INFECTION AND IMMUNITY, June 1995, p. 2120–2125 Vol. 63, No. 6
0019-9567/95/$04.00+0

Functional Expression of Falcipain, a *Plasmodium falciparum* Cysteine Proteinase, Supports Its Role as a Malarial Hemoglobinase

FIDEL SALAS,[1] JENNY FICHMANN,[1] GARSON K. LEE,[1] MARK D. SCOTT,[2] AND PHILIP J. ROSENTHAL[1]*

Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California 94143,[1] *and Division of Experimental Pathology, Albany Medical College, Albany, New York 12208*[2]

Received 13 December 1994/Returned for modification 15 February 1995/Accepted 6 March 1995

Erythrocytic malaria parasites degrade hemoglobin as a principal source of amino acids for parasite protein synthesis. We have previously shown that a *Plasmodium falciparum* trophozoite cysteine proteinase, now termed falcipain, is required for hemoglobin degradation, and we have hypothesized that this proteinase is responsible for initial cleavages of hemoglobin. To further evaluate the biological role of falcipain, we expressed the enzyme in bacterial and viral expression systems. After expression in the baculovirus system, falcipain was enzymatically active and had biochemical properties very similar to those of the native proteinase. Recombinant falcipain rapidly hydrolyzed both denatured and native hemoglobin. Hemoglobin hydrolysis was blocked by cysteine proteinase inhibitors but not by inhibitors of other classes of proteinases. Our results support our hypothesis that falcipain is a critical malarial hemoglobinase that is responsible for both initial cleavages of hemoglobin and the subsequent hydrolysis of globin into small peptides.

Malaria, one of the most important infectious disease problems in the world, is responsible for hundreds of millions of illnesses and over one million deaths per year (40). As *Plasmodium falciparum*, the cause of most of the severe cases of malaria, is increasingly resistant to available drugs, there is an urgent need to identify new targets for chemotherapy (16).

Potential targets for antimalarial chemotherapy include enzymes involved in the processing of hemoglobin. Erythrocytic malaria parasites degrade hemoglobin as a principal source of amino acids for parasite protein synthesis (14, 31). Hemoglobin degradation takes place in an acidic food vacuole, where the heme component of hemoglobin is processed into malarial pigment and globin is hydrolyzed to free amino acids (31). We have shown that a *P. falciparum* trophozoite cysteine proteinase, now termed falcipain, is required for hemoglobin degradation, as incubation of parasites with cysteine proteinase inhibitors blocked globin hydrolysis (24, 25) and for a series of inhibitors, the degree of inhibition of globin hydrolysis correlated with the inhibition of falcipain (27). We have hypothesized that falcipain is responsible for initial cleavages of globin in the malarial food vacuole, and we have shown that the proteinase degrades denatured hemoglobin in vitro (24). Malarial aspartic proteinases have also been shown to degrade hemoglobin in vitro and been hypothesized to function as hemoglobinases (4, 8–10, 38).

To better characterize the role of falcipain in malarial globin hydrolysis, we have expressed the proteinase in bacterial and viral expression systems. After functional expression in the baculovirus system, falcipain had biochemical properties very similar to those of the native enzyme and rapidly cleaved denatured and native hemoglobin. Thus, as predicted by our earlier studies with intact parasites and proteinase inhibitors, falcipain is a potent hemoglobinase. We hypothesize that this proteinase initiates globin hydrolysis by malaria parasites and thus is a promising therapeutic target.

* Corresponding author. Mailing address: Box 0811, University of California, San Francisco, CA 94143. Phone: (415) 206-8845. Fax: (415) 206-6015. Electronic mail address: rosnthl@itsa.ucsf.edu.

MATERIALS AND METHODS

Expression of falcipain in *E. coli*. For expression of the mature form of falcipain, the portion of the falcipain gene predicted to encode the mature proteinase was amplified with PCR using primers overlapping with the position of the most N-terminal amino acid (Val-333 [26]) and with the stop codon (Fig. 1). The 5′ primer also included an *Nco*I cleavage site, and the 3′ primer included a *Bam*HI cleavage site. The sequence of this construct (and other PCR-amplified constructs discussed below) was confirmed by DNA sequencing. The fragment was cleaved with appropriate restriction enzymes, purified, and ligated into pET-9d.

For expression of a construct encoding profalcipain, a 482-bp fragment extending from the codon for Ile-53 (the predicted N-terminal amino acid of profalcipain) to a *Bam*HI site at nucleotide 709 was PCR amplified (Fig. 1). The 5′ PCR primer included a *Sac*I restriction site immediately upstream of an *Nco*I site. A *Sac*I-*Bam*HI fragment from a preprofalcipain pBluescript construct was then replaced by the PCR-amplified profragment. The fragment encoding profalcipain was then ligated into pRSET utilizing the *Sac*I site and a *Hin*dIII site in the pBluescript polylinker immediately downstream of the profalcipain gene. Two constructs encoding smaller portions of the falcipain proform were also constructed, one extending from an *Xmn*I site (nucleotide 315; Leu-82 codon) and the other extending from the *Bam*HI site (nucleotide 709; Asp-214 codon). These constructs were ligated into pRSET utilizing *Pvu*II and *Bam*HI 5′ restriction sites, respectively, and the *Hin*dIII 3′ restriction site.

Purified plasmids were used to transform *Escherichia coli* BL21 (DE3) by standard methods (30). Transformants were selected with kanamycin (pET-9) or ampicillin (pRSET). To evaluate expression, log-phase bacteria were grown with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.4 mM) for 3 h, collected, and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antiserum production and Western blotting (immunoblotting). To generate antisera directed against falcipain, bacterial lysates containing the expressed mature form of the enzyme were electrophoresed in a 12.5% SDS–PAGE gel and the gel was stained with 0.05% Coomassie blue in water for 10 min and then washed extensively in water (12). The stained falcipain band was then cut from the gel, stored at 4°C, emulsified, and injected into rabbit lymph nodes following protocols of Babco, Richmond, Calif. Approximately 300 μg of recombinant falcipain was injected in the initial immunization, and 100 μg was injected in each of three booster immunizations at 3-week intervals. The rabbit serum used in our experiments was collected 10 days after the third booster immunization.

For Western blotting, proteins were blotted onto nitrocellulose membranes, blocked with 1% bovine serum albumin, incubated with antifalcipain serum ($1:10^4$ dilution) for 1 h, washed extensively in Tris-buffered saline, incubated with biotinylated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch; $1:10^6$

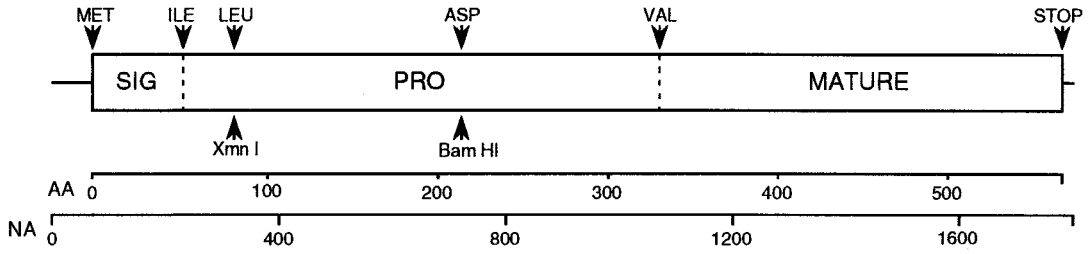

FIG. 1. Map of the falcipain gene. The nucleic acids (NA) of the original clone (26) and the amino acids (AA) of the protein are numbered. The predicted signal sequence (SIG), pro sequence, and mature proteinase sequence are labelled. Sites for cloning of *E. coli* and baculovirus constructs, as discussed in the text, are indicated by arrows.

dilution) for 1 h, washed extensively in Tris-buffered saline, incubated with streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch; 1:10^6 dilution), and developed with the ECL detection kit (Amersham). Identical conditions provided equivalent labeling of native and recombinant falcipain.

Expression of falcipain in the baculovirus system. To create a fragment encoding preprofalcipain, the portion of the gene predicted to encode the signal sequence (Fig. 1) was PCR amplified using a falcipain clone (26) as a template. The 5′ primer included an *Nhe*I site to facilitate cloning into the transfer vector. The amplified fragment was ligated to the full profalcipain clone described above at the site overlapping the Ile-53 codon. The expected sequences of the PCR-amplified portion of the clone and the insertion junction were verified by DNA sequencing. The preprofalcipain clone was then treated sequentially with *Hin*dIII, Klenow polymerase, and *Nhe*I, and the preprofalcipain gene was subcloned into a pBlueBac2 transfer vector that had been treated with *Bam*HI, Klenow polymerase, and *Nhe*I.

For expression, the pBlueBac2 vector containing preprofalcipain was cotransfected with linear wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Invitrogen) into *Trichoplusia ni* insect cells (High-5; Invitrogen) using cationic liposomes according to the vendor's instructions. Recombinant viruses were identified as blue plaques lacking polyhedra and purified by plaque purification (35). The presence and integrity of the preprofalcipain gene in purified recombinant baculovirus were verified by PCR and restriction enzyme analysis (17). For high-level expression, *T. ni* cells were adapted for growth in suspension in protein-free medium (Sf900 II SFM; Gibco-BRL) and infected with the recombinant baculovirus. *T. ni* cultures were maintained in 500-ml spinner flasks at 28°C at a density of $\sim 2 \times 10^6$ cells per ml. Large-scale expression was carried out in T225 (Corning) flasks by infection of $\sim 2 \times 10^8$ cells at a multiplicity of infection of 5 to 10. The titers of viral stocks were determined by endpoint dilution (17). To study the timing of expression, culture media were collected every 12 to 16 h and the media were analyzed by Western blotting and gelatin substrate SDS-PAGE.

To purify recombinant falcipain, culture media from cells harvested 90 h after infection were centrifuged ($1,000 \times g$, 15 min, 4°C) and the supernatants were collected and concentrated 100-fold with a stir-cell concentrator (Amicon). The concentrate was passed over Sephadex G-25 equilibrated with Tris HCl (20 mM, pH 7.5) and then a MacroPrep High Q (BioRad) anion exchange column, using a Waters protein purification system. Bound material was eluted with a 0 to 0.8 M NaCl gradient, and fractions with activity were identified with the fluorogenic substrate Z-Phe-Arg-AMC (7-amino-4-methyl coumarin) as described below. Active fractions were pooled, passed over a Sephadex G-25 column to change the buffer to 20 mM sodium acetate, pH 5.5, and then passed over a Resource S (Pharmacia) cation-exchange column. Fractions were assessed for activity with Z-Phe-Arg-AMC, gelatin substrate PAGE (see below), and Western blotting with antifalcipain serum. Under the conditions noted, bound proteinase corresponded to a baculovirus activity that was identical to that in wild-type virus preparations. Proteinase that did not bind corresponded by its size, immunoreactivity, and biochemical characteristics to falcipain. The unbound protein was then passed over a Mono Q (Pharmacia) anion-exchange column in 20 mM Tris, pH 7.5. Protein was eluted with a 0 to 0.8 M NaCl gradient, and active fractions were identified by the assays noted above and stored at −70°C.

Proteinase activity assays. Gelatin substrate PAGE was performed as previously described (25). In brief, proteins were electrophoresed in nonreducing SDS sample buffer in gels copolymerized with 0.1% gelatin, SDS was removed from the gels by incubation with 2.5% Triton X-100 (two 30-min washes), and the gels were incubated overnight at 37°C in 0.1 M sodium acetate (pH 5.5, 10 mM dithiothreitol) and then stained with Coomassie blue. For inhibitor studies, the inhibitor was added to the sample before electrophoresis and also to the buffer in which the gel incubated overnight after electrophoresis. Proteinases were identified as clear bands against the dark-staining background of the gel.

For analysis of the cleavage of fluorescein isothiocyanate (FITC)-casein, falcipain was incubated with inhibitors or appropriate solvent controls for 30 min at room temperature in 50 μl of buffer (0.1 M sodium acetate, 10 mM dithiothreitol [pH 5.5]), FITC-casein (1 mg/ml) was added, and the solution was incubated shaking at 37°C for 4 h. Ice-cold trichloroacetic acid (100 μl) was then added, the sample was incubated at 4°C for 1 h and then centrifuged ($16,000 \times g$ for 5 min), the supernatant was collected, Tris HCl (200 μl, 0.5 M, pH 8.0) was added, and fluorescence, representing cleaved casein peptides, was assayed (excitation, 485 nM; emission, 538 nM) with a Labsystems spectrofluorometer. Proteinase inhibitors were from Sigma except for Z-Phe-Arg-CH_2F, which was a gift from Prototek Inc., Dublin, Calif.

Assays of the hydrolysis of peptide-AMC substrates (Enzyme Systems Products, Livermore, Calif.) by falcipain were as previously described (25), except that a 96-well format (350-μl assay volumes) was utilized for spectrofluorometry, as described elsewhere (22). For inhibitor studies, inhibitors were incubated with the enzyme for 1 h before substrate was added. For kinetic analyses, rates of hydrolysis of different concentrations of substrates by falcipain were determined, the falcipain concentration was determined by titration with the stoichiometric inhibitor E-64 (3), and kinetic parameters were extrapolated from Lineweaver-Burk plots.

Hemoglobin hydrolysis assays. The hydrolysis of [^{14}C]hemoglobin (DuPont-NEN; 0.02 mCi/mg; 0.2 mg/ml) by falcipain was studied as previously described (24). For the evaluation of the hydrolysis of native hemoglobin, the protein was purified from human erythrocytes by DEAE-cellulose chromatography (20). The maintenance of native conformation by hemoglobin was confirmed by the demonstration that its oxygen affinity was that expected for native hemoglobin. Hemoglobin was incubated at 37°C with recombinant falcipain in buffer (0.1 M sodium acetate, 10 mM dithiothreitol [pH 5.5]). After the incubation, the reaction was stopped by immersion in dry ice-ethanol, reducing SDS-PAGE sample buffer was added, and the reaction mixture was stored at −20°C until electrophoresis on 15% polyacrylamide gels. After electrophoresis, gels were stained with Coomassie blue. For the quantitative comparison of globin hydrolysis, the densities of individual protein bands on the gels were integrated with a UVP Gel Documentation System.

RESULTS

Expression of falcipain in *E. coli*. The gene encoding falcipain was previously cloned by a PCR-based strategy (26). As an initial approach toward heterologous expression, the portion of the gene encoding the predicted mature form of falcipain (Fig. 1) was cloned into the *E. coli* expression vector pET-9d. Abundant recombinant protein was expressed by transformed bacteria as insoluble, enzymatically inactive inclusion bodies (Fig. 2). This protein was used to generate rabbit antisera directed against falcipain. As proteinase proforms may be necessary for appropriate protein folding (32, 34, 41), we also cloned and expressed constructs including portions of the falcipain pro region (Fig. 1). These constructs were also abundantly expressed as insoluble inclusion bodies (not shown). With all constructs expressed in the *E. coli* vectors pET-9d and pRSET, however, initial attempts at standard solubilization and refolding protocols (13) did not generate enzymatically active falcipain.

Expression of enzymatically active falcipain in the baculovirus system. Due to the limitations of *E. coli* expression, we utilized the baculovirus system for the expression of active falcipain. A construct including the entire falcipain open reading frame was constructed, inserted into the pBlueBac2 transfer vector, and cotransfected with baculovirus DNA into *T. ni* insect cells. Insect cells were then infected with purified recombinant virus. Recombinant falcipain was identified in cul-

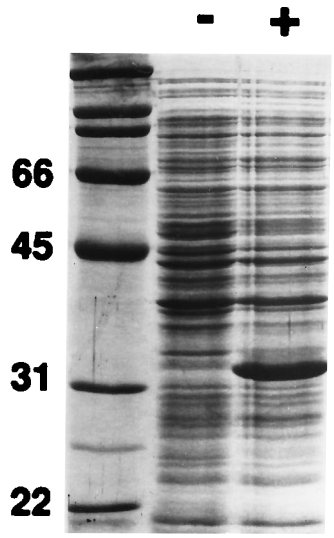

FIG. 2. Expression of falcipain in *E. coli*. *E. coli* BL21 (DE3) was transformed with the pET-9d plasmid containing an insert representing the predicted coding region for mature falcipain. Log-phase bacteria were grown for 3 h without (−) or with (+) 0.4 mM IPTG to induce expression, bacteria from 100 μl of culture were solubilized in reducing SDS sample buffer, and proteins were fractionated by SDS-PAGE (12.5% gel) and stained with Coomassie blue. Molecular mass markers are labelled in kilodaltons. The expressed 32,000-M_r protein seen in the + lane was used for the generation of antiserum.

ture media beginning 60 h (identified by Western blotting) and 72 h (identified by gelatin substrate SDS-PAGE) after infection, and activity was maximal at 96 to 120 h of growth (Fig. 3). Media from control cultures containing uninfected insect cells had no demonstrable proteinase activity. Media from control cells infected with wild-type virus contained proteinase activity (migrating as a dimer at an M_r of 110,000 to 140,000 on gelatin substrate SDS-PAGE gels), but the activity was clearly distinguished from falcipain based on its size, biochemical properties, and lack of reactivity with antifalcipain antibody (29).

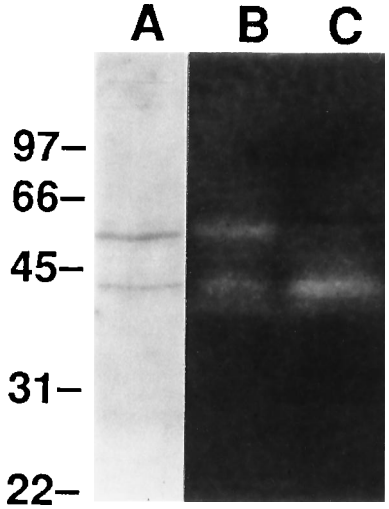

FIG. 3. Expression of falcipain in baculovirus. *T. ni* insect cells were infected with recombinant baculovirus containing the intact coding region of falcipain. Aliquots of culture media were collected and analyzed by Western blotting with antiserum against *E. coli*-expressed falcipain (lane A, 96 h after infection) and gelatin substrate PAGE (lane B, 96 h; lane C, 120 h). For these and multiple other time points, gelatinolytic activity consistently corresponded with Western blot positivity. The positions of molecular mass markers are indicated in kilodaltons.

TABLE 1. Effect of proteinase inhibitors on falcipain activity[a]

Inhibitor	Class	Concn	% of control activity		
			Casein	ZFR-AMC	^{14}C-HB
E-64	Cysteine	10 μM	2.3	0	6.6
Z-Phe-Arg-CH_2F	Cysteine	1 μM	2.2	0	6.5
Pepstatin	Aspartic	10 μM	93.1	48.8	95.3
PMSF[b]	Serine	1 mM	91.7	87.3	89.8
1,10-Phenanthroline	Metallo	1 mM	21.1	40.6	39.1

[a] The hydrolysis of the substrates FITC-casein (Casein), Z-Phe-Arg-AMC (ZFR-AMC), and [^{14}C]hemoglobin (^{14}C-HB) by falcipain was studied in the presence of inhibitors of different classes of proteinases. Each assay was performed in triplicate, and the results shown are the mean percent control activities from two or three (for FITC-casein) experiments.
[b] PMSF, phenylmethylsulfonyl fluoride.

Falcipain was processed during the viral developmental cycle to apparently active forms with M_rs of 55,000 and 43,000 (Fig. 3). We cannot rule out the possibility, however, that one or both of these forms were enzymatically inactive but that processing of falcipain to smaller, active forms occurred during the overnight incubation of the gel that preceded gel staining. The identified forms were larger than those seen upon purification of falcipain from cultured parasites (M_r, 28,000 [24]), suggesting altered posttranslational modification by the baculovirus system or incomplete processing of the proteinase. Additional processing to smaller forms was not seen despite incubation of recombinant falcipain under conditions (37, 40, and 60°C at pH 4.0 to 6.0 for 0 to 16 h) at which other expressed cysteine proteinases were processed to mature forms (6, 34, 39). Of note, a number of apparently active proforms of native falcipain with M_rs of 32,000 to 45,000 have been identified on gelatin substrate PAGE gels after variations in culture and parasite processing conditions (21).

Recombinant falcipain had the biochemical properties of native falcipain. Recombinant falcipain was purified from insect cell supernatants by anion-exchange (Mono-Q) and cation-exchange (Mono-S) chromatography. Active fractions (identified with the fluorogenic substrate Z-Phe-Arg-AMC) were evaluated for inhibition by standard inhibitors of different classes of proteinases. With gelatin substrate SDS-PAGE, gelatinase activity was completely ablated by the cysteine proteinase inhibitor E-64 (100 μM) (data not shown). With the FITC-casein assay, another nonspecific proteinase assay, activity was strongly inhibited by inhibitors of cysteine proteinases and much less inhibited by inhibitors of other proteinase classes (Table 1). Partial inhibition by the chelator 1,10-phenanthroline suggests a requirement for metal ions for optimal enzyme activity and that partial inhibition of falcipain might contribute to the antimalarial effects of the iron chelator desferrioxamine (18). In any event, the strong inhibition by cysteine proteinase inhibitors of activity against nonspecific substrates argues against significant contamination of falcipain with proteinases of other classes. The purification also completely separated recombinant falcipain from a baculovirus cysteine proteinase activity (migrating as a dimer at an M_r of 110,000 to 140,000) that we identified (29). This proteinase, perhaps encoded by a baculovirus cysteine proteinase gene that has recently been identified (19), was readily separated from falcipain due to its differential binding to the cation-exchange column.

Activity against the generic substrate casein and the specific

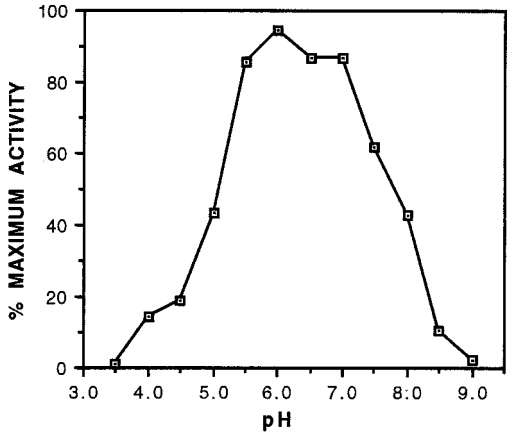

FIG. 4. pH profile of activity of recombinant falcipain. The activity of recombinant falcipain was measured as the rate of hydrolysis of Z-Phe-Arg-AMC over 30 min at different pHs. Buffers used were sodium acetate, 0.1 M (pH 3.5 to 6.0), potassium phosphate, 0.1 M (pH 6.5 to 7.5), and Tris HCl, 0.1 M (pH 8.0 to 9.0). All assays included 10 mM dithiothreitol. Results are the mean percentages of maximum activity from three experiments, each of which included duplicate points at each pH.

cysteine proteinase substrate Z-Phe-Arg-AMC was maximal at acid pH (Fig. 4) and was markedly stimulated by the reducing agent dithiothreitol. Recombinant falcipain also had the substrate preferences of the native enzyme, with a strong preference for the cathepsin L substrate Z-Phe-Arg-AMC over cathepsin B (Z-Arg-Arg-AMC) and cathepsin H (Z-Arg-AMC) substrates (activities of 7 to 9 nM recombinant falcipain against 1 to 50 μM Z-Arg-Arg-AMC and Z-Arg-AMC were below the limits of detection over a 30-min assay period). As seen with the native enzyme (25), Z-Val-Leu-Arg-AMC was a more effective substrate than Z-Phe-Arg-AMC (Table 2). Recombinant falcipain differed somewhat from the native enzyme (25) in that its pH optimum was somewhat less acidic and its preference for Z-Val-Leu-Arg-AMC over Z-Phe-Arg-AMC was more pronounced. In summary, recombinant falcipain had the general biochemical properties of the native proteinase, though, as has been seen with other comparisons of native and recombinant enzymes (e.g., cathepsin L [34]), some differences in biochemical properties were noted.

Falcipain is a potent hemoglobinase. To assess the hydrolysis of denatured hemoglobin by recombinant falcipain, we evaluated the cleavage of [^{14}C]hemoglobin. Falcipain readily cleaved [^{14}C]hemoglobin. In a typical 100-μl reaction mixture including falcipain (50 nM) and hemoglobin (0.2 μM), approximately 100 ng of hemoglobin was degraded to trichloroacetic acid-soluble peptides during the 4-h incubation. [^{14}C]hemoglobin hydrolysis was markedly inhibited by the cysteine proteinase inhibitor E-64 and the specific falcipain inhibitor Z-Phe-Arg-CH_2F (Table 1).

The conformation of hemoglobin after transport to the malarial food vacuole is unknown, but the protein probably maintains significant tertiary structure. To assess the hydrolysis of native hemoglobin by falcipain, we purified human hemoglobin by DEAE-cellulose chromatography (20) and incubated it with the recombinant proteinase. Falcipain rapidly cleaved native hemoglobin under conditions approximating those of the malarial food vacuole (pH 5.5, 10 mM dithiothreitol) (Fig. 5). Efficient hydrolysis of globin required a reducing environment, as is provided in erythrocytes by glutathione (15), which also stimulated the activity of native falcipain (24). As expected for falcipain activity, globin hydrolysis was strongly inhibited by E-64 and Z-Phe-Arg-CH_2F and was only minimally inhibited by inhibitors of other classes of proteinases (Fig. 5). To provide a measure of the kinetics of hemoglobin degradation, the hydrolysis of native hemoglobin by falcipain was followed over time. In a typical 50-μl reaction mixture including falcipain (7 nM) and hemoglobin (2 μM), approximately 100 ng of native hemoglobin was degraded per min and the half-life for hemoglobin hydrolysis was approximately 10 min (Fig. 6).

TABLE 2. Kinetics of cleavage of peptide substrates by falcipain[a]

Substrate	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (sec^{-1} M^{-1})
Z-Val-Leu-Arg-AMC	0.25	3.7	68,000
Z-Phe-Arg-AMC	0.018	28	640

[a] Kinetic parameters shown were generated from Lineweaver-Burk plots after assays of the hydrolysis of multiple concentrations of the substrates by falcipain. Results shown are the means from duplicate experiments.

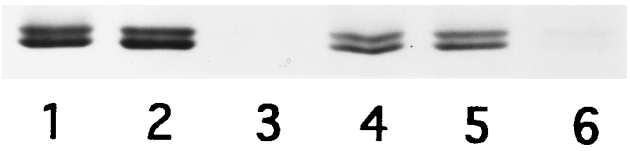

FIG. 5. Hydrolysis of native hemoglobin by falcipain. Native hemoglobin (6 μg/50 μl reaction mixture) was incubated in sodium acetate (0.1 M, pH 5.5) with (lanes 2 and 3) or without (lane 1) 7 nM falcipain and with (lanes 1 and 3) or without (lane 2) 10 mM dithiothreitol for 4 h at 37°C. After the reactions, samples were electrophoresed under reducing conditions in a 15% SDS–PAGE gel (such that globin dimers migrated at an Mr of ~15,000) and then stained with Coomassie blue. In the reaction mixture including falcipain and dithiothreitol, hemoglobin was hydrolyzed to peptides that were too small to be resolved (lane 3). Reactions as in lane 3 were also performed with the addition of the cysteine proteinase inhibitor E-64 (100 μM; lane 4), the specific falcipain inhibitor Z-Phe-Arg-CH_2F (1 μM; lane 5), and inhibitors of serine (phenylmethylsulfonyl fluoride, 1 mM), aspartic (pepstatin, 100 μM), and metallo- (1,10-phenanthroline, 1 mM) proteinases (lane 6). Only cysteine proteinase inhibitors markedly blocked hemoglobin degradation.

DISCUSSION

We previously showed that cysteine proteinase inhibitors completely blocked the hydrolysis of globin by cultured malaria parasites and that this inhibition of globin hydrolysis correlated with the inhibition of falcipain (24, 27). We therefore hypothesized that falcipain is required for malarial globin hydrolysis and that it cleaves native hemoglobin after this molecule is transported to the malarial food vacuole (24). Direct studies of the cleavage of hemoglobin by falcipain have been limited by the availability and stability of the native enzyme, though soluble lysates of malarial trophozoites and partially purified preparations of falcipain cleaved native hemoglobin in a man-

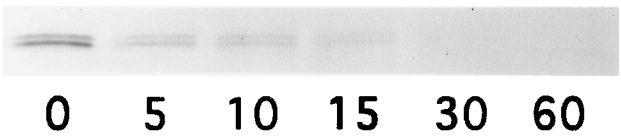

FIG. 6. Time course of hydrolysis of native hemoglobin by falcipain. The hydrolysis of native hemoglobin (2 μg/50-μl reaction mixture) by falcipain (7 nM) in sodium acetate (0.1 M, pH 5.5) and dithiothreitol (10 mM) at 37°C was followed over time. Samples were solubilized in reducing SDS sample buffer after 0, 5, 10, 15, 30, and 60 min of incubation, and the globin dimer was resolved on a 15% SDS–PAGE gel and stained with Coomassie blue. Hemoglobin was rapidly degraded with a half-life of about 10 min.

ner that was fully inhibited by specific falcipain inhibitors (7). To further characterize the biological role of falcipain, we expressed the functional proteinase in a baculovirus system. Functional expression was complicated by the coexpression of a baculovirus cysteine proteinase and apparently limited secretion and incomplete processing of falcipain. Despite these limitations, however, recombinant falcipain had biochemical properties that were very similar to those of the native enzyme. Quantitative expression in the baculovirus system did not approach the magnitude of expression in *E. coli*, but it provided ample falcipain for the desired studies of hemoglobin degradation. Through the use of the recombinant proteinase, we have now confirmed our hypothesis that falcipain is a potent hemoglobinase.

The degradation of hemoglobin to free amino acids and heme in the malarial food vacuole probably involves multiple enzymes, including endopeptidases (see reference 14 for a review), exopeptidases (36), and one or more heme-processing enzymes (33). At least two aspartic proteinases (9, 10, 38) and one cysteine proteinase (9, 24) have been identified in trophozoites and isolated from food vacuoles. The three food vacuole proteinases may all play a role in globin hydrolysis. Gluzman et al. (9) recently showed that, in their in vitro system, one of the malarial aspartic proteinases, but not the other food vacuole proteinases, degraded native hemoglobin. This result suggested that the aspartic proteinase was responsible for initial cleavages of hemoglobin, but it was difficult to reconcile with observations that specific cysteine proteinase inhibitors completely blocked globin hydrolysis in intact parasites (1, 5, 24, 37). We have now shown, using recombinant falcipain and simple assays for the hydrolysis of denatured and native hemoglobin, that falcipain is a potent hemoglobinase. We hypothesize that this enzyme functions as a major malarial hemoglobinase and is likely responsible for initial cleavages of native hemoglobin. In addition, our results show that falcipain is capable of multiple cleavages of globin into peptides that are too small to be either precipitated by trichloroacetic acid or resolved on a 15% SDS–PAGE gel. The efficiency of globin hydrolysis is most likely augmented by the interaction of multiple enzymes, probably including the two identified aspartic proteinases, at least one of which can also cleave native hemoglobin.

Falcipain rapidly degraded native hemoglobin. In our in vitro assay, nanomolar concentrations of falcipain degraded native hemoglobin at the rate of ~0.1 μg/min. The concentration of falcipain studied was considerably lower than the micromolar concentrations estimated for trophozoite food vacuoles based on quantitation (by titration with the stoichiometric proteinase inhibitor E-64 [3]) of falcipain harvested from cultured parasites (21). Thus, it appears likely that quantities of falcipain available in the trophozoite food vacuole are readily capable of cleaving hemoglobin at the rate (1 to 3 pg per parasite per h) that would be required to cleave the 25 to 75% of total erythrocyte hemoglobin (~7.5 to 22.5 pg per erythrocyte) that is cleaved per parasite, primarily during the trophozoite segment of the erythrocytic malarial life cycle (2, 11, 28). It further appears that the rate-limiting factor in hemoglobin hydrolysis is most likely hemoglobin delivery, and not the hydrolytic capacity of the parasite. This simple analysis does not consider the complex interplay of malarial enzymes that is likely required for the complete hydrolysis of hemoglobin to free amino acids. However, it further supports our hypothesis that falcipain is a critical malarial hemoglobinase that participates in initial cleavages of hemoglobin upon the transport of erythrocyte cytosol to the malarial food vacuole. Falcipain is thus a promising target for antimalarial chemotherapy. Indeed, we have already shown that falcipain inhibitors that block globin hydrolysis have potent antimalarial effects in vitro (27) and in vivo (23).

ACKNOWLEDGMENTS

We thank Joel Ernst for a critical review of the manuscript.

This work was supported by grants from the NIH (AI35800 and HL53066), the Advanced Research Projects Agency (MDA-972-91-J1013), the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (920079), and the American Heart Association. P.J.R. is an Established Investigator of the American Heart Association.

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