physiological conditions (pH 5.5, 1 mM glutathione), falcipain-2 hydrolyzed both native hemoglobin and denatured globin. Our results suggest that falcipain-2 can initiate cleavage of native hemoglobin in the *P. falciparum* food vacuole, that, following initial cleavages, the protease plays a key role in rapidly hydrolyzing globin fragments, and that a drug discovery effort targeted at this protease is appropriate.
INTRODUCTION

Malaria remains one of the most important infectious diseases in the world (1). A key factor contributing to our continued inability to control this disease is the increasing resistance of malaria parasites to available drugs (2). The identification and characterization of new targets for antimalarial chemotherapy is thus an urgent priority. Among potential new targets for chemotherapy are proteases that degrade hemoglobin, a principal source of amino acids (3). In *Plasmodium falciparum*, the most virulent human malaria parasite, erythrocytic parasites transport hemoglobin to an acidic food vacuole, where the protein is hydrolyzed (4, 5). Enzymes that appear to participate in hemoglobin degradation include aspartic (6, 7), cysteine (8), and metallo (9) proteases. Enzymes of each of these mechanistic classes are potential chemotherapeutic targets. However, our understanding of the precise roles of plasmodial proteases in hemoglobin degradation is incomplete. In particular, studies of the roles of cysteine proteases in this process have been limited by the lack of straightforward purification schemes and difficulties with heterologous expression. Further characterization of plasmodial cysteine proteases and their roles in hemoglobin degradation should aid in the development of inhibitors of this process as antimalarial drugs.

Cysteine protease activity was originally identified in extracts of trophozoites, the erythrocytic parasite stage during which most hemoglobin degradation occurs (8, 10). A critical role for a cysteine protease hemoglobinase was suggested when it was demonstrated that cultured malaria parasites failed to develop when incubated with cysteine protease inhibitors (8, 11, 12). Morphological examination of cysteine protease
inhibitor-treated parasites revealed abnormally swollen, dark-staining food vacuoles, and biochemical evaluation indicated that the abnormality was caused by an accumulation of undigested hemoglobin in the food vacuole (8, 12-15). These results suggested a central role for a cysteine protease in early steps in hemoglobin degradation by *P. falciparum*. However, other studies yielded conflicting results, suggesting that the trophozoite cysteine protease cannot hydrolyze native hemoglobin, but rather that this protease hydrolyzes only denatured hemoglobin fragments produced by the action of vacuolar aspartic proteases (16, 17).

Until now, trophozoite cysteine protease activity has been attributed to the product of the single-copy falcipain gene (now renamed falcipain-1) (18). However, although recombinant falcipain-1 was shown to degrade hemoglobin (19), the lack of adequate expression and purification schemes has prevented the definitive characterization of the falcipain-1 gene product. We now report the purification of a major *P. falciparum* cysteine protease by affinity chromatography. This purification has yielded the unexpected result that the principal trophozoite cysteine protease, as determined with peptide substrates, is not the product of the falcipain-1 gene, but rather the product of a newly identified gene, falcipain-2. Our analysis of native and recombinant falcipain-2 strongly suggests that this protease plays a key role in the hydrolysis of both native and denatured hemoglobin by malaria parasites.

**EXPERIMENTAL PROCEDURES**

*Materials:* Benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl coumarin (Z-
Phe-Arg-AMC\(^1\) and glycyl-phenylalanyl-glycyl-semicarbazone were from Bachem. Human cathepsin B and cathepsin L were from Calbiochem. Recombinant cruzain was provided by Dr. Elizabeth Hansell (University of California, San Francisco). All other synthetic peptide substrates were a gift from Dr. David Tew (SmithKline Beecham). Z-Phe-Arg-fluoromethyl ketone (FMK) was a gift from Dr. Robert Smith (Prototek). All other biochemical reagents were from Sigma.

*Parasite culture* *P. falciparum* strain It or W2 parasites were cultured with human erythrocytes at 2% hematocrit in RPMI 1640 medium (20) supplemented with 0.5% AlbuMAX (Gibco BRL) or 10% human serum. Synchrony was maintained by serial sorbitol treatment (21).

*Preparation of parasite and subcellular fractions* Infected erythrocytes were washed with ice-cold phosphate buffered saline (PBS), treated with 0.1% saponin in ice-cold PBS for 5 min to lyse erythrocyte membranes (22), centrifuged (12,000 \(\times g\), for 10 min at 4\(^\circ\)C), and washed three times with ice-cold PBS. For trophozoite extracts, pellets were suspended in extraction buffer (20 mM bis TrisCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 10 \(\mu\)M pepstatin, pH 6.0), subjected to two freeze-thaw cycles to lyse parasite membranes, and repeatedly extracted in this buffer until protease activity was undetectable. Extracts were pooled and stored at -70\(^\circ\)C.

For preparation of the crude vacuolar and cytosolic fractions, \(2 \times 10^8\) trophozoites were prepared by lysis of erythrocyte membranes as described above, incubated with 5 volumes of 5% \(\alpha\)-sorbitol for 10 min on ice and centrifuged at 650 \(\times g\)
for 7 min (16). The pellet was resuspended in extraction buffer and subjected to two freeze-thaw cycles to prepare the crude vacuole extract, which was stored at -70°C; the supernatant was the cytosolic fraction. Purification of food vacuoles by density-gradient centrifugation was carried out exactly as previously described (23).

Preparation of affinity column

Glycyl-phenylalanyl-glycyl-semicarbazone (25 mg) was dissolved in 1 ml of methanol and combined with 5 ml of pre-swollen cyanogen bromide (CNBr)-activated aminohexyl-Sepharose 4B beads in a final volume of 50 ml of sodium carbonate buffer (100 mM, pH 8.0) as previously described (24). The mixture was rocked overnight at room temperature (RT), the beads were washed with 1 liter each of 50% methanol and deionized water, and unreactive sites were blocked with 6% ethanolamine for 4 h at RT. The beads were then washed with 1 liter each of deionized water and 20 mM TrisCl, pH 7.0, and the column was stored at 4°C.

Purification of native falcipain-2

Purification of falcipain-2 was carried out at 4°C using ice-cold buffers. The glycyl-phenylalanyl-glycyl-Sepharose column (~3 ml bed volume) was pre-equilibrated in binding buffer (extraction buffer with 2 mM dithiothreitol (DTT), 100 mM NaCl). Trophozoite extract (from ~3 × 10¹⁰ trophozoites per experiment) was then adjusted to the same concentrations of NaCl and DTT as binding buffer and applied to the column. The flow-through was reapplied to the column, which was then washed sequentially with 10 bed volumes binding buffer and 6 bed volumes of 20 mM sodium acetate buffer (NaOAc), 1 mM EDTA, pH 5.5. For elution, 3 volumes of elution buffer (20 mM TrisCl, 1 mM EDTA, 1 mM 2,2’-dipyridyl disulfide, pH 7.0) was passed though the column, the flow was stopped, and the
column was left overnight at 4°C. Elution was resumed with 9 additional bed volumes of elution buffer. The eluted enzyme was concentrated using Centricon 10 concentrators (Amicon) to a final volume of 200-300 µl. An equal volume of glycerol was added, and the enzyme was stored at -20°C.

For sequencing, falcipain-2 was electrophoresed on a 12.5% SDS-PAGE gel, transferred to an Immobilon-P SQ membrane (Millipore) and evaluated by Edman sequencing before or after treatment with CNBr. For mass spectrometry analysis, falcipain-2 was excised from a Coomassie Blue-stained SDS-PAGE gel and digested overnight with trypsin. Peptides were extracted from the gel slices with three 50% acetonitrile/5% formic acid washes. The combined supernatant was desalted with a C18 ZipTip (Millipore) and analyzed using a PE BioSystems Voyager Elite MALDI-TOF mass spectrometer.

Isolation of DNA and RNA: Genomic DNA was isolated from schizont-stage parasites (prepared as discussed above) by treatment with lysis buffer (100 µg/ml proteinase K, 10 mM TrisCl, 100 mM EDTA, 0.5% SDS, pH 8.0) followed by phenol extraction and isopropanol precipitation. Total RNA was extracted from parasites using Trizol as recommended by the manufacturer (Gibco BRL). The RNA pellet was suspended in sterile H2O (with 0.1% diethylpyrocarbonate) and treated with DNAse I. Purity and quantitation of nucleic acids were assessed spectrophotometrically. RNA samples free of genomic DNA (tested by PCR with falcipain-2 primers) were reverse transcribed using the SuperScript Preamplification System (Gibco BRL) as
recommended by the manufacturer. Briefly, 10-15 µg of total RNA was annealed with
the oligo(dT)12-18 primer and extended with Super Script II reverse transcriptase at
42°C for 50 min. Samples were treated with RNase H before PCR amplification.

*Amplification, cloning, and analysis of the falcipain-2 gene*  The falcipain-2
open reading frame (ORF) was amplified with forward (5´-
GTGTATTTTATTTGTAGCAAGAACGTGTTG-3´) and reverse (5´-
TGACAAGCTTTATTATAATGGAATGAATGCATCAGTACC-3´) primers based on
the Sanger Centre *P. falciparum* genomic sequence database using Taq DNA
polymerase (Gibco BRL) and W2-strain genomic DNA or trophozoite-stage cDNA.
PCR products were gel purified using the Qiagen gel extraction kit, ligated into the
pCR2.1-TOPO vector (pTOP-FP2) and transformed into TOPO10 *Escherichia coli*
(Invitrogen). Clones was sequenced in both directions by dideoxy sequencing at the UCSF
Biomolecular Resource Center. Analysis of sequence data was performed using the
Basic Local Alignment Search Tool (BLAST) program of the National Center for
Biotechnology Information (25), the DNASTAR program, and the PRED-TMR
algorithm for transmembrane segment determination (26).

*Southern and Northern blotting*  For Southern blotting, 10 µg aliquots of *P.
falciparum* genomic DNA were digested with restriction endonucleases,
electrophoresed on 0.7% agarose gels, and transferred onto nylon membranes
(Amersham). The membranes were hybridized overnight at 55°C with an α[^32P]-
labeled probe (Multiprime DNA labeling system; Amersham) that encoded the most
carboxy-terminal 35 amino acids of the prodomain and the complete mature domain of
falcipain-2 (-35FP2) in hybridization buffer (5 X SSPE (1 × = 0.15 M NaCl, 0.01 M NaH₂PO₄, 1 mM EDTA), 0.5% SDS, 5 × Denhardt's solution). Membranes were then subjected to serial washes under high stringency (2 × SSC (1 × = 0.15M NaCl, 0.15 M sodium citrate), 0.1% SDS, RT; 1 × SSC, 0.1% SDS, 55°C; 0.2 × SSC, 0.1% SDS, 60°C; each for 15 min), or low stringency (omission of last wash) conditions. For probing the same membrane with α [³²P]-labeled falcipain-1 (18), the membrane was washed twice with 0.1% SDS, 95°C for 15 min, once with 1 × SSC, RT for 5 min, and then exposed on X-ray film to confirm probe stripping. The membrane was then hybridized with a falcipain-1 probe (the full coding sequence) and washed as described for falcipain-2.

For Northern analysis, 10 µg total RNA samples from multiple life cycle stages were electrophoresed on a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The membrane was hybridized overnight at 55°C with α [³²P]-labeled -35FP2 in hybridization buffer and washed with 2 × SSC, 0.1% SDS, RT; 1 × SSC, 0.1% SDS, 42°C; and 0.2 × SSC, 0.1% SDS, 55°C, each for 15 min.

*Expression and purification of recombinant falcipain-2.* The -35FP2 fragment was amplified from the pTOP-FP2 plasmid using Vent DNA polymerase (New England Biolabs) and primers (forward 5´-

ATAGTTGGATCCGGGAAAGAATTAAACAGATTTGCC-3´; reverse 5´-

TGACAAGCTTATTCAATTAATGGAATGAATGCATCAGTACC-3´) incorporating internal restriction endonuclease cleavage sites. The PCR product was digested with *Bam* HI and *Hind* III, gel purified, and ligated into the pQE-30 expression vector (which
encodes an amino-terminal 6-His tag; Qiagen) to produce expression construct pQ-35FP2. This construct was used to transform M15(pREP4)-strain *E. coli*, its sequence was confirmed, and transformants were analyzed for expression of the protein by SDS-PAGE.

For large-scale expression, bacteria containing pQ-35FP2 were grown to mid-log phase and induced with isopropylthio-β-galactoside (IPTG, 0.25 mM) for 5 h at 37°C. Cells were harvested, washed with ice-cold 100 mM TrisCl, 10 mM EDTA, pH 7.4, sonicated (12 cycles of 10 sec each, with cooling for 10 sec between the cycles), and centrifuged at 12,000 × g for 30 min at 4°C. The pellet was washed twice with 2.5 M urea, 20 mM TrisCl, 2.5% Triton X-100, pH 8.0, centrifuged at 17,000 × g for 30 min at 4°C, solubilized in 6 M guanidine HCl, 20 mM TrisCl, 250 mM NaCl, 20 mM imidazole, pH 8.0 (5 ml/g of inclusion body pellet) at RT for 60 min with gentle stirring, and insoluble material was separated by centrifuging at 27,000 × g for 30 min at 4°C. For purification of the recombinant protein, the supernatant was incubated for 60 min at RT with a nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) equilibrated with the same buffer. The resin was loaded on a column and washed with 10 bed volumes each of 6 M guanidine HCl, 20 mM TrisCl, 250 mM NaCl, pH 8.0; 8 M urea, 20 mM TrisCl, 500 mM NaCl, pH 8.0; and 8 M urea, 20 mM TrisCl, 30 mM imidazole, pH 8.0. Bound protein was eluted with 8 M urea, 20 mM TrisCl, 1 M imidazole, pH 8.0, and quantified by the Bradford dye binding assay (27).

Refolding and additional purification of recombinant falcipain-2. The Ni-NTA purified protein was reduced with 10 mM DTT at 37°C for 45 min. Refolding of
recombinant falcipain-2 to an active protease was then assessed by a microtiter rapid
dilution method using 214 refolding buffers, all including 100 mM TrisCl, 1 mM EDTA,
pH 8.0, and differing in the ratio of reduced glutathione (GSH) to oxidized glutathione
(GSSG) and various additives (glycerol, sucrose, L-arginine, KCl, non-detergent
sulfo betaine-256 (NDSB-256), Triton X-100, and polyethylene glycol (PEG)). Each well,
including a 100-fold dilution of recombinant falcipain-2 (to 10 µg/ml) in 350 µl of ice-
cold refolding buffer, was incubated at 4°C for 20 h, and refolding efficiency was
evaluated by assaying 20 µl samples for hydrolysis of Z-Phe-Arg-AMC, as described
below.

Large scale refolding was carried out in optimized refolding buffer (100 mM
TrisCl, 1 mM EDTA, 20% glycerol, 250 mM L-arginine, 1 mM GSH, 1 mM GSSG, pH
8.0) with a 100-fold dilution of recombinant falcipain-2 (to 10 µg/ml) into 500 ml of the
ice-cold buffer, incubation with moderate stirring at 4°C for 24 h, and concentration to
20 ml using a stirred cell with a 10 kDa cut-off membrane (Amicon) at 4°C. The sample
was then filtered using a 0.22 µm syringe filter, dialyzed against a 100 × volume of
binding buffer, and further purified by affinity chromatography using a glycyl-
phenyl alanyl-glycyl-Sepharose column, as described earlier for native falcipain-2.

Assays of falcipain-2 activity For substrate gel analysis, samples were mixed
with SDS-PAGE sample buffer lacking 2-mercaptoethanol and electrophoresed in a
polyacrylamide gel copolymerized with 0.1% gelatin (8). The gel was then washed twice
(30 min, RT) with 2.5% Triton X-100 and incubated overnight at 37°C in 100 mM
NaOAc, 10 mM DTT, pH 5.5 before staining with Coomassie Blue.
Fluorimetric assays of native and recombinant falcipain-2 activity and inhibition were carried out as previously described (11, 28) in 100 mM NaOAc, 10 mM DTT, pH 5.5 (or with changes in pH or reductant as described) in a final volume of 0.35 ml. Fluorogenic substrates were added, and the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation 355 nm; emission 460 nm) over 30 min at RT with a Labsystems Fluoroskan II spectrofluorometer. Activities were compared as fluorescence over time.

**Enzyme kinetics** Enzyme concentrations were determined by titration with Z-Phe-Arg-fluoromethyl ketone (native and recombinant falcipain-2, cruzain) or trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; papain, cathepsin B and cathepsin L) (29). Rates of hydrolysis of peptide-AMC substrates were determined in the presence of constant enzyme concentrations for each substrate [native falcipain-2 (0.60 - 0.70 nM), recombinant falcipain-2 (1.4 nM), cruzain (0.16 nM), papain (0.02 - 0.04 nM), cathepsin B (0.04 - 0.21 nM), and cathepsin L (0.14 - 0.21 nM)]. The enzymes were assayed as described for native falcipain-2 (except at pH 6.0 for cathepsin B). The kinetic constants $K_m$ and $V_{max}$ were determined using the Enzfitter program (30).

**Production of antibodies, immunoblotting, and immunofluorescence** Female Balb/c mice (6 weeks old) were immunized intraperitoneally with an emulsified polyacrylamide gel containing Ni-NTA purified recombinant falcipain-2 in complete (on day 0) or incomplete (days 14 and 28) Freund’s adjuvant. Mice were sacrificed on day 35 and sera were pooled.

For immunoblotting, parasite extracts collected as described above and refolded
falcipain-2 were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight with 5% non-fat milk in PBS at 4°C, washed with PBS containing 0.05% Tween-20, incubated with a 1:350 dilution of immune serum for 1 h at RT, washed, incubated with goat anti-mouse IgG-alkaline phosphatase at 1:1000 dilution for 1 h at RT, and washed again. The reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma).

For immunofluorescence, air dried *P. falciparum* culture smears were fixed with ice-cold methanol for 20 min, air dried, and incubated for 90 min at RT with immune serum (1:100 in PBS). Slides were washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (IgG/IgA/IgM; 1:250 dilution in PBS). Slides were washed with PBS, air dried, and examined by fluorescence microscopy.

*Hemoglobin assays* To assess the biochemical requirements for hemoglobinase activity of native falcipain-2, the enzyme was added at a final concentration of 19 nM to 20 µl reactions containing 2 µg hemoglobin or 3 µg acid denatured globin (31) in 100 mM NaOAc, and concentrations of reductants, protease inhibitors, and reaction pH were varied as noted. Reactions were carried out at 37°C for 2 h and stopped by addition of 10 µl of 3 × SDS-PAGE sample buffer. Similarly, recombinant falcipain-2 (20 nM) was added to 30 µl reactions containing 3 µg human hemoglobin or 5 µg acid denatured globin in 100 mM NaOAc, pH 5.5, with or without 1 mM GSH and protease inhibitors, and after a 60 min incubation at 37°C reactions were stopped by adding
SDS-PAGE sample buffer.
RESULTS

Falcipain-2 is a principal P. falciparum trophozoite cysteine protease. Affinity chromatography of trophozoite extracts on a glycyl-phenylalanyl-glycyl-Sepharose column resulted in the purification to homogeneity of a protease that stained well with Coomassie blue but poorly with silver and demonstrated proteolytic activity when analyzed by gelatin substrate SDS-PAGE (Fig. 1). The protease migrated as a doublet when unreduced, although activity was only seen at $M_r$ 26,000. It migrated as a single band of $M_r$ 31,000 under reducing conditions. Amino-terminal sequencing of the purified protease (QMNYEVEIKKYRGEE) and internal peptide fragments generated by CNBr cleavage identified the protein as the product of the previously unidentified falcipain-2 gene. The protease was pure, as evidenced by the detection of only a single amino-terminal sequence in both the non-reduced and reduced protease samples. Furthermore, mass spectrometric analysis of a tryptic digest of the purified protease confirmed the presence of only predicted falcipain-2 digestion products.

In a series of seven purifications, 80-95% of soluble trophozoite cysteine protease activity bound to the affinity ligand. Re-application of flowthrough fractions to the affinity column allowed additional binding. After three applications, binding was complete, and residual cysteine protease activity, measured against the fluorogenic substrate Z-Phe-Arg-AMC, was 7% of initial activity. Whether the unbound activity represented additional falcipain-2 or other proteases is unknown. In any event, falcipain-2, the only protease purified by our affinity approach, appears to be the principal trophozoite cysteine protease. Purified falcipain-2 was stable, retaining
greater than 95% of its activity after storage in 50% glycerol at -20°C for 30 days.

*Falcipain-2 is concentrated in the parasite food vacuole* Crude food vacuole preparations were markedly enriched for cysteine protease activity (Table 1). Falcipain-2 was purified from the crude vacuolar fraction (Fig. 1D), and 85% of protease activity in density-gradient purified food vacuoles bound to the affinity ligand. Falcipain-2 was also present in cytosolic fractions, albeit with a much lower specific activity (Table 1). This activity may have been due to leakage of the protease from vacuoles during their preparation or the presence of the enzyme (possibly as an inactive proform that was activated during preparation) in the cytosol.

*Identification and analysis of the falcipain-2 gene* The amino-terminal sequence of falcipain-2 matched the deduced amino acid sequence encoded by a 1455 bp ORF from the Sanger Centre *P. falciparum* genomic sequence database (within fragment pfg11_624). A portion of this ORF also matched a *P. falciparum* erythrocytic-stage expressed sequence tag (Genbank N97987 (32)). The falcipain-2 gene was amplified from *P. falciparum* W2-strain trophozoite-stage cDNA and genomic DNA using primers spanning the ORF. The PCR products included identical uninterrupted ORFs which differed from the database sequence at 30 nucleotides (Fig. 2). The start codon for the falcipain-2 gene was assigned to the first ATG codon following a stop codon based on the first ATG rule (33, 34), the presence of a consensus lower eukaryote initiation sequence (AXX-ATG-G (33, 35)), and markedly increased AT content upstream of the start codon, a feature of non-coding *P. falciparum* DNA (36).

The falcipain-2 gene predicts a fairly typical papain-family cysteine protease,
with a large prodomain, a mature domain of 27 kDa, and conservation of active site amino acids (Fig. 3). A BLAST search showed falcipain-2 to be most similar among GenBank sequences to falcipain-1 (18), although identity between predicted mature domains (37%) was only slightly higher than that between falcipain-2 and many other cysteine proteases, including plant, viral, and mammalian enzymes. Falcipain-2 has some unusual features for a papain-family enzyme (37), including (a) lack of a typical mature protease processing site, (b) more predicted disulfide bonds (four) than many members of this family, (c) a 17 amino acid insert between the mature protease processing site and a highly conserved amino-terminal region, and (d) an insert between catalytic His and Asn residues at the location of a larger insert in falcipain-1. As also seen with falcipain-1, the falcipain-2 prodomain is unusually large, with minimal amino-terminal sequence conservation, but modest conservation of the most carboxy-terminal sequence (for the 79 carboxy-terminal amino acids of the falcipain-2 prodomain, identity was 34% between falcipain-1 and falcipain-2 and 33% between falcipain-2 and papain). Falcipain-2 lacks a typical signal sequence, but does contain a 20 amino acid hydrophobic stretch that is predicted by the PRED-TMR algorithm to represent a transmembrane domain (26).

Digests of *P. falciparum* W2 strain genomic DNA were probed with the falcipain-2 gene. The probe hybridized strongly with two DNA fragments generated by each of 5 restriction endonucleases (Fig. 4). Three of these enzymes had no restriction sites within the -35FP2 probe, and for the other two enzymes, *Eco* RI and *Bgl* II, which had cleavage sites near the 5′ terminus of the probe, a third DNA fragment was
recognized under lower stringency conditions (not shown). Probing the same blot under high stringency with a falcipain-1 probe yielded a completely different hybridization pattern (not shown). These results suggest the presence of a second gene that is closely related to falcipain-2. The gene, which is not falcipain-1, may be a third putative cysteine protease gene (named falcipain-3) which was also identified in a genomic database search (within Sanger Centre fragment pfg11_624) and has not yet been fully sequenced. Falcipain-3 is more similar in its mature domain sequence to falcipain-2 than is falcipain-1, but its predicted amino-terminal sequence differs from that of falcipain-2, and it was not detected in the cysteine protease activity purified from trophozoites (Fig. 3).

Expression, refolding, and processing of recombinant falcipain-2. A fragment corresponding to the most carboxy-terminal 35 amino acids of the prodomain and the complete mature domain of falcipain-2 was amplified, cloned into the expression vector pQE-30, and used to transform M15(pREP4) E. coli. Falcipain-2 was abundantly expressed in an insoluble form (Fig. 5). In order to generate active enzyme, inclusion bodies were solubilized and purified by metal chelate affinity chromatography, and multiple refolding conditions (38, 39) were evaluated in a microplate format. As measured by hydrolysis of Z-Phe-Arg-AMC, optimal refolding of recombinant falcipain-2 was obtained in 100 mM TrisCl, 1 mM EDTA, 20% glycerol, 250 mM L-arginine, 1 mM GSH, 1 mM GSSG, pH 8.0.

Refolding was accompanied by processing of falcipain-2, with a shift in migration from $M_r$ 32,000 to $M_r$ 27,000 (Fig. 6). Additional purification of active
recombinant falcipain-2 was achieved by affinity purification with Gly-Phe-Gly-Sepharose, as described for native falcipain-2. Gelatin substrate SDS-PAGE confirmed that recombinant falcipain-2 was active and that the refolded protease was processed to a smaller species. Processing was blocked by the cysteine protease inhibitor leupeptin, but not inhibitors of other classes of proteases, consistent with autohydrolysis during refolding (Fig. 6). The amino-terminal sequence of refolded falcipain showed microheterogeneity, with identification of four different amino-terminal sequences, NSKYL-, SKYLL-, YLLDQ-, and QMNYE-.

*Falcipain-2 is an acidic cysteine protease with unique substrate specificity*

Under our conditions of study, the hydrolysis of the synthetic peptide substrate Z-Phe-Arg-AMC by native falcipain-2 was optimal at acid pH and was markedly stimulated by reducing agents, as is typical for papain-family proteases (Fig. 7). Falcipain-2 was inhibited by the cysteine protease inhibitors E-64 and leupeptin, but not by inhibitors of other protease classes. These properties were very similar to those determined earlier for the cysteine protease activity of trophozoite extracts (8, 11), supporting the conclusion that falcipain-2 is the principal cysteine protease of trophozoites. The properties of recombinant falcipain-2 were very similar to those of the native enzyme (Fig. 7).

To begin to characterize the substrate specificity of falcipain-2 and to compare it with other papain-family enzymes, we evaluated the hydrolysis of a panel of synthetic peptide substrates. Native falcipain-2 cleaved 10 of 25 tested substrates; all of the cleaved substrates had arginine or lysine at the P1 position (Table 2). Both native and
recombinant falcipain-2 showed a marked preference for leucine or phenylalanine at the P$_2$ position. The P$_2$ specificity of native falcipain-2 was compared with that of other papain-family proteases, using a panel of Z-X-Arg-AMC substrates (Fig. 8). Falcipain-2 shared with cathepsin L, papain, and cruzain the preference for hydrophobic but not charged amino acids at the P$_2$ position. The strong falcipain-2 preference for leucine at P$_2$ was unique among the proteases tested.

*Stage-specific expression and localization of falcipain-2* To evaluate transcription of the falcipain-2 gene, Northern blotting was performed with a falcipain-2 probe and stage specific *P. falciparum* RNA. A 2.5 kb message was detected principally in late ring and trophozoite RNA, with maximal expression in early trophozoites (Fig. 9A). Immunoblotting with a murine antiserum against recombinant falcipain-2 identified maximal protein expression in trophozoites and early schizonts (Fig. 9B). Immunoblotting identified two proteins, an $M_r$ 27,000 species that comigrated with recombinant mature falcipain-2, and a larger species of approximately the size predicted for unprocessed profalcipain-2. A comparison of the cysteine protease activity of parasites of different stages identified maximal hydrolysis of gelatin in early trophozoites (Fig. 9C). To investigate the subcellular localization of falcipain-2, methanol fixed trophozoites were probed with antiserum against the protease. The antiserum diffusely stained trophozoites, but not host erythrocytes (Fig. 9D). Staining of the parasite food vacuole was evident, although the signal was attenuated in the vicinity of hemozoin. This result suggests that falcipain-2 localizes to the food vacuole,
as shown biochemically (Fig. 1 and Table 1), but also that the enzyme is present in the cytosol, probably as an inactive proform.

_Falcipain-2 cleaves hemoglobin under physiological conditions._

Hemoglobin is degraded in the _P. falciparum_ food vacuole, an acidic (pH~5.2), lysosome-like hydrolytic organelle (40, 41). The spontaneous denaturation of hemoglobin is very slow at this pH (15, 42). Native and recombinant falcipain-2 cleaved hemoglobin at acid pH. This activity required reducing agents, but physiological concentrations (1 mM) of GSH that did not appreciably denature hemoglobin (not shown) were adequate to support efficient hydrolysis of hemoglobin (Fig. 10). Denatured globin was more rapidly hydrolyzed than hemoglobin in a reducing environment (not shown), and also was slowly hydrolyzed under nonreducing conditions.

**DISCUSSION**

A principal cysteine protease of _P. falciparum_ trophozoites was purified using an affinity chromatography protocol, which was adapted from a method for the purification of cathepsin B (24). Our purification provided, for the first time, a definitive amino-terminal sequence of this protease. Surprisingly, this sequence was not encoded by the falcipain-1 gene, but rather by a previously unidentified cysteine protease gene that we have named falcipain-2. In retrospect, it appears that the conclusion that falcipain-1 encodes the principal trophozoite cysteine protease (18) was based on cross-reactive antisera. Indeed, a rabbit antiserum raised against recombinant falcipain-1 reacted with purified falcipain-2 (data not shown). Falcipain-2 was the
only protease purified by our affinity technique. Importantly, both sequencing and mass spectrometry analyses of purified protein did not detect the presence of falcipain-1 or falcipain-3. The biological roles of these other putative \textit{P. falciparum} proteases are unknown. Falcipain-2 constituted at least 93\% of soluble trophozoite cysteine protease activity, as measured with the substrate Z-Phe-Arg-AMC, but some activity was not accounted for by this enzyme, and activities may differ against different substrates. It is thus possible that the other plasmodial cysteine proteases are also active in trophozoites, and play ancillary roles in hemoglobin degradation. Falcipain-2 activity was optimal at acidic pH and concentrated in the acidic food vacuole, as would be expected for a trophozoite hemoglobinase.

The facile heterologous expression and refolding of falcipain-2 was somewhat surprising, as the expression of \textit{P. falciparum} enzymes has often been problematic, and as our expression construct encoded only a small part of the prodomain. An extensive literature has documented the essential nature of protease prodomains in the expression and refolding of active enzymes \textsc{(43-45)}. The prodomains presumably mediate folding and act as protease inhibitors \textsc{(46-48)}. For multiple proteases, including \textit{\alpha}-lytic protease \textsc{(49)}, subtilisins \textsc{(50)}, yeast carboxypeptidase \textsc{(51)}, thermolysin \textsc{(52)}, and papain-family cysteine proteases \textsc{(53)}, failure to include the prodomain in expression constructs prevented the refolding of active protease. Our falcipain-2 construct included the most 35 carboxy-terminal residues of the 243 amino acid prodomain. This small portion of the prodomain was adequate to support successful refolding of falcipain-2, apparently because it is the carboxy-terminal portion of cysteine protease
prodomains that mediate enzyme inhibition and/or refolding (54-56). The refolding of falcipain-2 was accompanied by processing to the mature active protease. This processing was autocatalytic, as is the case with other papain-family proteases including papain (57), cathepsin B (58), cathepsin L (59), cathepsin K (60), and cruzain (61). Surprisingly, the processing occurred in refolding buffer at an alkaline pH (8.0) at which mature falcipain-2 has minimal activity against peptide substrates or hemoglobin. For other papain-family enzymes, acidic conditions appear to be required to unfold the prodomain and allow autoactivation (62). Processing of profalcipain-2 may have been expedited by the fact that our expression construct included a truncated prodomain. This domain was adequate to facilitate refolding, but may not have interacted with the active site in the manner shown for full prodomains of related enzymes (54-56). Thus, for expressed truncated profalcipain-2, the active site may have been free to cleave the truncated proregion under alkaline conditions. Of note, cleavages during refolding occurred at the native processing site and three sites 4-7 amino acids upstream of the native site, with the majority of molecules cleaved at sites containing P$_2$ leucine, a feature of the most favored peptide substrates identified for falcipain-2.

Falcipain-2 has some unusual features when compared to other papain-family cysteine proteases, including an unusually large prodomain. Interestingly, the prodomains of falcipain-1 (18) and its analogues from other plasmodial species (63, 64) are even larger. The carboxy-terminal portions of the prodomains of the plasmodial cysteine proteases share modest homology with other papain-family enzymes and
likely mediate refolding in a manner analogous to that of other papain-family enzymes, as supported by the successful refolding of falcipain-2 containing only a truncated prodomain. The amino-terminal portions of the plasmodial cysteine proteases have unknown functions. Of interest, the prodomains of other plasmodial proteases, including the food vacuole aspartic proteases plasmepsin I and plasmepsin II (6, 7), also have unusually large prodomains for their respective protease classes. Amino-terminal prodomain sequences may mediate trafficking of the plasmodial proteases, as is the case in some plant (65), yeast (66) and protozoan (67) proteases. Hydrophobic domains that are present in the prodomains of falcipain-2, falcipain-1, and the plasmepsins do not appear to be typical signal sequences, but may play a role in trafficking to the parasite food vacuole. However, immunofluorescence studies suggest that falcipain-2 is present in both the parasite cytosol and food vacuole, suggesting that transit of profalcipain-2 to the food vacuole is relatively slow, and that a reservoir of the proenzyme is present in the cytosol.

Analysis of the specificity of falcipain-2 indicated that, at least with peptide substrates, the amino acid at the P2 position plays a key role in mediating substrate specificity. This is also the case with other cathepsin L-like papain family proteases, but not with cathepsin B, which accepts chemically disparate P2 amino acids in its substrates. Falcipain-2 showed a preference for the bulky hydrophobic amino acids leucine and phenylalanine over smaller hydrophobic (valine) or charged (arginine, glutamic acid) P2 amino acids. The marked preference for leucine and poor acceptance
of valine distinguished falcipain-2 from cathepsin L. The P2 specificity of falcipain-2 was more similar to that of cathepsin K and cathepsin S, two other mammalian papain-family cysteine proteases (68, 69). Although results with peptide substrates may not predict specificity against physiological substrates, these data are consistent with a preference for P2 leucine in inhibitors with potent antimalarial activity (22, 28). The identification of lead falcipain-2 inhibitors may be expedited by efforts to develop cathepsin K inhibitors as treatments for osteoporosis (70).

Multiple *P. falciparum* food vacuole enzymes, including aspartic, cysteine, and metallo proteases, appear to participate in hemoglobin degradation, although the precise role of each protease is not well understood (5). It has been proposed that hemoglobin degradation is an ordered process in which aspartic proteases initiate hemoglobin hydrolysis, and a cysteine protease subsequently hydrolyzes denatured hemoglobin peptides (5, 16). This model is appealing in its simplicity, but it cannot easily explain the observation with cultured parasites that the inhibition of falcipain-2 causes the food vacuole to fill with intact globin (8), at least a portion of which is native hemoglobin (15). The model for the ordered degradation of hemoglobin was based on experiments performed in the absence of reducing agents; consideration of the effects of the physiological reductant GSH on falcipain-2 activity suggests that this protease also hydrolyzes native hemoglobin.

Our data show that falcipain-2 is a potent hemoglobinase under strongly reducing conditions, but also that, under physiological conditions (1 mM GSH), falcipain-2 effectively hydrolyzes native hemoglobin. Sufficient reducing strength to activate
Falcipain-2 appears to be present in the food vacuole, as erythrocyte cytoplasm, which contains millimolar GSH (71), is transported into the food vacuole in large quantity, and parasites maintain millimolar concentrations of GSH via synthetic pathways (72-74) and the export of oxidized glutathione (72). Our results agree with earlier findings (17) that falcipain-2 more efficiently cleaves hemoglobin after its denaturation. These data support a model in which falcipain-2 participates in initial cleavages of hemoglobin and plays a key role in the hydrolysis of globin fragments after initial cleavages cause denaturation of the substrate.

Falcipain-2 and other *P. falciparum* hemoglobinases are promising chemotherapeutic targets. Indeed, inhibitors of both falcipain-2 (12, 22, 28, 75) and the plasmepsins (6, 76-78) are potent antimalarials, and combinations of inhibitors of both classes of proteases yield synergistic antimalarial activity in vitro (14, 79) and in vivo (79). The availability of large quantities of active recombinant falcipain-2 offers hope that additional biochemical and structural characterization of the protease will benefit ongoing drug discovery efforts directed toward this enzyme.

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REFERENCES

1. Olliaro, P., Cattani, J., and Wirth, D. (1996) JAMA 275, 230-233
2. White, N. J. (1998) Br Med Bull 54, 703-715
3. Olliaro, P. L. and Yuthavong, Y. (1999) Pharmacol. Ther. 81, 91-110
4. Rosenthal, P. J. and Meshnick, S. R. (1996) Mol. Biochem. Parasitol. 83, 131-139
5. Francis, S. E., Sullivan, D. J., and Goldberg, D. E. (1997) Annu. Rev. Micro. 51, 97-123
6. Francis, S. E., Gluzman, I. Y., Oksman, A., Knickerbocker, A., Mueller, R., Bryant, M. L., Sherman, D. R., Russell, D. G., and Goldberg, D. E. (1994) EMBO J. 13, 306-317
7. Hill, J., Tyas, L., Phylip, L. H., Kay, J., Dunn, B. M., and Berry, C. (1994) FEBS Lett. 352, 155-158
8. Rosenthal, P. J., McKerrow, J. H., Aikawa, M., Nagasawa, H., and Leech, J. H. (1988) J. Clin. Invest. 82, 1560-1566
9. Eggleson, K. K., Dufin, K. L., and Goldberg, D. E. (1999) J. Biol. Chem. 274, 32411-32417
10. Rosenthal, P. J., Kim, K., McKerrow, J. H., and Leech, J. H. (1987) J. Exp. Med. 166, 816-821
11. Rosenthal, P. J., McKerrow, J. H., Rasnick, D., and Leech, J. H. (1989) Mol. Biochem. Parasitol. 35, 177-184
12. Rosenthal, P. J., Wollish, W. S., Palmer, J. T., and Rasnick, D. (1991) J. Clin. Invest. 88, 1467-1472
13. Dluzewski, A. R., Rangachari, K., Wilson, R. J. M., and Gratzer, W. B. (1986) Exp. Parasitol. 62, 416-422
14. Bailly, E., Jambou, R., Savel, J., and Jaureguiberry, G. (1992) J. Protozool. 39, 593-599
15. Gamboa de Domínguez, N. D. and Rosenthal, P. J. (1996) Blood 87, 4448-4454
16. Gluzman, I. Y., Francis, S. E., Oksman, A., Smith, C. E., Duffin, K. L., and Goldberg, D. E. (1994) J. Clin. Invest. 93, 1602-1608
17. Francis, S. E., Gluzman, I. Y., Oksman, A., Banerjee, D., and Goldberg, D. E. (1996) Mol. Biochem. Parasitol. 83, 189-200
18. Rosenthal, P. J. and Nelson, R. G. (1992) Mol. Biochem. Parasitol. 51, 143-152
19. Salas, F., Fichmann, J., Lee, G. K., Scott, M. D., and Rosenthal, P. J. (1995) *Infect. Immun.* 63, 2120-2125
20. Trager, W. and Jensen, J. B. (1976) *Science* 193, 673-675
21. Lambros, C. and Vanderberg, J. P. (1979) *J. Parasitol.* 65, 418-420
22. Rosenthal, P. J., Lee, G. K., and Smith, R. E. (1993) *J. Clin. Invest.* 91, 1052-1056
23. Goldberg, D. E., Slater, A. F. G., Cerami, A., and Henderson, G. B. (1990) *Proc. Natl. Acad. Sci. USA* 87, 2931-2935
24. Rich, D. H., Brown, M. A. and Barrett, A. J. (1986) *Biochem. J.* 235, 731-734
25. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403-410
26. Pasquier, C., Promponas, V. J., Palaios, G. A., Hamodrakas, J. S., and Hamodrakas, S. J. (1999) *Protein Eng.* 12, 381-385
27. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248-254
28. Rosenthal, P. J., Olson, J. E., Lee, G. K., Palmer, J. T., Klaus, J. L., and Rasnick, D. (1996) *Antimicrob. Agents Chemother.* 40, 1600-1603
29. Barrett, A. J. and Kirschke, H. (1981) *Meth. Enzymol.* 80, 535-561
30. Leatherbarrow, R. (1987) *Enzfitter: A Nonlinear Regression Data Analysis Program for the IBM PC* (Elsevier, Amsterdam)
31. Ascoli, F., Fanelli, M. R., and Antonini, E. (1981) *Methods Enzymol.* 76, 72-87
32. Chakrabarti, D., Reddy, G. R., Dame, J. B., Almira, E. C., Laips, P. J., Ferl, R. J., Yang, T. P., Rowe, T. C., and Schuster, S. M. (1994) *Mol. Biochem. Parasitol.* 66, 97-104
33. Kozak, M. (1989) *J. Cell Biol.* 108, 229-241
34. Kozak, M. (1995) *Proc. Natl. Acad. Sci. U S A* 92, 7134
35. Saul, A. and Battistutta, D. (1990) *Mol. Biochem. Parasitol.* 42, 55-62
36. Saul, A. and Battistutta, D. (1988) *Mol. Biochem. Parasitol.* 27, 35-42
37. Berti, P. J. and Storer, A. C. (1995) *J. Mol. Biol.* 246, 273-283
38. Rudolph, R. and Lilie, H. (1996) *FASEB J.* 10, 49-56
39. Mukhopadhyay, A. (1997) *Adv. Biochem. Eng. Biotechnol.* 56, 61-109
40. Yayon, A., Cabantchik, Z. I., and Ginsburg, H. (1984) *EMBO J.* 3, 2695-2700
41. Krogstad, D. J., Schlesinger, P. H., and Gluzman, I. Y. (1985) *J. Cell Biol.* 101, 2302-
42. Gabay, T. and Ginsburg, H. (1993) *Exp. Parasitol.* **77**, 261-272
43. Ikemura, H. and Inouye, M. (1988) *J. Biol. Chem.* **263**, 12959-12963
44. Baker, D., Shiau, A. K., and Agard, D. A. (1993) *Curr. Opin. Cell. Biol.* **5**, 966-970
45. Peters, R. J., Shiau, A. K., Sohl, J. L., Anderson, D. E., Tang, G., Silen, J. L., and Agard, D. A. (1998) *Biochemistry* **37**, 12058-12067
46. Baker, D., Silen, J. L., and Agard, D. A. (1992) *Proteins* **12**, 339-344
47. Fox, T., de Miguel, E., Mort, J. S., and Storer, A. C. (1992) *Biochemistry* **31**, 12571-12576
48. Li, Y., Hu, Z., Jordan, F., and Inouye, M. (1995) *J. Biol. Chem.* **270**, 25127-25132
49. Silen, J. L., Frank, D., Fujishige, A., Bone, R., and Agard, D. A. (1989) *J. Bacteriol.* **171**, 1320-1325
50. Ikemura, H., Takagi, H., and Inouye, M. (1987) *J. Biol. Chem.* **262**, 7859-7864
51. Ramos, C., Winther, J. R., and Kielland-Brandt, M. C. (1994) *J. Biol. Chem.* **269**, 7006-7012
52. Marie-Claire, C., Ruffet, E., Beaumont, A., and Roques, B. P. (1999) *J. Mol. Biol.* **285**, 1911-1915
53. Smith, S. M. and Gottesman, M. M. (1989) *J. Biol. Chem.* **264**, 20487-20495
54. Coulombe, R., Grochulski, P., Sivaraman, J., Menard, R., Mort, J. S., and Cygler, M. (1996) *EMBO J.* **15**, 5492-5495
55. Podobnik, M., Kuhelj, R., Turk, V., and Turk, D. (1997) *J. Mol. Biol.* **271**, 774-88
56. LaLonde, J. M., Zhao, B., Janson, C. A., D’Alessio, K. J., McQueney, M. S., Orsini, M. J., Debouck, C. M., and Smith, W. W. (1999) *Biochemistry* **38**, 862-869
57. Taylor, M. A., Pratt, K. A., Revell, D. F., Baker, K. C., Sumner, I. G., and Goodenough, P. W. (1992) *Protein Eng.* **5**, 455-459
58. Rowan, A. D., Mason, P., Mach, L., and Mort, J. S. (1992) *J. Biol. Chem.* **267**, 15993-15999
59. Mason, R. W., Gal, S., and Gottesman, M. M. (1987) *Biochem. J.* **248**, 449-454
60. McQueney, M. S., Amegadzie, B. Y., D’Alessio, K., Hanning, C. R., McLaughlin, M. M., McNulty, D., Carr, S. A., Ijames, C., Kurdyla, J., and Jones, C. S. (1997) *J. Biol. Chem.*
61. Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H., and Craik, C. S. (1992) *J. Biol. Chem.* **267**, 7411-7420
62. Jerala, R., Zerovnik, E., Kidric, J., and Turk, V. (1998) *J. Biol. Chem.* **273**, 11498-11504

63. Rosenthal, P. J. (1993) *Biochem. Biophys. Acta* **1173**, 91-93
64. Rosenthal, P. J., Ring, C. S., Chen, X., and Cohen, F. E. (1994) *J. Mol. Biol.* **241**, 312-316
65. Vitale, A. and Chrispeels, M. J. (1992) *Bioessays* **14**, 151-160
66. Johnson, L. M., Bankaitis, V. A., and Emr, S. D. (1987) *Cell* **48**, 875-885
67. Huete-Perez, J. A., Engel, J. C., Brinen, L. S., Mottram, J. C., and McKerrow, J. H. (1999) *J. Biol. Chem.* **274**, 16249-16256

68. Bromme, D., Bonnaue, P. R., Lachance, P., and Storer, A. C. (1994) *J. Biol. Chem.* **269**, 30238-30242
69. Bossard, M. J., Tomaszek, T. A., Thompson, S. K., Amegadzie, B. Y., Hanning, C. R., Jones, C., Kurdyla, J. T., McNulty, D. E., Drake, F. H., Gowen, M., and Levy, M. A. (1996) *J. Biol. Chem.* **271**, 12517-12524
70. Thompson, S. K., Halbert, S. M., Bossard, M. J., Tomaszek, T. A., Levy, M. A., Zhao, B., Smith, W. W., Abdel-Meguid, S. S., Janson, C. A., D’Alessio, K. J., McQueney, M. S., Amegadzie, B. Y., Hanning, C. R., DesJarlais, R. L., Briand, J., Sarkar, S. K., Huddleston, M. J., Ijames, C. F., Carr, S. A., Garnes, K. T., Shu, A., Heys, J. R., Bradbeer, J., Zembryki, D., Lee-Rykaczewski, L., James, I. E., Lark, M. W., Drake, F. H., Gowen, M., Gleason, J. G., and Veber, D. F. (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 14249-14254

71. Mills, B. J. and Lang, C. A. (1996) *Biochem. Pharmacol.* **52**, 401-406
72. Atamna, H. and Ginsburg, H. (1997) *Eur. J. Biochem.* **250**, 670-679
73. Ayi, K., Cappadoro, M., Branca, M., Turrini, F., and Arese, P. (1998) *FEBS Lett.* **424**, 257-261
74. Atamna, H., Pascarmona, G., and Ginsburg, H. (1994) *Mol Biochem Parasitol* **67**, 79-89
75. Olson, J. E., Lee, G. K., Semenov, A., and Rosenthal, P. J. (1999) *Bioorg. Med. Chem.*
76. Silva, A. M., Lee, A. Y., Gulnik, S. V., Majer, P., Collins, J., Bhat, T. N., Collins, P. J.,
Cachau, R. E., Luker, K. E., Gluzman, I. Y., Francis, S. E., Oksman, A., Goldberg, D. E.,
and Erickson, J. W. (1996) Proc. Natl. Acad. Sci. USA 93, 10034-10039
77. Moon, R. P., Bur, D., Loetscher, H., D'Arcy, A., Tyas, L., Oefner, C., Grueninger-
Leitch, F., Mona, D., Rupp, K., Dorn, A., Matile, H., Certa, U., Berry, C., Kay, J., and
Ridley, R. G. (1998) Adv. Exp. Med. Biol. 436, 397-406
78. Haque, T. S., Skillman, A. G., Lee, C. E., Habashita, H., Gluzman, I. Y., Ewing, T. J.,
Goldberg, D. E., Kuntz, I. D., and Ellman, J. A. (1999) J. Med. Chem. 42, 1428-1440
79. Semenov, A., Olson, J. E., and Rosenthal, P. J. (1998) Antimicrob. Agents.
Chemother. 42, 2254-2258
FOOTNOTES

1 The abbreviations used are: Z-Phe-Arg-AMC, benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl coumarin; FMK, fluoromethyl ketone; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; CNBr, cyanogen bromide; RT, room temperature; DTT, dithiothreitol; NaOAc, sodium acetate buffer; ORF, open reading frame; BLAST, basic local alignment search tool; IPTG, isopropylthio-β-galactoside; Ni-NTA, nickel-nitrilotriacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; NDSB-256, non-detergent sulfobetaine-256; PEG, polyethylene glycol; AMC, 7-amino-4-methyl coumarin; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; FITC, fluorescein isothiocyanate.
FIGURE LEGENDS

FIG. 1. Purification of native falcipain-2 from trophozoite extract and food vacuoles. Samples of trophozoite extract (TE), food vacuoles (FV) and affinity-purified protein (AP) were analyzed by 12.5% SDS-PAGE under reducing (r) or nonreducing (n) conditions, and stained with Coomassie Blue (A & D) or silver (B). Protease activities were analyzed by gelatin substrate SDS-PAGE (C). Molecular mass markers are labeled in kilodaltons.

FIG. 2. Nucleotide and deduced amino acid sequence of falcipain-2. The full coding sequence is shown. The sequences of oligonucleotide primers used for amplifying the gene are italicized. Upstream and downstream in-frame stop codons are labelled with asterisks. A predicted transmembrane sequence is underlined, the mature protease processing site is labeled with an arrow, the mature protease domain is shaded, and conserved papain-family active site amino acids are highlighted.

FIG. 3. Alignment of falcipain-2 with other papain-family proteases. The sequences of falcipain-1 (FP1), falcipain-2 (FP2), falcipain-3 (FP3) and papain (Pap) were aligned using the DNASTAR program (CLUSTAL method). Numbering is for the known or predicted mature sequence of each protease, dashes represent gaps required for optimal alignment, conserved active site amino acids are highlighted, and identities with falcipain-2 are shaded.

FIG. 4. Southern analysis of falcipain-2. *P. falciparum* genomic DNA (10 µg/lane) was digested with the restriction endonucleases shown, electrophoresed in a 0.7% agarose gel, transferred to a nylon membrane, hybridized with the -35FP2 probe,
washed under high stringency conditions, and evaluated by autoradiography. The positions of size markers are shown in kbp.

**Fig. 5. Expression and purification of recombinant falcipain-2.** The -35FP2 fragment, corresponding to truncated profalcipain, was expressed in *E. coli* and evaluated by reducing 12% SDS-PAGE and staining with Coomassie blue (A). The lanes show uninduced cells (1), cells induced with IPTG (2), soluble (3) and insoluble (4) cellular fractions, falcipain-2 purified by Ni-NTA affinity chromatography (5), expressed material after refolding by the protocol described in the text (6), and recombinant falcipain-2 additionally purified by Gly-Phe-Gly-Sepharose affinity chromatography (7). Refolded material corresponding to lane 6 was also evaluated by 12% gelatin substrate SDS-PAGE (B). The positions of molecular weight markers are labeled in kDa.

**Fig. 6. Processing of falcipain-2 during refolding.** Ni-NTA affinity purified falcipain-2 (lane 1) was refolded as described in the text in the presence of leupeptin (50 µM; lane 2), PMSF (1 mM; lane 3), pepstatin (50 µM; lane 4), or no inhibitor (lane 5), concentrated, and analyzed by reducing 12% SDS-PAGE (A) and non-reducing gelatin substrate SDS-PAGE (B) and Coomassie blue staining. The positions of molecular weight markers are labeled in kDa.

**Fig. 7. Effect of pH, reductant concentration and inhibitors on activity of native and recombinant falcipain-2.** The activity of native (open symbols) and recombinant (closed symbols) falcipain-2 was assayed against 50 µM Z-Phe-Arg-AMC in 100 mM NaOAc (pH 3.5-6.0) or sodium phosphate (pH 6.5-8.0) and 10 mM DTT (A), and in 100
mM NaOAc, pH 5.5 with different concentrations of DTT (circles), cysteine (triangles) or GSH (squares) (B). For inhibitor assays, native (shaded bars) and recombinant (filled bars) falcipain-2 were added to 50 µM Z-Phe-Arg-AMC in 100 mM NaOAc, pH 5.5 in the absence or presence of E-64 (10 µM), leupeptin (LEUP; 10 µM), pepstatin (PEP; 10 µM), PMSF (1 mM), 1,10 phenanthroline (PA; 1 mM), or EDTA (1 mM) (C). Release of AMC was continuously monitored for 30 min, slopes of fluorescence over time were calculated, and results were expressed as percent of maximum activity for each enzyme and each reductant.

**Fig. 8.** Substrate specificity of papain family proteases. $k_{\text{cat}}/K_m$ values are compared for substrates with the structure Z-X-R-AMC, with the P$_2$ amino acids indicated by the single-letter code, and the maximum value for each enzyme normalized to 1.0.

**Fig. 9.** Stage specificity of falcipain-2 expression. Highly synchronized W2-strain parasites (lanes: 1, early rings; 2, late rings; 3, early trophozoites; 4, mid-late trophozoites; 5, early-mid schizonts; 6, late schizonts/early rings) were collected every 8 hours and used for the purification of total RNA and for the extraction of soluble proteins. **A. Northern analysis.** RNA (10 µg/lane) was electrophoresed in a 1.0% agarose-formaldehyde gel, transferred to a nylon membrane, hybridized with the -35FP2 probe, and evaluated by autoradiography. The positions of size markers are labelled in kb. **B. Immunoblotting.** Soluble parasite extracts (from $1.8 \times 10^7$ parasites/lane) and recombinant falcipain-2 (lane 7) were electrophoresed in a
reducing 12% SDS-PAGE gel, blotted onto nitrocellulose, and their immunoreactivity with murine anti-falcipain-2 antiserum (1:350 dilution) was evaluated. The positions of molecular weight markers are labelled in kDa. **C. Gelatin substrate analysis.** Soluble parasite extracts (from $9 \times 10^6$ parasites/lane) and recombinant falcipain-2 (lane 7) were analyzed by 12% gelatin substrate SDS-PAGE. Protease activity is identified as clear bands against a Coomassie blue-stained background. **D. Localization of falcipain-2.** Erythrocytes infected with *P. falciparum* trophozoites were dried on a glass slide, fixed with methanol, and evaluated by immunofluorescence microscopy with murine anti-falcipain-2 antiserum. The same field is visualized by bright field microscopy (1) and fluorescence (2). Parasites within erythrocytes are visible with both modalities. Labeling of parasites, but not infected or uninfected erythrocyte cytosol, is apparent.

**Fig. 10. Hemoglobinase activity of native and recombinant falcipain-2.** Native falcipain-2 (nFP-2) was added to 20 µl reactions containing 2 µg hemoglobin (Hb) or 3 µg globin (Gb) in 100 mM NaOAc, pH 5.5 in the absence or presence of 1 mM DTT or GSH (A); in 100 mM NaOAc (pH 3.5-6.0) or sodium phosphate (pH 6.5-7.5) buffer with 1 mM GSH (B); or in 100 mM NaOAc, 10 mM DTT, pH 5.5 with or without (No Inh) protease inhibitors (labeled as in Fig. 7) or with the DMSO solvent alone (C). Recombinant falcipain-2 (rFP-2) was added to 20 µl reactions containing 3 µg hemoglobin or 5 µg globin in 100 mM NaOAc, pH 5.5 and 1 mM GSH, with or without protease inhibitors (FMK, Z-Phe-Arg-FMK; others labeled as in Fig. 7). The reactions were stopped by addition of SDS-PAGE sample buffer after incubation for 2h at 37 °C (1h at 37 °C for rFP-2), and products were resolved by 15% SDS-PAGE and stained.
with Coomassie Blue.
| Fraction            | Protein concentration (mg/ml) | Activity (U/ml) | Specific activity (U/mg protein) |
|---------------------|------------------------------|-----------------|---------------------------------|
| Trophozoite extract | 0.23                         | 2,366           | 10,286                          |
| Cytosolic fraction  | 0.14                         | 870             | 6,214                           |
| Vacuolar fraction   | 0.012                        | 570             | 47,500                          |

*Arbitrary units, determined from the hydrolysis of Z-Phe-Arg-AMC*
| Substrates     | Enzyme | $K_m$ ($\mu$M) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$M$^{-1}$) |
|---------------|--------|----------------|----------------------|----------------------------------|
| Z-LR-AMC      | nFP-2  | 6.35           | 1.34                 | 210,000                          |
|               | rFP-2  | 8.35           | 0.884                | 106,000                          |
| Z-FR-AMC      | nFP-2  | 17.3           | 0.704                | 40,600                           |
|               | rFP-2  | 9.13           | 0.412                | 45,100                           |
| Z-VR-AMC      | nFP-2  | 61.5           | 0.467                | 7,600                            |
|               | rFP-2  | 59.6           | 0.324                | 5,440                            |
| Z-ER-AMC      | nFP-2  | 112            | 0.128                | 1,140                            |
|               | rFP-2  | ND             | ND                   | ND                               |
| Z-RR-AMC      | nFP-2  | 173            | 0.055                | 318                              |
|               | rFP-2  | 39.8           | 0.230                | 578                              |
| Z-VLR-AMC     | nFP-2  | 6.50           | 0.310                | 47,700                           |
|               | rFP-2  | 3.07           | 0.190                | 61,900                           |
| Z-VVR-AMC     | nFP-2  | 51.1           | 0.029                | 568                              |
|               | rFP-2  | 6.70           | 0.027                | 4,030                            |
| H-PFR-AMC     | nFP-2  | 96.5           | 0.331                | 3,430                            |
|               | rFP-2  | 94.8           | 0.086                | 907                              |
| Boc-VLK-AMC   | nFP-2  | 1.73           | 0.133                | 76,900                           |
|               | rFP-2  | 3.36           | 0.167                | 49,700                           |
| Ac-KEKLR-AMC  | nFP-2  | 16.5           | 0.286                | 17,300                           |
|               | rFP-2  | ND             | ND                   | ND                               |

Substrates not cleaved by falcipain-2: G-AMC, R-AMC, Z-R-AMC, Bz-R-AMC, Ac-FR-AMC, H-AR-AMC, Boc-LGR-AMC, Boc-VGR-AMC, Boc-VPR-AMC, Bz-VGR-AMC, Bz-FVR-AMC, Boc-QAR-AMC, Z-GGR-AMC, Z-GPR-AMC; Z-benzzyloxycarbonyl; Bz-, benzolyl; Ac-, acetyl; Boc-, t-butyloxycarbonyl; single letter amino acid abbreviations used; ND, not done
A

|                  | + nFP-2 |               | - nFP-2               |               |
|------------------|---------|---------------|-----------------------|---------------|
| No -SH           | + GSH   | + DTT         | No -SH               | + GSH         |
| Hb               |         |               | Gb                    |               |

B

|                  | 3.5     | 4.0           | 4.5                   | 5.0           | 5.5           | 6.0           | 6.5           | 7.0           | 7.5           | 8.0           |
|------------------|---------|---------------|-----------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| pH               |         |               |                       |               |               |               |               |               |               |               |
| Hb               |         |               |                       |               |               |               |               |               |               |               |
| Gb               |         |               |                       |               |               |               |               |               |               |               |

C

|                  | No Inh  | + E-64        | + LEUP                | + PEP        | + PMSF        | + PA         | + DMSO       |
|------------------|---------|---------------|-----------------------|--------------|---------------|--------------|--------------|
| Hb               |         |               |                       |               |               |               |               |
| Gb               |         |               |                       |               |               |               |               |

D

|                  | - rFP-2 | + rFP-2       | + rFP-2               | + rFP-2 + GSH |               |               | + GSH        | - GSH        | + GSH + PMSF | + PEP        | + PA         | + LEUP       | FMK          | E-64        |
|------------------|---------|---------------|-----------------------|---------------|---------------|---------------|--------------|--------------|--------------|--------------|--------------|--------------|---------------|--------------|
| Hb               |         |               |                       |               |               |               |              |              |              |              |              |              |               |              |
| Gb               |         |               |                       |               |               |               |              |              |              |              |              |              |               |              |
Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of Plasmodium falciparum

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