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Order and Specificity of the *Plasmodium falciparum* Hemoglobin Degradation Pathway

Ilya Y. Gluzman,* Susan E. Francis,* Anna Oksman,* Christine E. Smith,† Kevin L. Duffin,‡ and Daniel E. Goldberg*

*Departments of Molecular Microbiology and Medicine, Washington University School of Medicine and The Jewish Hospital of St. Louis, St. Louis, Missouri 63110; and †Monsanto Corporate Research, Monsanto Company, St. Louis, Missouri 63198

Abstract

The human malaria parasite, *Plasmodium falciparum*, degrades nearly all its host cell hemoglobin during a short segment of its intraerythrocytic development. This massive catabolic process occurs in an acidic organelle, the digestive vacuole. Aspartic and cysteine proteases have been implicated in this pathway. We have isolated three vacuolar proteases that account for most of the globin-degrading activity of the digestive vacuole. One is the previously described aspartic hemoglobinase that initiates hemoglobin degradation. A second aspartic protease is capable of cleaving hemoglobin with an overlapping specificity, but seems to prefer acid-denatured globin. The third is a cysteine protease that does not recognize native hemoglobin but readily cleaves denatured globin. It is synergistic with the aspartic hemoglobinase, both by in vitro assay of hemoglobin degradation, and by isobologram analysis of protease inhibitor-treated parasites in culture. The cysteine protease is highly sensitive to chloroquine-heme complex, suggesting a possible mechanism of 4-aminoquinoline antimalarial action. The data suggest an ordered pathway of hemoglobin catabolism that presents an excellent target for chemotherapy. (*J. Clin. Invest.* 1994. 93:1602-1608.) Key words: plasmodium • protease • hemoglobin • chloroquine • aspartic

Introduction

Intraerythrocytic *Plasmodium* uses hemoglobin as a major nutrient source (1). In a short period of time it degrades virtually all the hemoglobin in its host cell. Catabolism takes place in an acidic organelle, the digestive vacuole (2-4). Recent work has suggested the involvement of aspartic and cysteine proteases in this process (5-7). A vacuolar aspartic hemoglobinase has been purified and characterized (6). It appears to make the initial attack on the hemoglobin molecule, cleaving in the hinge region that maintains quaternary structure. This cleavage is thought to unravel the hemoglobin, exposing it to further, efficient proteolysis. The hemoglobinase gene has been cloned; it encodes a protein with substantial homology to the mammalian aspartic proteases cathepsin D and renin (8). A peptidomimetic inhibitor selectively blocks the action of this enzyme and kills *P. falciparum* parasites in culture.

There appears to be more than one aspartic protease activity in the digestive vacuole (7, 8) and there is more than one aspartic protease gene (8). A cysteine protease has also been implicated in the hemoglobin degradation process, as cysteine protease inhibitors block the action of a 28-kD protease and cause accumulation of undigested hemoglobin and parasite death in culture (5, 9). A candidate gene for this enzyme has also been cloned (10) but the protein has not yet been purified, nor has its intracellular location been determined. This study describes the isolation and characterization of components of the proteolytic pathway. The proteases are shown to work in an ordered manner, synergistically, and with distinct specificities. In addition, the cysteine protease has properties that may shed light on the mechanism of chloroquine action.

Methods

Reagents. Hemoglobin and globin were from Sigma Chemical Co. (St. Louis, MO). DEAE-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Protein-Pak SW-200 and DEAE 5PW HPLC columns were from Waters Associates (Milford, MA). The hydroxylapatite HPHT column was from Bio-Rad Laboratories (Richmond, CA). [¹⁴C]Formaldehyde (46.7 mCi/mmol) and [³H]hypoxanthine (17.2 Ci/mmol) were from Dupont/NEN (Wilmington, DE).

Culture. *P. falciparum* clone HB-3 (a kind gift of Dr. W. Trager, Rockefeller University) was grown by the method of Trager and Jensen (11) using human plasma (12). Synchrony was maintained by sorbitol treatment (13).

Protease isolation. Isolated vacuoles from 1.2×10^{11} trophozoite-stage parasites were used as starting material. Vacuoles were prepared as previously described (14), omitting the final Percoll gradient step. These were then solubilized and fractionated by DEAE-Sepharose chromatography as previously described (6). The 0.5 M NaCl cut was used for purification of both aspartic proteases. The two enzymes were separated by hydroxylapatite HPLC using the system reported by Vander Jagt et al. (7). Two peaks of aspartic protease activity were obtained. The second peak was collected and rerun on a DEAE minicolumn (0.1 ml) using a gradient of NaCl in 20 mM Bis Tris, pH 7.0. This procedure yielded a homogeneous preparation of aspartic hemoglobinase I by SDS-PAGE; its identity was confirmed by NH₂-terminal sequence analysis. The enzyme was stable on ice for 1-2 mo but did not survive freezing. The procedure is a modification from that previously described (6).

The earlier-eluting hydroxylapatite peak was rerun on a Protein-Pak 5PW DEAE HPLC column (Waters Associates). A gradient of NaCl from 0 to 0.7 M in 20 mM Tris, HCl, pH 7.5, was used at 1 ml/min for elution. The isolated enzyme (aspartic hemoglobinase II) was stable on ice for 2-6 wk.

The cysteine protease was purified from the original 0.2 M NaCl DEAE fraction by further chromatography on DEAE HPLC (Protein Pak DEAE-5PW; Waters Associates) using a gradient of NaCl from 0 to 0.5 M in 20 mM Tris, pH 7.5, over 60 min with a flow rate of 0.5 ml/min. This was followed by HPLC chromatography on hydroxylapatite as described above. The purified enzyme could be stored at -70° for at least 3 mo.

[¹⁴C]Globin proteolysis assay. Human globin was labeled by reductive methylation using [¹⁴C]formaldehyde (15). The final specific ac-

Address correspondence to Dr. Daniel E. Goldberg, Washington University School of Medicine, Box 8230, 660 South Euclid Avenue, St. Louis, MO 63110.

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tivity was 47 $\mu\text{Ci}/\text{mg}$. 0.06 μCi was incubated with 10 μl of enzyme in 150 mM sodium acetate buffer, pH 5.0, in a final volume of 0.04 ml. After 2 h, the reactions were stopped by addition of 100 μl of globin carrier (2 mg/ml) followed by 150 μl cold 8% TCA. After 30 min on ice the tubes were centrifuged for 30 min at 13,000 g and the supernate was assayed for radioactivity. In crude fractions containing a mixture of proteases, aspartic protease activity was assessed by addition of 10 μM E-64 to block any cysteine protease action. Cysteine protease activity was measured in these fractions by including 10 μM pepstatin in the assay to block aspartic protease action. For hemin inhibition assays, hemin was made as a concentrated stock in 0.1 N NaOH. It was mixed with 1 mM chloroquine before enzyme addition. A similar amount of NaOH alone did not affect pH or enzyme activities. 1 U is defined as the amount of enzyme that will cleave 1 $\mu\text{g}/\text{h}$ of [^{14}C]globin to TCA-soluble fragments at pH 5.0, 37°C.

pH profile of cysteine protease activity. Purified cysteine protease (60 mU) was incubated for 2 h with [^{14}C]globin in 200 mM citrate-phosphate buffer at pH values from 4 to 6.5 (increments of 0.3–0.5 U). Proteolytic activity was measured by TCA assay (measured in pmol/h).

SDS-PAGE of hemoglobin degradation products. Incubations were performed as for the [^{14}C]globin assays but using unlabeled human globin or hemoglobin (3.2 μg) as substrate. Reactions were stopped by mixing with electrophoresis sample buffer and boiling for 5 min; products were separated on 12% SDS-PAGE (16). Gels were developed by silver staining (17) as were similar gels to assess protein purification.

Liquid chromatography/mass spectrometry (LC/MS).¹ Enzyme (20 mU) was incubated with 3.2 μg human hemoglobin (aspartic proteases) or human globin (cysteine protease) in 0.2 M ammonium acetate, pH 5.0, at 37°C for 15 h. Control incubations contained 10 μM SC-50083 (aspartic hemoglobinase I), pepstatin (aspartic hemoglobinase II), or E-64 (cysteine protease) to inhibit activity. SC-50083 is a selective aspartic hemoglobinase I inhibitor (8). After incubation, samples were acidified to pH 2 with trifluoroacetic acid, evaporated to dryness, and resuspended in 20 μl of aqueous 0.1% TFA. 5 μl was injected onto a 0.32-mm i.d. \times 15-cm capillary column packed with Vydac C-18 (300 Å) stationary phase. Peptide fragments were eluted from the column with 0–70% acetonitrile in 0.1% TFA over 60 min at a flow rate of 5 $\mu\text{l}/\text{min}$. Column eluate passed through a 50- μm \times 50-cm fused silica capillary to the electrospray interface of a Sciex (Thornhill, Ontario, Canada) API III triple quadrupole mass spectrometer. The quadrupole mass analyzer was scanned from 300 to 2,400 u in 0.3-u steps over \sim 3 s. Retention times of eluted peptides were determined by an increase in the total ion current of the mass chromatogram. Molecular weights of eluted peptides were determined after averaging together the mass spectral scans over which the peptide eluted. Average molecular weights of high mass peptides were determined by deconvoluting the multiply charged ion signals in the mass spectra.

NH₂-terminal sequence analysis. Automated Edman degradation chemistry was used to determine the NH₂-terminal protein sequence. A gas phase sequencer (470A; Applied Biosystems, Inc., Foster City, CA) was used for the degradations using the standard sequencer cycle, 03RPTH. The respective PTH-amino acid derivatives were identified by reverse-phase HPLC analysis in an on-line fashion using an analyzer (120A PTH; Applied Biosystems, Inc.) fitted with a Brownlee 2.1-mm i.d. PTH-C18 column.

Isobologram analysis. Late ring-stage cultures at 2% parasitemia were grown for 16 h in the presence of various combinations of the selective aspartic hemoglobinase I inhibitor SC-50083 (8) and the cysteine protease inhibitor E-64. At the end of this period, 1 μCi (17.2 Ci/mmol) of [^3H]hypoxanthine was added and the cultures were incubated for another 4 h. Parasites were harvested and [^3H]hypoxanthine incorporation was measured as previously described (18). As a control, the DMSO vehicle for the drug was diluted in RPMI 1640 to the same extent and added to a similar culture. This had no effect on parasite hypoxanthine incorporation. Parasitemia in the cultures paralleled hy-

Table I. Effect of Inhibitors on Digestive Vacuole Proteolysis

	cpm	Percent inhibition
No inhibitor	5,379	—
SC-50083	2,115	60.7
Pepstatin	1,406	73.9
E-64	3,967	26.3
E-64 + pepstatin	99	98.2
E-64 + SC-50083	1,077	80.0

Digestive vacuole extract was incubated with [^{14}C]globin for 2 h in the presence of various inhibitors (each at 10 μM). Extent of proteolysis was measured by the TCA assay. Results are averages of duplicates from a representative experiment.

poxanthine incorporation. The IC₅₀ for each inhibitor in the presence of sub-IC₅₀ doses of the other inhibitor was calculated and plotted (isobologram).

Results

Protease isolation. Our previous work had indicated a contribution of aspartic and cysteine protease activities to the degradation of hemoglobin in purified digestive vacuoles (6). Since not all proteases work efficiently on the hemoglobin tetramer, we incubated extract of purified vacuoles with acid-denatured [^{14}C]globin in the presence or absence of inhibitors. The selective aspartic hemoglobinase I inhibitor SC-50083 (8) blocked the majority of degradation (60–80%, depending on the vacuole preparation), and there was 10–20% more inhibition when the general aspartic protease inhibitor pepstatin was substituted (Table I). The combination of these inhibitors with the specific cysteine protease inhibitor E-64 stopped virtually all proteolysis, suggesting that aspartic and cysteine protease activities account for the great majority of the digestive vacuole globin degradation capability. The assay was repeated using unlabeled human hemoglobin as substrate to ensure a native conformation, and degradation was analyzed by SDS-PAGE (Fig. 1). Again, SC-50083 blocked the majority of hemoglobin degradation, pepstatin blocked nearly all degradation, and the combination of E-64 with pepstatin blocked all detectable proteolysis.

When vacuole extract was fractionated by hydroxylapatite HPLC using the procedure of Vander Jagt et al. (7), globin-degrading activity was separated into two pepstatin-inhibitable peaks and one E-64-inhibitable peak (Fig. 2 A). The high-salt aspartic protease activity (aspartic hemoglobinase I) was two orders of magnitude more susceptible to inhibitor SC-50083 (IC₅₀ = 500 nM) than the low salt aspartic protease activity

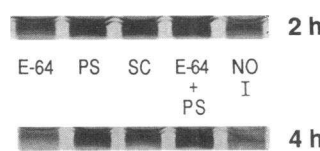


Figure 1. Effect of inhibitors on vacuolar hemoglobin degradation. Extract of purified digestive vacuoles was incubated with human hemoglobin at 37°C, pH 5.0, for 2 or 4 h in the presence of various inhibitors.

Remaining hemoglobin substrate was assessed by SDS-PAGE. Equal amounts of incubation mixture were loaded in each lane. The experiment was repeated three times with similar results. PS, pepstatin; SC, SC-50083; No I, no inhibitor added.

1. Abbreviations used in this paper: LC, liquid chromatography; MS, mass spectrometry.

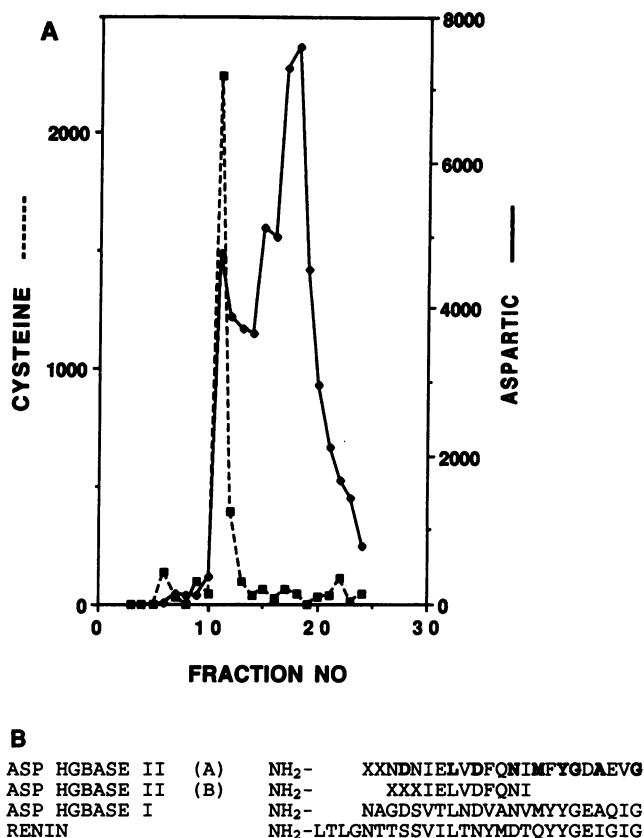


Figure 2. Vacuolar protease isolation and analysis. (A) Activity profile on hydroxylapatite HPLC chromatography. Vacuole extract was passed over DEAE-Sepharose and eluted with a NaCl gradient. The major peaks of aspartic and cysteine protease activity were pooled and fractionated on Biogel HPHT hydroxylapatite HPLC chromatography. Fractions were assayed for cysteine and aspartic protease activity. Activity measured in counts per minute product per hour. (B) Sequence analysis of aspartic hemoglobinase II. Purified enzyme was analyzed by SDS-PAGE, transferred to PVDF membrane, and subjected to NH₂-terminal sequencing. Aligned are the two NH₂ termini obtained, along with aspartic hemoglobinase I and human renin (6). Residues identical in the two *Plasmodium* protease sequences are emboldened. AH, aspartic hemoglobinase.

(aspartic hemoglobinase II), as reported elsewhere (8). The cysteine protease activity was insensitive to this agent.

We purified the cysteine and aspartic proteases from digestive vacuoles in order to characterize their properties, specificity, and interaction. The cysteine protease was purified by sequential chromatography on DEAE at two different pHs followed by hydroxylapatite. The final product migrated at 31 kD on SDS-PAGE and at 28 kD on gel filtration chromatography (not shown). Gelatin activity gel analysis generated a 28-kD band and synthetic substrate assays showed that Z-phe-arg was preferred over Z-arg and Z-arg-arg (not shown), similar to results obtained by Rosenthal et al. (5) in crude extracts. This suggests that the activity they have described may be the vacuolar cysteine protease. Purified vacuoles also gave the same band on activity gels and showed the same substrate preference, adding support for the vacuolar localization of this enzyme.

Purification of aspartic hemoglobinase I to apparent homogeneity was modified from the procedure described previously (6). Isolation of aspartic hemoglobinase II involved a combination of DEAE, hydroxylapatite, and repeated DEAE chromatography. When the aspartic hemoglobinase II preparation was

subjected to SDS-PAGE, a band at ~ 36 kD was observed (not shown). This was transferred to PVDF membrane and subjected to Edman degradation. The sequence obtained (Fig. 2 B, sequence A) is 43% identical to the mature NH₂ terminus of aspartic hemoglobinase I (6, 8) and therefore appears to be a related but distinct aspartic protease. An aspartic protease gene with identical sequence near the 5' end has recently been cloned (18a). A small amount of a second sequence (Fig. 2 B, sequence B) was obtained from the same sample. The two sequences are related and suggest a variable proenzyme cleavage or proteolysis during purification.

Protease specificity. To examine the activity of the vacuolar proteases against native hemoglobin, SDS-PAGE assays were performed. Equal [¹⁴C]globin-degrading units of aspartic hemoglobinase I and aspartic hemoglobinase II activities were incubated with unlabeled human hemoglobin at pH 5.0, the estimated physiological pH of the digestive vacuole (19, 20). Products were separated on a denaturing gel and silver stained. Aspartic hemoglobinase I was considerably more active in degrading native hemoglobin, but both enzymes were able to work on this substrate (Fig. 3 A). In contrast, the cysteine protease was unable to cleave native hemoglobin at pH 5.0, but was quite active against acid-denatured globin (Fig. 3 B). At lower pH values, the cysteine protease did have some activity against hemoglobin, which is likely due to substrate unfolding in acidic conditions (data not shown).

The cleavage sites of each protease on its native substrate, hemoglobin (globin for the cysteine protease), were determined by LC-MS analysis. Multiple fragments were obtained using each enzyme (Fig. 4). Table II shows the hemoglobin fragments assigned to each LC peak based on its molecular mass, deduced as previously reported (15). Interestingly, all three enzymes generated fragments corresponding to cleavage of α33phe/34leu, while other cleavages were specific to each particular protease (Table III). For aspartic hemoglobinase I, 12 substantial fragments were detected and 11 could be unambiguously identified, corresponding to 6 cleavages in the hemoglobin molecule. For aspartic hemoglobinase II there were five major fragments corresponding to four cleavages. The cysteine protease generated seven fragments, corresponding to five cleavages. Most of these cleavages could be confirmed by NH₂-terminal amino acid analysis (marked by asterisks in Table III). The most selective inhibitors available, SC-50083 for aspartic hemoglobinase I, pepstatin for aspartic hemoglobinase II, and E-64 for the cysteine protease, abolished the proteolytic fragmentation (Fig. 4, bottom panel of each pair). The α106-141 fragment previously reported (6) was also detected in the

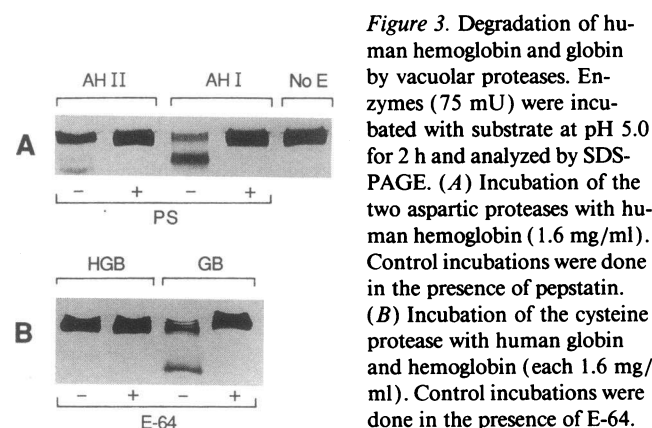


Figure 3. Degradation of human hemoglobin and globin by vacuolar proteases. Enzymes (75 mU) were incubated with substrate at pH 5.0 for 2 h and analyzed by SDS-PAGE. (A) Incubation of the two aspartic proteases with human hemoglobin (1.6 mg/ml). Control incubations were done in the presence of pepstatin. (B) Incubation of the cysteine protease with human globin and hemoglobin (each 1.6 mg/ml). Control incubations were done in the presence of E-64.

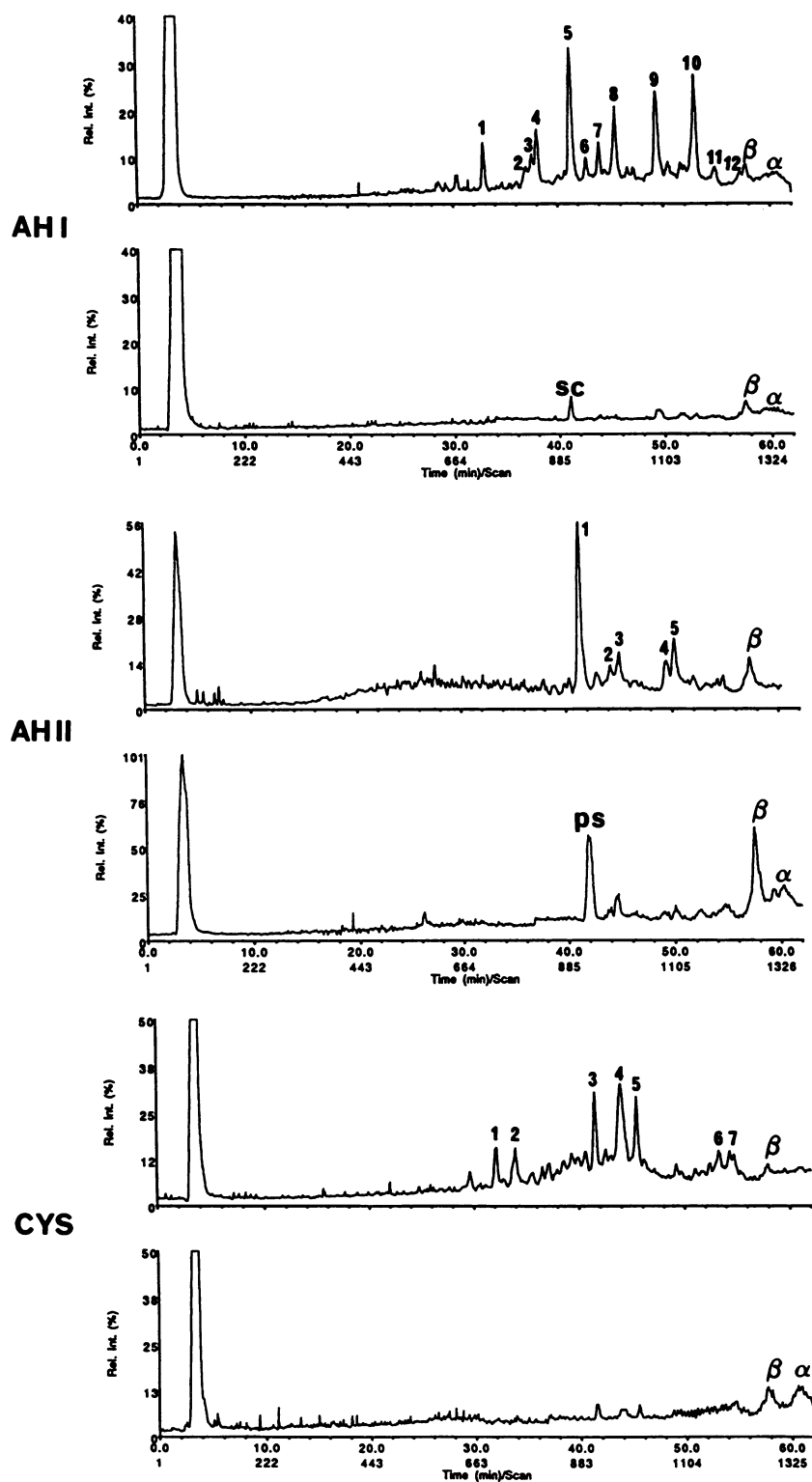


Figure 4. LC-MS analysis of proteolytic globin fragments generated by isolated vacuolar proteases. Vacuolar aspartic proteases (20 mU) were incubated with human hemoglobin; cysteine protease was incubated with human globin. Proteolysis was carried out at 37°C for 16 h. Products were separated by C-18 reverse-phase chromatography. Uncleaved α and β chains, as well as inhibitors pepstatin (*ps*) and SC-50083 (*sc*) are marked. In each pair of panels the top depicts an incubation without inhibitor, the bottom with inhibitor (10 μ M SC-50083, pepstatin, E-64 respectively).

aspartic hemoglobinase I digest, but was a minor peak. Trace exopeptidase contamination of aspartic hemoglobinase II may account for the minor fragments 110–136 and 109–134 observed along with the major and fuller length 109–136.

Synergism. To assess the contribution of the cysteine protease to hemoglobin degradation, native human hemoglobin was incubated with small amounts of aspartic hemoglobinase I and cysteine protease in the presence or absence of inhibitors. Degradation was monitored by disappearance of starting material on SDS-PAGE (Fig. 5 A). The combination of proteases

rapidly degraded hemoglobin. In contrast, during the time period used, little degradation was achieved when either enzyme was inhibited. Similar results were obtained using SC-50083 instead of pepstatin (not shown). The data suggest that the action of these enzymes is highly synergistic. This was further examined in culture. *P. falciparum* parasites (ring stage) were grown in medium supplemented with combinations of SC-50083 (to selectively inhibit aspartic hemoglobinase I) and E-64 (to inhibit the cysteine protease). [3 H]Hypoxanthine incorporation was measured after the control culture had

Table II. Assignment of Proteolysis Fragments after MS Analysis

	Fragment mass	Assigned peptide	Calculated mass
ASP HGBASE I			
1	1,869.5	β 130-146	1,869.2
2	1,308.0	β 32-41	1,308.6
3	3,491.3	Unassigned	N/A
4	1,586.0	α 34-46	1,585.8
5	3,475.2	α 1-33	3,475.0
6	3,276.4	β 1-31	3,275.7
7	5,469.8	α 47-98	5,469.2
8	7,037.9	α 34-98	7,037.0
9	4,565.9	α 1-41	4,566.3
10	9,467.5	β 42-146	9,467.9
11	11,318.5	β 42-146	11,319.0
12	14,016.3	β 1-129	14,016.1
ASP HGBASE II			
1	3,475.2	α 1-33	3,475.0
2	2,694.3	α 109-134	2,694.1
3	3,389.2	β 1-32	3,388.9
4	2,793.6	α 110-136	2,793.2
5	2,906.4	α 109-136	2,906.4
cys protease			
1	1,400.5	β 70-82	1,400.6
2	3,197.0	α 1-31	3,196.6
3	3,474.9	α 1-33	3,475.0
4	4,107.9	β 33-69	4,107.7
5	3,388.5	β 1-32	3,389.0
6	7,477.6	β 1-69	7,478.6
7	8,406.2	β 70-146	8,406.7

reached the schizont stage (24 h) to assess viability (18). An isobologram was constructed to assess interaction between the two drugs (Fig. 5 B). Strong synergism was exhibited, as small amounts of SC-50083 lowered the IC_{50} of E-64 substantially.

Table III. Summary of Cleavage Sites Deduced from the MS Analysis

Enzyme		Bond cleaved	Cleavage sequence
AH I	α	*33/34	RMF/LSF
		*46/47	PHF/DLS
		98/99	VNF/KLL
	β	*31/32	GRL/LVV
		*41/42	QRF/FES
		*129/130	QAA/YQK
AH II	α	*33/34	RMF/LSF
		*108/109	LVT/LAA
		*136/137	TVL/TSK
	β	32/33	RLL/VVY
Cysteine	α	31/32	LER/MFL
		*33/34	RMF/LSF
	β	*32/33	RLL/VVY
		*69/70	VLG/AFS
		82/83	LSA/LSD

* Cleavages that could be confirmed by NH_2 -terminal sequence analysis.

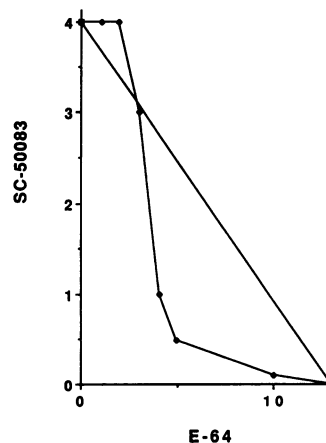
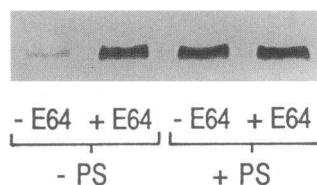


Figure 5. Synergistic action of aspartic hemoglobinase I and cysteine protease. (A) In vitro. Human hemoglobin was incubated with equal activities of the two enzymes (35 mU) in the presence or absence of aspartic and cysteine protease inhibitors. Products were analyzed by SDS-PAGE after a 1.5-h incubation. (B) In culture. Isobologram analysis of inhibitors of aspartic hemoglobinase I and cysteine protease. Parasites were cultured with combinations of SC-50083 and E-64 to determine the influence of one drug on the IC_{50} of the other drug. The diagonal line represents the curve expected for two drugs that are neither synergistic nor antagonistic. Axes show IC_{50} values (μM). With E-64 alone the IC_{50} was 13 μM ; with SC-50083 alone the IC_{50} was 4 μM . Data are averages of triplicate determinations.

Very small amounts of E-64 did not change the SC-50083 IC_{50} , but higher levels lowered it. Synergism has been shown previously for E-64 with the less selective aspartic protease inhibitor pepstatin (21).

Cysteine protease characterization. The purified cysteine protease showed a pH optimum of 5.0-5.3, similar to that of both aspartic proteases (not shown) and consistent with the physiological pH of the digestive vacuole (19, 20). Activity was inhibited by low concentrations of E-64 and leupeptin; PMSF was inhibitory in a DTT-reversible manner characteristic of cysteine proteases (Table IV). *O*-Phenanthroline was inhibitory, but preabsorption of the chelator with Cu^{2+} failed to block this inhibition and addition of a wide variety of metals could not restore activity, suggesting that phenanthroline was acting by a metal-independent mechanism. Consistent with this, EDTA was actually stimulatory. Chloroquine-hemin complex was inhibitory at low concentrations (Fig. 6); this complex also inhibited both aspartic proteases (as first reported by Vander Jagt et al. workers [22]), but was more than an order of magnitude less potent than in their study.

Discussion

P. falciparum hemoglobin degradation is a massive process that appears to be essential to the organism's growth and development. Selective blockade of the aspartic hemoglobinase impairs hemoglobin degradation and leads to parasite death (8). Though the mechanism of action of chloroquine and other 4-aminoquinolines is poorly understood, it is clear that these potent antimalarials concentrate and probably act in the digestive vacuole, on aspects of the hemoglobin catabolism pathway (reviewed in reference 4). It is therefore of biological and therapeutic interest to understand the proteolytic enzymes involved in hemoglobin breakdown. We have found that three enzymes are important in hemoglobin/globin degradation in the digestive vacuoles. All three activities have previously been de-

Table IV. Inhibition of Cysteine Protease Activity

Inhibitor	Concentration	Percent inhibition
E-64	1 μ M	96.1
	10 μ M	98.8
Leupeptin	1 μ M	99.2
	10 μ M	100
PMSF	1 mM	72.9
PMSF + DTT	1 mM/10 mM	10.4
Pepstatin	10 μ M	0.6
Phenanthroline	1 mM	82.8
	.25 mM	28.6
Phenanthroline + Co ²⁺	1 mM/2 mM	79.0
Co ²⁺	2 mM	0.0
EDTA	0.5 mM	(70.8)
DTT	10 mM	(45.6)

Values in parentheses represent stimulation rather than inhibition. Results are averages of duplicates from a representative experiment.

scribed in extracts, but only aspartic hemoglobinase I had been purified before this work (6). The gene for this hemoglobinase has been cloned (8), and a candidate gene for the vacuolar cysteine protease has also been cloned (10). A gene encoding the same NH₂-terminal sequence as aspartic hemoglobinase II has also recently been cloned (18a). An additional minor aspartic protease activity has been described in the particulate fraction of a digestive vacuole sonicate (7). Whether this is a third vacuolar aspartic protease or a modification of one of the others has not been established.

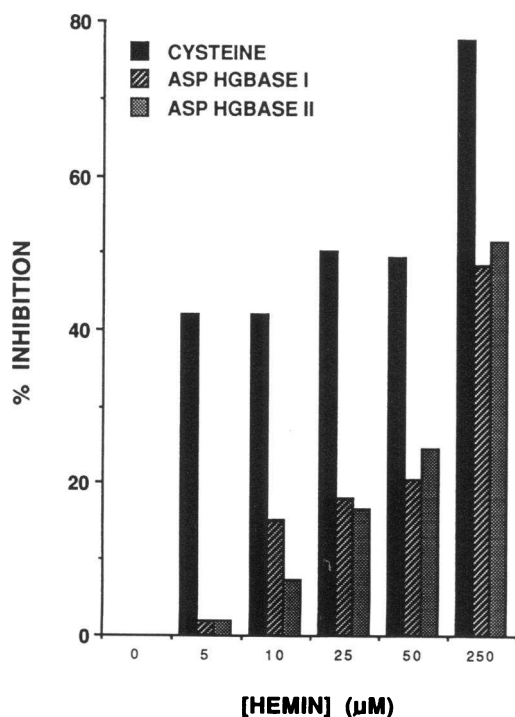


Figure 6. Inhibition of vacuolar proteases by chloroquine-hemin complex. Isolated proteases were incubated with [¹⁴C]globin in the presence of varying amounts of hemin and 1 mM chloroquine. Activity was measured by TCA assay. Data are combined from two different experiments done in duplicate.

Isolation of aspartic hemoglobinases I and II and the vacuolar cysteine protease has allowed us to begin to understand their specificities and their roles in hemoglobin degradation. Both aspartic proteases are capable of degrading hemoglobin. The cleavages made by aspartic hemoglobinase I suggest a preference for phenylalanine at the P1 position; aspartic hemoglobinase II appears to prefer a leucine at P1' (Table III). For both the major cleavage is the α 33Phe-34Leu bond in the hinge region that appears vital for the integrity of the hemoglobin tetramer (23). Our previous aspartic hemoglobinase report (8) described an initial cleavage at α 33/34, and two major early secondary cleavages at 105/106 and 108/109. In our current analysis both enzymes cleaved at 33/34, hemoglobinase I cleaved at 105/106, and hemoglobinase II cleaved at 108/109. This suggests that the previous aspartic hemoglobinase I preparation may have contained some aspartic hemoglobinase II activity. This could not have been detected in our previous work because the selective inhibitor SC-50083 was not available and pepstatin inhibited all cleavages of both enzymes.

Why there should be two distinct enzymes so similar in their specificity is unclear. This seems to be another case of a redundant *Plasmodium* function, similar to the dual merozoite invasion systems (24), the multiple endothelial sequestration receptors (25, 26) and the dual sporozoite heparan sulfate binding proteins (27–29). The second aspartic protease is not enough to sustain the required hemoglobin degradation, however. Selective blockade of aspartic hemoglobinase I by SC-50083 halts the bulk of hemoglobin degradation, and the parasites die (8). Aspartic hemoglobinase II may work in concert with aspartic hemoglobinase I to increase the efficiency of hemoglobin breakdown. Perhaps aspartic hemoglobinase II also has a second function. It could serve as a processing enzyme, activating hemoglobinase I and/or other vacuolar enzymes when acidic pH is achieved. Biosynthesis experiments may help sort this out.

The cysteine protease is incapable of degrading native hemoglobin at physiological vacuolar pH. It will, however, rapidly degrade the denatured molecule. It is also synergistic with aspartic hemoglobinase I, and inhibitors of both proteases in culture display potent synergism. We conclude that *Plasmodium* hemoglobin degradation is an ordered pathway, wherein aspartic hemoglobinase I acts first to cleave native hemoglobin at the hinge region, unravelling the molecule. Aspartic hemoglobinase II appears to be able to start working on some of the native hemoglobin but prefers the denatured molecule. The cysteine protease recognizes only the unwound or fragmented molecule; once the aspartic proteases have initiated proteolysis, it can make further attacks and proteolysis can proceed efficiently. Interestingly, the cysteine protease is capable of cleaving denatured globin at the same α 33/34 site recognized on native hemoglobin by both aspartic proteases. This peptide bond is wound up in an α helix. This suggests that the aspartic proteases are capable of inducing enough unwinding to make the site accessible for cleavage. The pH 5 environment may also help unwinding, but this does not seem to be enough to expose the site on native hemoglobin for the cysteine protease.

The cleavages that these enzymes make are infrequent enough that there must be exopeptidases to finish the proteolysis, generating free amino acids for the parasite's nutrition. Little is known about these steps, though an aminopeptidase activity has been detected in *Plasmodium* (30, 31).

The cysteine protease is very sensitive to heme. Heme accumulates during chloroquine action, either by direct chloro-

quine sequestration (32), or by heme polymerase inhibition (33). It has been proposed that the heme-chloroquine complex generated is damaging to membranes (34) as well as to aspartic proteases (22). Our data, using the millimolar chloroquine levels estimated to accumulate during therapy (35, 36), suggest that the vacuolar cysteine protease is actually most sensitive to free or chloroquine-complexed heme. The cysteine protease may be the ultimate target of chloroquine action, then. It should be noted that Vander Jagt et al. (7) have reported an IC_{50} of 5–15 μ M for chloroquine-heme inhibition of aspartic protease activities. This is an order of magnitude lower than our values. The reason for the discrepancy is unclear, but could have to do with differences in the pH or assay used.

In addition to being indirect targets for quinoline action, the proteases described here should be excellent direct targets. Already, inhibitors of the aspartic hemoglobinase I and cysteine protease have been described that are potent agents against *P. falciparum* in culture (8, 9) and in animal models (37). More selectivity is needed to avoid host toxicity. The specificity studies described here offer evidence that the substrate preference of these enzymes is different enough from those of homologous host enzymes that selectivity should be achievable. The way is clear to develop a new class of antimalarials that interfere with this essential catabolic pathway.

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