A Malarial Cysteine Proteinase Is Necessary for Hemoglobin Degradation by *Plasmodium falciparum*

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Abstract

To obtain free amino acids for protein synthesis, trophozoite stage malaria parasites feed on the cytoplasm of host erythrocytes and degrade hemoglobin within an acid food vacuole. The food vacuole appears to be analogous to the secondary lysosomes of mammalian cells. To determine the enzymatic mechanism of hemoglobin degradation, we incubated trophozoite-infected erythrocytes with peptide inhibitors of different classes of proteinases. Leupeptin and L-transepoxy-succinyl-leucyl-amido-(4-guanidino)-butane (E-64), two peptide inhibitors of cysteine proteinases, inhibited the proteolysis of globin and caused the accumulation of undegraded erythrocyte cytoplasm in parasite food vacuoles, suggesting that a food vacuole cysteine proteinase is necessary for hemoglobin degradation. Proteinase assays of trophozoites demonstrated cysteine proteinase activity with a pH optimum similar to that of the food vacuole and the substrate specificity of lysosomal cathepsin L. We also identified an M_r 28,000 proteinase that was trophozoite stage–specific and was inhibited by leupeptin and E-64. We conclude that the M_r 28,000 cysteine proteinase has a critical, perhaps rate-limiting, role in hemoglobin degradation within the food vacuole of *Plasmodium falciparum*. Specific inhibitors of this enzyme might provide new means of antimalarial chemotherapy.

Introduction

The human malaria parasite *Plasmodium falciparum* has a 48-h life cycle within host erythrocytes that is responsible for all of the clinical manifestations of falciparum malaria. During the erythrocytic cycle the intracellular parasite develops from a ring stage into a more metabolically active trophozoite, divides asexually and becomes a schizont, and finally ruptures the host erythrocyte, releasing daughter merozoites that invade other erythrocytes to reinitiate the cycle. Several lines of evidence indicate that the degradation of host erythrocyte hemoglobin provides an important reservoir of amino acids for protein synthesis by intraerythrocytic malaria parasites. First, malaria parasites have a limited capacity for synthesizing amino acids, and the quantity of free amino acids within erythrocytes is not

sufficient for the needs of the parasite (1). Second, the hemoglobin content of infected erythrocytes decreases 25–75% during the life cycle of erythrocytic parasites (2, 3), and the concentration of free amino acids is greater in infected erythrocytes than in uninfected erythrocytes (4). Third, the composition of the amino acid pool of infected erythrocytes is similar to the amino acid composition of hemoglobin (5–7). Fourth, the infection of erythrocytes containing radiolabeled hemoglobin is followed by the appearance of labeled amino acids in parasite proteins (8–10).

Hemoglobin degradation occurs predominantly during the trophozoite stage of the erythrocytic life cycle of *P. falciparum*. Trophozoites ingest erythrocyte cytoplasm and transport it within vesicles to a large central food vacuole (11, 12), where the hemoglobin-rich cytoplasm is degraded. In the food vacuole the heme moiety precipitates and is a major component of malarial pigment (13), and globin is hydrolyzed to its constituent free amino acids. The food vacuole of *P. falciparum* is an acidic (14, 15), membrane-bound (11) compartment where proteins are degraded, and therefore it appears to be analogous to the secondary lysosomes of mammalian cells, where multiple proteinases hydrolyze proteins at acid pH (16, 17).

The enzymatic mechanism of globin degradation within the malarial food vacuole is unknown. In previous studies, aspartic proteinases that degraded denatured hemoglobin were isolated from malaria parasites (18–20). However, denatured hemoglobin is a nonspecific substrate that can be hydrolyzed by many proteinases, and the biological role of the aspartic proteinases cannot be determined from these studies. To determine the enzymatic mechanism of globin degradation in the trophozoite food vacuole we first studied the effects of class-specific proteinase inhibitors on intact parasites. We found that two peptide inhibitors of cysteine proteinases blocked globin degradation in the food vacuole of *P. falciparum* trophozoites. We also identified a cysteine proteinase of trophozoites that was inhibited by the same two proteinase inhibitors and had biochemical properties that were similar to those of the lysosomal cysteine proteinase cathepsin L. Our results suggest that the cysteine proteinase we identified has a critical role in hemoglobin degradation in the food vacuole of *P. falciparum* trophozoites.

Methods

Reagents. Leupeptin, chymostatin, pepstatin A, L-transepoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64),[1] N-ethylmaleimide (NEM), PMSF, 1,10-phenanthroline, DTT, cysteine, and glutathione were from Sigma Chemical Co. (St. Louis, MO). Azocoll,

1. *Abbreviations used in this paper:* AMC, amino-4-methyl-coumarin; E-64, L-transepoxy-succinyl-leucylamido-(4-guanidino)-butane; EM, electron microscopy; NEM, N-ethylmaleimide; Z-V-L-R-AMC, valine-leucine-arginine AMC; Z, benzyloxycarbonyl.

an insoluble diazonium dye-collagen complex that releases soluble diazo-containing fragments with proteolysis, was purchased from Calbiochem-Behring Corp. (La Jolla, CA). [^{14}C]Methemoglobin (30 mCi/g) was from DuPont New England Nuclear (Wilmington, DE). The following flurogenic peptide substrates were purchased from Enzyme Systems Products (Livermore, CA): benzyloxycarbonyl (Z)-Phe-Arg-7-amino-4-methyl-coumarin (AMC); Z-Val-Leu-Arg-AMC; Z-Arg-Arg-AMC; Z-Leu-AMC; Z-Phe-Pro-Arg-AMC; Z-Phe-AMC; Z-Ala-Arg-Arg-AMC.

Parasites and culture. Cloned parasites from a Brazilian isolate (It) were cultured in human erythrocytes and media containing RPMI-1640 (Gibco Laboratories, Grand Island, NY), 25 mM Hepes, 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ), 0.27% sodium bicarbonate, and 10% human serum (21). The cultures were synchronized by sequential treatments with 5% (wt/vol) sorbitol in PBS (22). Leukocytes and platelets were exhaustively removed from erythrocytes used for culture as previously described (23).

Transmission electron microscopy (EM) and EM autoradiography. Synchronized parasite cultures were incubated with 100 μM leupeptin, 140 μM E-64, or no inhibitor for 12 h and the trophozoite-infected erythrocytes were then concentrated by suspension in 0.5% (wt/vol) gelatin (Type I; Sigma Chemical Co.) in RPMI-1640 (24). The infected erythrocytes were then washed in PBS and incubated for 1 h in 2% glutaraldehyde, 0.05 M phosphate, and 4% sucrose at pH 7.4. Samples were postfixed in 1% osmium tetroxide in Millonig's phosphate buffer, dehydrated in ethanol, and embedded in Spurr. Thin sections were stained with uranyl acetate, then lead citrate, before EM.

For EM autoradiography, leupeptin was tritium labeled by microwave discharge activation (25) and was a gift from Dr. C. T. Peng of the University of California, San Francisco. Synchronized parasite cultures were incubated with [^{3}H]leupeptin for 12 h and the trophozoite-infected erythrocytes were then concentrated, fixed, postfixed, and embedded as described above. 400–600-Å-thick sections were placed on Formvar and carbon-coated copper grids and stained with uranyl acetate and lead citrate. The grids were covered with a monolayer of emulsion (Ilford, Knutsford, Cheshire, England) by the wire loop method (26). The emulsion-covered grids were kept in the dark at 4°C for 6 wk, developed in Microdol X (Eastman-Kodak Co., Rochester, NY) for 2 min, and fixed in Kodak rapid fixer for 2 min. The grids were washed in 1 N NaOH for 5 min and examined by EM.

Determination of the globin content of parasites. Erythrocytes infected with synchronized parasites were incubated with 100 μM leupeptin, 140 μM E-64, or no inhibitor for 15 h. The trophozoite-infected erythrocytes were then washed in PBS and lysed with 0.1% (wt/vol) saponin (Sigma Chemical Co.) in PBS. Saponin is a neutral detergent that lyses the erythrocyte membrane but not the parasite membrane. The parasites (within erythrocyte ghosts) were washed four times in ice-cold PBS to remove all free hemoglobin. Equal numbers of parasites were solubilized with electrophoresis sample buffer [5% (wt/vol) SDS, 62.5 mM Tris HCl, 5% (vol/vol) 2-mercaptoethanol, pH 6.8] and electrophoresed in a 15% acrylamide gel (27). Proteins were identified by staining the gel with Coomassie Blue.

Preparation of parasite extracts for proteinase assays. Synchronized cultures of trophozoites were concentrated to 50–70% parasitemias with 0.5% gelatin/RPMI 1640 as described above. The infected erythrocytes were treated with 0.1% saponin in PBS, washed three times with ice cold PBS, and incubated (in water) in a nitrogen cavitation bomb at 4°C and 3,500 kPa for 1 h. The suspension was centrifuged (13,000 g, 10 min, 4°C) and the supernatant was collected. Uninfected erythrocyte controls were treated identically.

Proteinase assays. Assays with Azocoll were performed by incubating 25 μl extract with 0.9 ml Azocoll suspension (5 mg/ml in 0.1 M sodium acetate buffer, adjusted to the desired pH). The samples were incubated for 6 (pH profile) or 18 (inhibitor profile) h at 37°C with continuous shaking and centrifuged (13,000 g for 2 min). The supernatant was collected and its absorbance at 540 nm was determined.

Assays with [^{14}C]methemoglobin were performed by incubating

25-μl aliquots of extract with 2 μl [^{14}C]methemoglobin (30 mCi/g, 0.17 mg/ml), 10 μl inhibitors and/or sulfhydryl compounds, 50 μl of 0.1 M sodium acetate adjusted to the desired pH, and water to give a final volume of 100 μl. After 4 h of incubation at 37°C, 50 μl BSA (3 mg/ml) and 50 μl of 50% trichloroacetic acid were added. Samples were incubated an additional 30 min at 4°C and centrifuged (13,000 g). 50-μl aliquots of the supernatant were added to 7-ml samples of scintillation fluid and radioactivity was determined with a scintillation counter. Controls without extract were processed simultaneously.

The rates of hydrolysis of AMC-peptide substrates were determined by measuring at different time points the fluorescence (at 460 nm with a 380-nm excitation wavelength) caused by the liberation of AMC. Trophozoite extract (10–50 μl) was added to 1.8 ml of buffer (0.1 M sodium acetate, pH 6.0) with 20 μl of inhibitors or sulfhydryl compounds and water to give a 2.0-ml volume. After a 10-min incubation at 37°C, 20 μl of substrate was added from a 5-mM stock solution in DMSO. The slopes of fluorescence over time were compared for different substrates and different inhibitors and sulfhydryl compounds. At least six time points were obtained for each assay.

Gelatin substrate PAGE. Trophozoite-infected erythrocytes were solubilized with electrophoresis sample buffer lacking mercaptoethanol, and electrophoresed in a 12% acrylamide gel copolymerized with 0.1% gelatin (28). After electrophoresis, the gel was washed in 2.5% (vol/vol) Triton X-100 (two 30-min washes) to remove SDS. The gel was then incubated in buffer (0.1 M glycine, 0.002 M CaCl$_2$, pH 6.0 or 7.0) at 37°C for 18 h to allow digestion of gelatin by the proteinase before staining the gel with Coomassie Blue. Proteinase activity was detected as a clear band against the blue background of the gel. To determine the effect of inhibitors on proteinase activity, the inhibitor was incubated with trophozoite-infected erythrocytes for 10 min before the addition of electrophoresis sample buffer, and the inhibitor was also added to the glycine buffer in which the gel was incubated after electrophoresis.

Results

Effects of proteinase inhibitors on intact parasites. When trophozoite-infected erythrocytes were incubated with the cysteine proteinase inhibitors leupeptin (20–100 μM) or E-64 (140 μM) for 6 or more hours, the appearance of the parasite food vacuole differed greatly from that of control parasites. Both light microscopy of Giemsa-stained parasites (not shown) and transmission EM (Fig. 1) showed that the food vacuole of leupeptin- or E-64-treated parasites was completely filled with erythrocyte cytoplasm. A similar effect of leupeptin on the *P. falciparum* food vacuole was recently reported by Dluzewski et al. (29). The food vacuole abnormality was not observed when trophozoite-infected erythrocytes were incubated with peptide inhibitors of serine proteinases (80 μM chymostatin) or aspartic proteinases (75 μM pepstatin), though pepstatin did cause the parasites to become pyknotic after 8 h of incubation.

Incubation of the parasites with leupeptin or E-64 for 24 h inhibited parasite differentiation and multiplication. Very few parasites developed into mature, multinucleated schizonts, and new ring stage parasites indicative of successful erythrocyte rupture and reinvasion were rarely seen. The effect of leupeptin on the parasite food vacuole was reversible. After removal of leupeptin from a culture the parasites regained a normal morphology and the subsequent ring parasitemia was 85% that of control parasites (mean of two experiments). The effect of E-64 on the food vacuole was not reversible, and after removal of E-64 the subsequent ring parasitemia was only 17% that of control parasites.
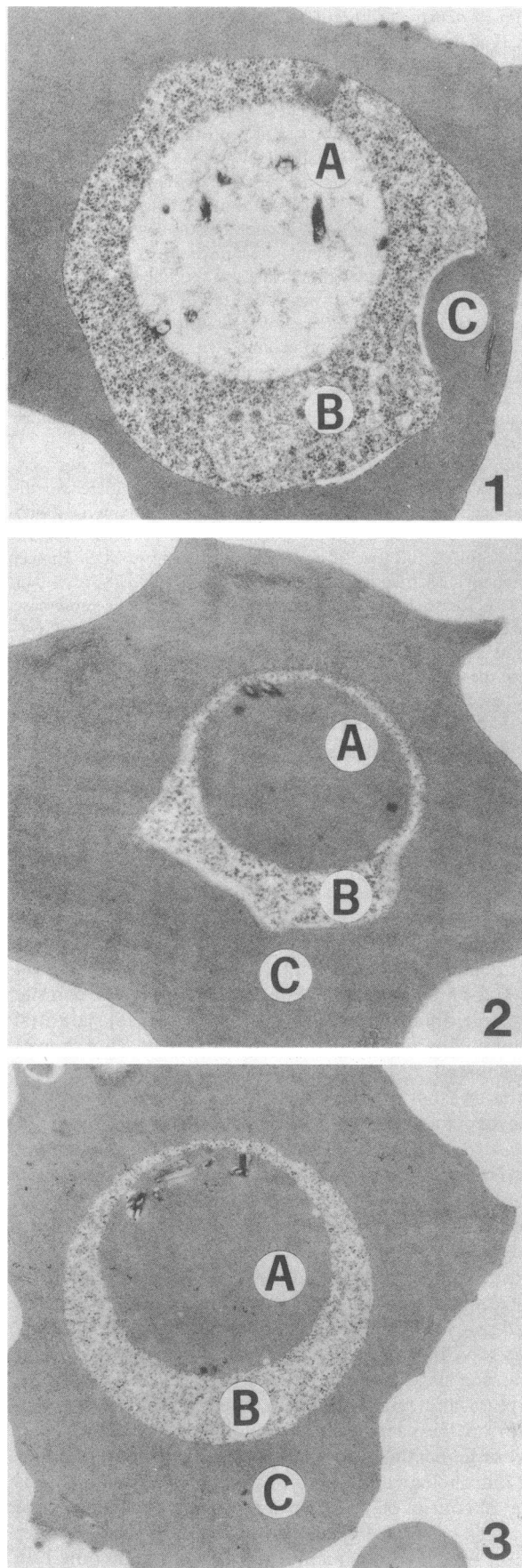
Localization of [³H]leupeptin. To test whether leupeptin could enter the *P. falciparum* food vacuole we performed EM autoradiography after incubating trophozoite-infected and uninfected erythrocytes with [³H]leupeptin. [³H]Leupeptin was observed in infected, but not uninfected, erythrocytes (50 of each were counted), and was consistently localized within the parasite food vacuole (Fig. 2). Additional [³H]leupeptin was observed in the cytoplasm of infected erythrocytes and in the parasite cytoplasm. Within the parasite cytoplasm, the [³H]leupeptin appeared to be localized to vesicles containing erythrocyte cytoplasm, suggesting that leupeptin reached the food vacuole by pinocytosis and vesicular transport rather than by diffusion.

Inhibition of globin degradation by leupeptin and E-64. The observed accumulation of erythrocyte cytoplasm within the food vacuoles of parasites incubated with leupeptin and E-64 suggested that the two proteinase inhibitors had blocked the degradation of globin within the food vacuole. To test this hypothesis, we incubated trophozoite-infected erythrocytes with leupeptin, E-64, or no inhibitor, lysed erythrocyte membranes with 0.1% saponin to release erythrocyte hemoglobin, and assayed for parasite-contained globin with PAGE. Control parasites contained no detectable globin, but parasites that had been incubated with leupeptin or E-64 contained abundant undegraded globin (Fig. 3).

Proteinase activity of P. falciparum trophozoites. The accumulation of globin in parasites incubated with leupeptin and E-64 suggested that trophozoites contain a cysteine proteinase with a critical role in globin degradation. We therefore prepared extracts from *P. falciparum* trophozoites by nitrogen cavitation and assayed the extracts for proteinase activity with three different proteolytic substrates. With Azocoll, proteinase activity was maximal at pH 6.0 (Fig. 4), was stimulated by cysteine, was inhibited by the cysteine proteinase inhibitors leupeptin and NEM, and was not inhibited by inhibitors of other classes of proteinases (Table I). No proteinase activity was detected with extracts prepared from uninfected erythrocytes (Fig. 4).

The results with Azocoll indicated that trophozoites contain substantial cysteine proteinase activity. To substantiate this observation we used as a proteolytic substrate the synthetic peptide Z-Val-Leu-Arg-AMC. This peptide was chosen because its structure is similar to those of leupeptin and E-64, both of which contain a terminal arginine side chain adjacent to leucine. Z-Val-Leu-Arg-AMC was rapidly hydrolyzed by the trophozoite extract. The hydrolysis of this substrate was also markedly stimulated by sulfhydryl reagents and selectively inhibited by inhibitors of cysteine proteinases at pH 6.0 (Table I).

With the substrate [¹⁴C]methemoglobin, an oxidized, denatured derivative of hemoglobin, somewhat different results were obtained (Table I). The proteinase activity of the trophozoite extract was again stimulated by sulfhydryl reagents and inhibited by cysteine proteinase inhibitors at pH 6.0, but the

Figure 1. Effect of cysteine proteinase inhibitors on the trophozoite food vacuole. Transmission EMs of trophozoite-infected erythrocytes after 12 h of incubation (*1*) with no inhibitor (×26,000), (*2*) with 100 μM leupeptin (×34,000), or (*3*) with 140 μM E 64 (×22,000). In each micrograph the food vacuole (*A*), parasite cytoplasm (*B*), and erythrocyte cytoplasm (*C*) are labeled.

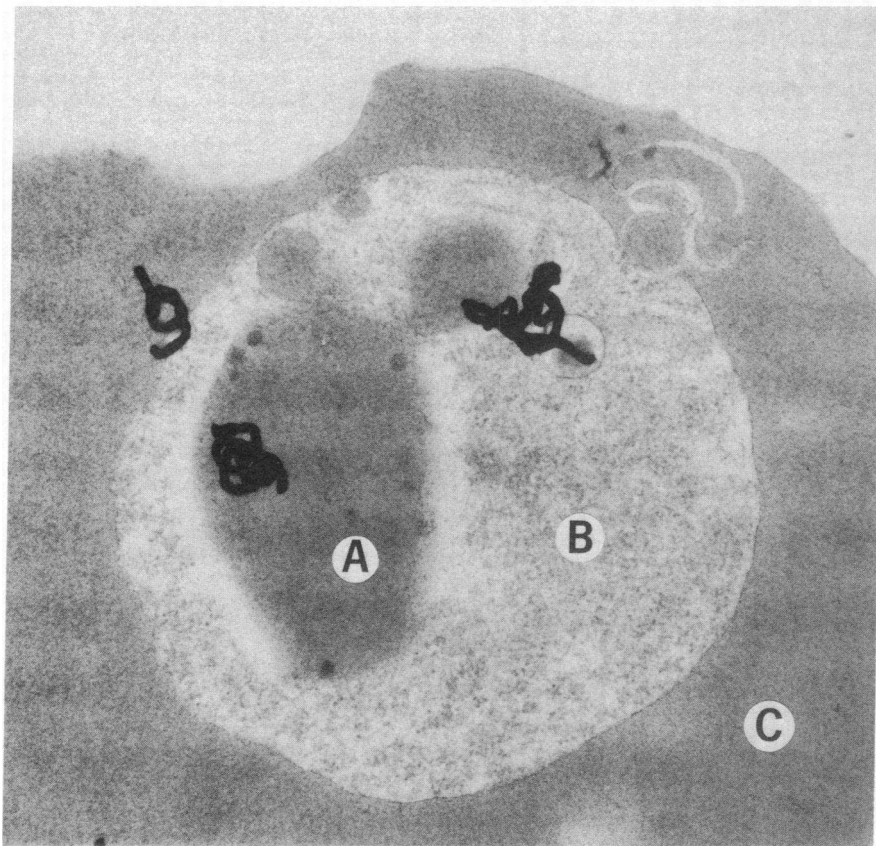*Figure 2.* Localization of [³H]leupeptin. EM autoradiograph of a trophozoite-infected erythrocyte after a 12-h incubation with [³H]-leupeptin (×40,000). Electron-dense filaments composed of silver grains indicate the localization of [³H]leupeptin to erythrocyte cytoplasm (*C*) and the trophozoite food vacuole (*A*). Additional silver grains appear to indicate vacuolar transport of leupeptin (with erythrocyte cytoplasm) through parasite cytoplasm (*B*) to the food vacuole.

inhibition was not as complete as with Azocoll or Z-Val-Leu-Arg-AMC. Furthermore, the proteolysis of [¹⁴C]methemoglobin was inhibited in part by pepstatin, indicating that trophozoites also contain aspartic proteinase activity. Leupeptin, E-64, and pepstatin also inhibited the degradation of [¹⁴C]methemoglobin by the trophozoite extract at pH 5.3 and pH 4.5 (not shown). At each pH studied, the combination of E-64 and pepstatin was more inhibitory than either compound alone (Table I).

Substrate preference of trophozoite proteinase activity. Substrate preference was determined by comparing the rates of

hydrolysis of different peptide-AMC substrates at pH 6.0 (Table II). Substrates with arginine at the cleavage site adjacent to a hydrophobic amino acid (Phe or Leu) were preferred over substrates with arginine adjacent to a charged amino acid or substrates lacking arginine.

Identification of an M_r 28,000 cysteine proteinase of trophozoites. Proteins of *P. falciparum* trophozoites were extracted with nonreducing electrophoresis sample buffer and
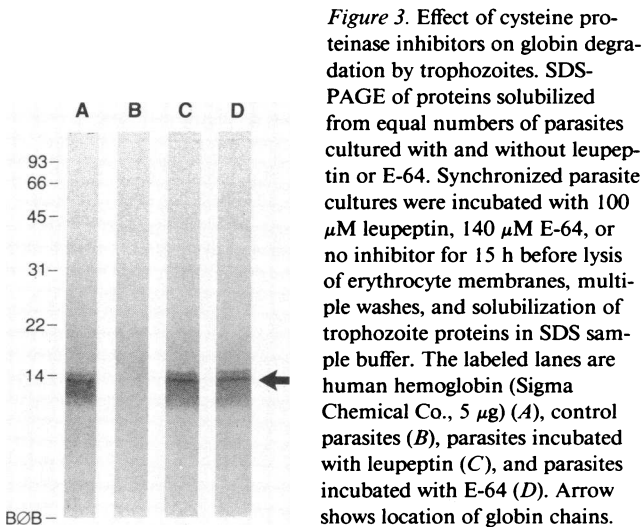

Figure 3. Effect of cysteine proteinase inhibitors on globin degradation by trophozoites. SDS-PAGE of proteins solubilized from equal numbers of parasites cultured with and without leupeptin or E-64. Synchronized parasite cultures were incubated with 100 μM leupeptin, 140 μM E-64, or no inhibitor for 15 h before lysis of erythrocyte membranes, multiple washes, and solubilization of trophozoite proteins in SDS sample buffer. The labeled lanes are human hemoglobin (Sigma Chemical Co., 5 μg) (*A*), control parasites (*B*), parasites incubated with leupeptin (*C*), and parasites incubated with E-64 (*D*). Arrow shows location of globin chains.
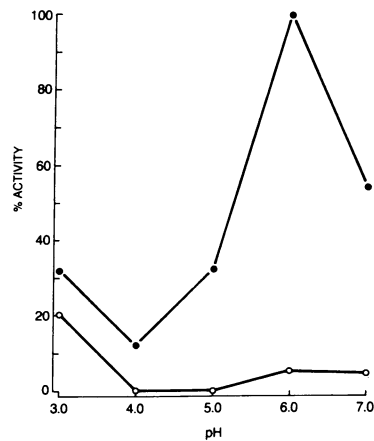

Figure 4. pH profile of trophozoite proteinase activity. The proteinase activities of a trophozoite extract (●) and an uninfected erythrocyte extract (○) were assayed with Azocoll at different pHs. Results shown are for a single experiment, but multiple assays yielded nearly identical results. Maximum activity (3.78 μg substrate hydrolyzed/ mg trophozoite extract per min) was observed with trophozoite extract at pH 6.0. Activity of other samples is reported as a percent of the maximum activity.

Table I. Proteinase Activity of Trophozoite Extract
with Different Substrates: Effect of Proteinase Inhibitors
and Sulfhydryl Compounds*

	Substrate		
Compound added	Azocoll	Z-V-L-R-AMC[‡]	[14C]Methemoglobin[‡]
None (Control)	100	100	100
Leupeptin (10 μM)	8.5	1.5	49.5
E-64 (10 μM)	ND[§]	8.2	49.7
NEM (5 mM)	29.1	9.6	90.9
Pepstatin (10 μM)	127.7	74.3	28.6
Pep./E-64[‖] (10 μM)	ND	ND	16.9
PMSF (1 mM)	136.5	96.8	102.0
1,10-Phen. (1 mM)	118.8	98.4	86.9
DTT (10 mM)	104.2	828.0	155.2
Cysteine (10 mM)	367.4	864.2	200.3
Glutathione (5 mM)	ND	398.4	176.6

* Results (expressed as % of control activity) of assays of the proteo-
lytic activity of trophozoite extract. The extract was prepared by ni-
trogen cavitation of trophozoites after the lysis of host erythrocytes.
Results shown for each substrate were from a single experiment, but
equivalent results were obtained in three or more assays. Control ac-
tivity for the three substrates was as follows: Azocoll, 2.69 μg sub-
strate hydrolyzed/mg trophozoite extract per min; Z-valine-leucine-
arginine-AMC (Z-V-L-R-AMC), 3.95 μmol substrate hydrolyzed/mg
trophozoite extract per min; [14C]methemoglobin, 0.00109 μg sub-
strate hydrolyzed/mg trophozoite extract per min. All assays were
performed at pH 6.0.
‡ For the Z-V-L-R-AMC assay, trophozoite extract was incubated
with 10 mM cysteine for the control and inhibitor (leupeptin, E-64,
NEM, pepstatin, PMSF, and 1,10-phenanthroline) assays. For the
[14C]methemoglobin assay, trophozoite extract was incubated with
10 mM DTT for the control and inhibitor assays. For the sulfhydryl
reagent (DTT, cysteine, and glutathione) assays, the controls were in-
cubated with buffer alone.
§ Not done.
‖ Pepstatin and E-64 (each 10 μM).

electrophoresed in a gelatin substrate polyacrylamide gel. A
clear band indicating proteinase activity was detected at M_r
28,000. This activity was completely inhibited by incubating
the gel with 280 μM E-64 before staining (Fig. 5). Incubation
of the gel with 100 μM leupeptin greatly decreased proteinase
activity (not shown). Identical results were obtained at pH 7.0
and pH 6.0.

Discussion

We report evidence that a malarial cysteine proteinase has a
critical role in hemoglobin degradation by P. falciparum tro-
phozoites. We observed that the incubation of trophozoites
with two inhibitors of cysteine proteinases (leupeptin and
E-64) caused undigested erythrocyte cytoplasm to accumulate
in the parasite food vacuole. We conclude that the effects of
leupeptin and E-64 were due to the specific inhibition of a food
vacuole cysteine proteinase rather than an unrelated toxicity
for the following reasons. (a) Leupeptin at micromolar con-
centrations inhibits proteinase activity without altering other
biochemical processes in mammalian cells (30). (b) The accu-
mulation of erythrocyte cytoplasm in the food vacuole was

Table II. Proteinase Activity of Trophozoite Extract
with Different AMC Peptide Substrates*

AMC peptide	Relative activity
Z-Phe-Arg	100
Z-Val-Leu-Arg	36.8
Z-Arg-Arg	19.4
Z-Leu	7.0
Z-Phe-Pro-Arg	6.7
Z-Phe	3.6
Z-Ala-Arg-Arg	3.1

* Results (normalized to 100 for the most effective substrate) of rep-
resentative assays of the hydrolysis by trophozoite extract of the
AMC-peptide fluorogenic substrates listed. Activity of the most effec-
tive substrate = 3.86 μmol substrate hydrolyzed/mg trophozoite ex-
tract per min.

caused only by inhibitors of cysteine proteinases. E-64 is a
highly specific cysteine proteinase inhibitor (31). (c) [3H]-
Leupeptin was shown to enter the food vacuole. (d) The effect
of leupeptin on the parasites was reversible and the effect of
E-64 was not reversible. These observations correlate with the
reversible inhibition of mammalian proteinases by leupeptin
and the irreversible inhibition of mammalian proteinases by
E-64 (30). (e) Intact globin accumulated in parasites incubated
with leupeptin and E-64, indicating that the hydrolysis of glo-
bin was indeed inhibited.

As predicted by the inhibitor studies, we detected cysteine
proteinase activity in trophozoite extracts and identified an M_r
28,000 cysteine proteinase that appeared to account for this
activity. We previously showed that the activity of the M_r
28,000 proteinase is expressed only at the trophozoite stage of
the life cycle (23) when the food vacuole can be identified
morphologically and hemoglobin degradation occurs. The
molecular size of the trophozoite proteinase and the acid pH
optimum, inhibitor profile, and substrate preference of the
proteinase activity are similar to the biochemical properties of
the lysosomal cysteine proteinase cathepsin L (16). The simi-
larities between the malarial enzyme and a lysosomal enzyme
further support our hypothesis that the M_r 28,000 proteinase is
a food vacuole enzyme since the food vacuole appears to be
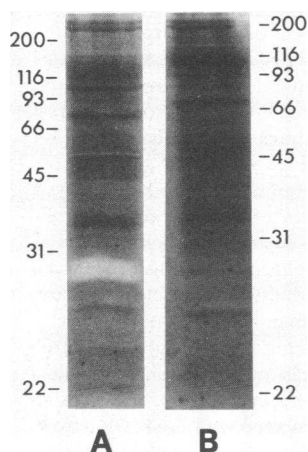analogous to secondary lysosomes. Although lysosomes con-

Figure 5. Identification of a tro-
phozoite cysteine proteinase. Gel-
atin substrate PAGE of tropho-
zoite proteins was performed. In
control trophozoites (lane A) a
clear band at M_r 28,000 represents
proteinase activity. In lane B,
E-64 was incubated with tropho-
zoites before the addition of sam-
ple buffer and was also added to
the buffer in which the gel lane in-
cubated after electrophoresis. No
evidence of proteinase activity was
seen in this lane or with extracts
from uninfected erythrocytes (not
shown).

tain multiple acid hydrolases, including cysteine and aspartic proteinases (16, 17), lysosomal cysteine proteinases appear to play a key role in the degradation of some proteins (16). For example, the cysteine proteinase inhibitor leupeptin inhibited 70% of endogenous protein degradation in rat liver cells (30) and caused undegraded protein to accumulate in lysosomes (32–34). Pepstatin, a potent inhibitor of aspartic proteinases, inhibited only 15–20% of protein degradation (30). The food vacuole of *P. falciparum* may also contain multiple proteinases with different mechanisms of action and different substrate specificities. Indeed, aspartic proteinases have been isolated from several species of *Plasmodium* (18–20), and we found that pepstatin partially inhibited the proteinase activity of trophozoites when the substrate was [^{14}C]methemoglobin. However, pepstatin did not cause the accumulation of erythrocyte cytoplasm in the food vacuole that we observed with leupeptin and E-64. The correlation of the effects of leupeptin and E-64 on the food vacuole of intact parasites with the inhibition of the trophozoite proteinase by the same inhibitors suggests that the M_r 28,000 cysteine proteinase has a critical, perhaps rate-limiting, role in hemoglobin degradation by malaria parasites.

We propose the following model for hemoglobin degradation in the food vacuole of *P. falciparum*. After erythrocyte cytoplasm has been ingested and transported to the food vacuole, the proteolysis of globin chains is initiated by a cysteine proteinase that cleaves peptide bonds adjacent to arginine residues on the surface of hemoglobin. We suggest that the M_r 28,000 proteinase has this function. It is intriguing that the sequence leucine-arginine, a preferred substrate of trophozoite proteinase activity, occurs twice in the beta-globin chain at positions that are susceptible to cleavage by the cysteine proteinase clostripain and that have been mapped to exposed sites on intact globin (35, 36). The cysteine proteinase that initiates hemoglobin degradation might be stimulated by the sulfhydryl compound glutathione, which is present in high concentrations in erythrocyte cytoplasm (37), and which stimulated trophozoite proteinase activity. The cleavage of exposed peptide bonds and the acid pH of the food vacuole may denature hemoglobin, making internal hydrophobic residues accessible to cleavage by aspartic proteinases. Cathepsin D, an aspartic proteinase of mammalian lysosomes, has little activity against native proteins, but has considerable activity against denatured proteins, particularly at hydrophobic residues (16). Although the complete proteolytic degradation of hemoglobin is probably a cooperative process requiring multiple proteinases, this model suggests that cleavage by a cysteine proteinase is required to initiate the process. Such a requirement would explain our observation that leupeptin and E-64 caused intact globin to accumulate in the food vacuole. The ensuing inhibition of parasite differentiation and multiplication may have been due to decreased availability of free amino acids for parasite protein synthesis. Specific inhibitors of the M_r 28,000 cysteine proteinase may provide new means of antimalarial chemotherapy.

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