Hemoglobin Degradation in the Human Malaria Pathogen *Plasmodium falciparum*: A Catabolic Pathway Initiated by a Specific Aspartic Protease

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Summary

Hemoglobin is an important nutrient source for intraerythrocytic malaria organisms. Its catabolism occurs in an acidic digestive vacuole. Our previous studies suggested that an aspartic protease plays a key role in the degradative process. We have now isolated this enzyme and defined its role in the hemoglobinolytic pathway. Laser desorption mass spectrometry was used to analyze the proteolytic action of the purified protease. The enzyme has a remarkably stringent specificity towards native hemoglobin, making a single cleavage between α33Phe and 34Leu. This scission is in the hemoglobin hinge region, unraveling the molecule and exposing other sites for proteolysis. The protease is inhibited by pepstatin and has NH_2-terminal homology to mammalian aspartic proteases. Isolated digestive vacuoles make a pepstatin-inhibitable cleavage identical to that of the purified enzyme. The pivotal role of this aspartic hemoglobinase in initiating hemoglobin degradation in the malaria parasite digestive vacuoles is demonstrated.

The intraerythrocytic malaria parasite develops within a cell that contains a single major cytosolic protein, hemoglobin. The organism avidly ingests host hemoglobin and degrades it in a specialized proteolytic organelle called the digestive vacuole (1–4). As the parasite has a limited capability for de novo synthesis (5, 6) or exogenous uptake (7) of amino acids, the hemoglobin is catabolized to provide amino acids for growth and maturation (6, 8, 9). The process of hemoglobin breakdown releases heme, which is detoxified by polymerization into a crystalline pigment, hemozoin (10, 11). It has been estimated that 25–75% of hemoglobin in the infected erythrocyte is degraded (12–15). To account for this enormous amount of protein breakdown that occurs in just a few hours of the parasite's trophozoite stage, we have proposed that there is an efficient and specific pathway for hemoglobin proteolysis in the digestive vacuole (16). Several putative malaria hemoglobinases have been described (17–26), but it has been difficult to ascribe a physiological role in hemoglobinolysis to any of these activities. We have recently reported the purification and characterization of *Plasmodium falciparum* digestive vacuoles in which the degradation of hemoglobin occurs (16). The isolated vacuoles were capable of cleaving hemoglobin to small fragments, and required action of an endogenous aspartic protease to cleave intact hemoglobin before other proteolytic activities could function efficiently. We have now defined the role of this aspartic hemoglobinase in the process of hemoglobin catabolism within the digestive vacuole.

Materials and Methods

Materials. Saponin, Triton X-100, and bovine spleen cathepsin D were from Sigma Chemical Co. (St. Louis, MO); Centricon filter units were from Amicon Corp. (Danvers, MA); Immobilon-P (PVDF) membranes were from Millipore Corp. (Bedford, MA); DEAE-Sephacel was from Pharmacia Fine Chemicals (Piscataway, NJ); the 200 SW gel filtration column came from Waters Chromatography Division (Milford, MA); Ultrogel-HA was a product of LKB Instruments, Inc. (Gaithersburg, MD); Guanidine HCl was from Pierce Chemical Co. (Rockford, IL). ^{3}H-Hemoglobin was prepared by metabolic labeling of rat reticulocytes with L-3,4, 5-^{3}H-leucine (New England Nuclear, Boston, MA) as described (16). Human hemoglobin was purified from normal peripheral blood (27) and stored at −70°C until use.

Parasite Culture. P. falciparum clone HB-3 was cultured in A⁺ human erythrocytes by the method of Trager and Jensen (28). Culture synchrony was maintained by sorbitol treatment (29), and cells were harvested at the late trophozoite stage.

Parasite Extract Preparation. Cultures (600 ml, 5% hematocrit, 15% parasitemia) were harvested by centrifugation for 10 min at 1,500 g, washed twice with PBS, and the parasites freed of their erythrocytes by incubation with an equal volume of 2 mg/ml saponin in PBS for 15 min at 37°C. The mixture was diluted with iced PBS, centrifuged for 10 min at 1,500 g, and the free parasite pellet was washed twice with ice-cold PBS. The pellet was frozen at −70°C until used (within 2 wk). When enough parasite pellets had accumulated (usually three to four), they were suspended in 10 mM Bis Tris buffer, pH 7.0, containing 1 mM PMSF, 1 mM 1,10-phenanthroline, 100 µM leupeptin, and were sonicated for 15 s three times on ice. The sonicate was centrifuged at 100,000 g

for 1 h (4°C). The pellet was then resuspended in 10 ml of 10 mM Bis Tris buffer, pH 7.0, with 1% Triton X-100, 1 mM PMSF, 1 mM 1,10-phenanthroline, 100 μM leupeptin using a Dounce homogenizer (Wheaton Scientific, Millville, NJ) (10 strokes). After 30 min on ice, the extract was centrifuged at 25,000 g for 30 min, the pellet reextracted with 4 ml more buffer, and the supernates combined.

DEAE-Sephacel Chromatography. The extract was passed over a 1.5 × 7-cm column of DEAE-Sephacel equilibrated in 20 mM Bis Tris buffer, pH 7.0. The column was washed with 16 ml of equilibration buffer, and eluted with a step of gradient of NaCl from 0.1 to 0.7 M in 0.1 M increments. Two 8-ml fractions were collected at each step. Fractions were assayed for aspartic protease activity as described below. Activity eluted between 0.3 and 0.5 M. These fractions were pooled and concentrated with Centricon 10 ultrafiltration membranes to ~1 ml. 0.25 volume of 1 M sodium acetate buffer, pH 4.5, was added. The preparation was incubated on ice for 20 min and centrifuged at 4°C for 20 min at 14,000 rpm. The supernate was immediately neutralized with 0.125 volume 1 M Tris HCl buffer, pH 8.8.

Hydroxylapatite Chromatography. The neutralized pH 4.5 supernate was diluted to 12 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 10 μM CaCl$_2$, and was loaded onto a 0.8-ml tuberculin syringe column of Ultrogel-HA equilibrated with the dilution buffer. The column was washed with this buffer and eluted with a gradient of sodium phosphate buffer, pH 7.0, from 0 to 0.15 M containing 10 μM CaCl$_2$. 0.6-ml fractions were collected. Active fractions were pooled, diluted 1:1 with 10 mM Bis Tris buffer, pH 7.0, and passed over a 0.1-ml tuberculin syringe column of DEAE-Sephacel equilibrated in 10 mM Bis Tris buffer, pH 7.0, as before. The column was eluted straight away with 0.3 ml of 0.75 M NaCl in 10 mM Bis Tris buffer, pH 7.0.

200SW Gel Filtration. 0.2 ml of the 0.75-M DEAE eluate was injected onto a Waters 200SW column equilibrated with PBS, using an FPLC unit (Pharmacia Fine Chemicals). 0.4-ml fractions were collected at a flow rate of 0.8 ml/min. The purified enzyme was stable for longer than 1 wk on ice. Protease purification was followed by 12% SDS-PAGE. Proteins were stained with silver (30).

Hemoglobinase Assay. Details of the assay have been described previously (16). Briefly, ^{3}H-leucine–labeled rat hemoglobin was incubated with enzyme for 30 min at 37°C in 100 mM citrate-phosphate buffer, pH 5.0, using a reaction volume of 30 μl. The incubation was stopped by addition of 1 ml of iced 6 M guanidine HCl, and the mixture was centrifuged through a Centricon 10 ultrafiltration device (Amicon Corp.). Fragments <10 kD were detected by assaying the filtrate for radioactivity. An inhibitor cocktail containing 1 mM PMSF, 1 mM 1,10-phenanthroline, and 100 μM leupeptin was added to routine assays. Omission of this mixture had no effect on total activity after the hydroxylapatite step.

Laser Desorption Mass Spectrometry. For this analysis, unlabeled human hemoglobin (4 μM) was incubated with purified enzyme as described for the hemoglobinase assay. Protease inhibitors were omitted, except as specifically noted. In some experiments, isolated human globin α chain was prepared from the same hemoglobin (31), and used as substrate. In other experiments, a similar amount of cathepsin D was substituted for the malaria enzyme. For analysis of hemoglobin degradation within digestive vacuoles, *P. falciparum* vacuoles were purified as previously described (16), and were subjected to two cycles of freeze-thaw using a dry ice-acetone bath. This preparation was then used as the enzyme source. Where noted, pepstatin was added to a final concentration of 10 μM. The reactions were stopped by rapid freezing in a dry ice-acetone bath. The samples were held at −70°C until used. Mass spectrometry was

performed as has been described (32–35). Briefly, the sample to be analyzed was mixed with laser desorption matrix material (saturated 3,5-dimethoxy,4-hydroxy-trans-cinnamic acid in 30% acetonitrile/0.1% trifluoroacetic acid), dried on the metal probe tip, and inserted into the spectrometer. The time of flight mass spectrometer consisted of a Lumonics HY400 neodymium/yttrium/aluminum garnet laser (Lumonics, Inc., Ontario, Canada), the third harmonic of which was focused onto the sample through a fused silica window. The product ions so formed were accelerated by a 30-kV static potential, and were detected at the end of a 2-m tube under vacuum, using a TR88288D transient recorder (TR88288D; LeCroy Research Systems Corp., Spring Valley, NY). Peak centroid determination and data reduction were carried out with a VAX workstation.

NH$_2$-Terminal Sequencing. Human hemoglobin was incubated with the purified enzyme for 30 min as above, and the products run on an 18% reducing SDS-PAGE gel (36). Proteins were blotted from the gel onto an Immobilon-P membrane electrophoretically (37). After visualization of the bands with Coomassie R250, the two peptide bands migrating faster than globin were excised and used for sequence analysis (37). 10 residues were determined for each peptide, allowing their positions in the hemoglobin molecule to be assigned. This experiment was repeated twice to confirm the results. For analysis of the aspartic hemoglobinase sequence, partially purified enzyme was subjected to electrophoresis on a 12% reducing SDS-PAGE gel, blotted, the 40-kD band excised, and sequenced.

Results

Aspartic Protease Purification and Properties. An aspartic protease was isolated from an extract of *P. falciparum* trophozoites by conventional chromatography on DEAE, hydroxylapatite, and gel filtration columns. The resultant peak of activity was 282-fold purified over the starting material (Table 1) and migrated as a single major band of M_r 40,000 by SDS-PAGE and gel filtration (Fig. 1). Using a saturating amount of substrate, the purified enzyme cleaved rat hemoglobin with a pH optimum of 4.5–5.0 (Fig. 2). The specific activity of the purified enzyme towards hemoglobin was approximately twice that of cathepsin D assayed under identical conditions at pH 4.5 (data not shown). The IC$_{50}$ for the inhibitor pepstatin was ~5 nM, indicating that the enzyme belongs to the aspartic protease family. PMSF (1 mM), o-phenanthroline (1 mM), and leupeptin (100 μM), inhibitors of serine, metallo-,

Table 1. *Purification of the Malaria Aspartic Hemoglobinase*

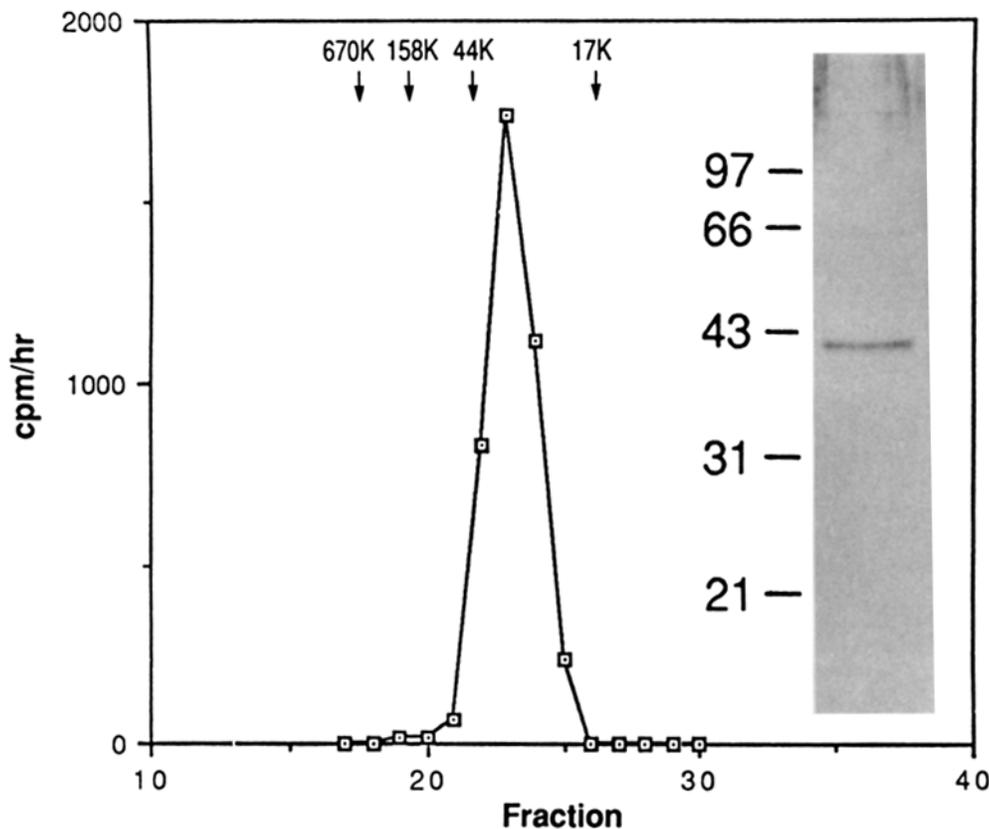| Step	Protein	Activity	Specific Activity	Purity	Yield
	mg	*nmol/min*	*nmol/min/mg*	*fold*	*%*
Triton extract	39.1	5.56	0.142	1	100
DEAE concentrate	2.13	6.92	3.25	23	124
pH 4.5 cut	0.375	2.61	6.96	49	47
Hydroxyl-apatite	0.041	0.97	23.66	167	17
200 SW	0.011	0.44	40.00	282	8

Figure 1. Gel filtration on a 200SW column. The hydroxylapatite-purified enzyme preparation was injected onto a 200SW column (Waters Instruments Inc.) using a FPLC apparatus (Pharmacia Fine Chemicals). The column was eluted isocratically with PBS. 0.4-ml fractions were collected and assayed for aspartic protease activity as described in Materials and Methods. The column was calibrated before and after the sample run with molecular weight standards (Bio-Rad Laboratories). Peak absorbance of each standard at A_{280} is shown by arrows at the top of the figure. Inset: SDS-PAGE analysis. A 50-μl aliquot of the gel filtration peak was subject to electrophoresis on a 12% SDS-PAGE gel under reducing conditions. The gel was developed by silver staining.

and cysteine proteases, respectively, had no effect on the reaction. NH$_2$-Terminal sequencing revealed 9 of 22 residues identical to the most specific mammalian aspartic protease renin. This is greater homology than observed with most fungal proteases in similar comparisons, equivalent to the homology between mammalian proteases, and much better than the homology of the retroviral proteases to mammalian or plasmodial aspartic proteases (Table 2).

Enzyme Specificity. To define the specificity of the *Plasmodium* aspartic protease towards its natural substrate hemoglobin, the enzyme was incubated with substrate for varying periods of time. Reactions were stopped by flash freezing and analyzed promptly by laser desorption mass spectrometry. This technique yields the precise molecular mass of peptides (34, 35), which, when derived from a protein of known sequence, allows the position of the peptides in that protein to be determined. Fig. 3 shows a time course of the reaction between hemoglobin and the protease. At early times, two peptide products are apparent, with mass/charge (m/z) of 3,473 and 11,670 daltons. These molecular masses correspond precisely to those predicted for hemoglobin α chain peptides 1–33 and 34–141. It should be noted that this technique is not quantitative; there is variability in ion yield between samples and between different peptides in the same sample (34). With increasing time, the two primary cleavage products accumulated, and other fragments appeared with masses and assignments summarized in Table 3. At late time points the α34–141 peptide signal diminished, while that of α1–33 persisted. Note that all the secondary cleavage products are fragments of the original α1–33 and 34–141 peptides. Cleavage

within these sequences occurred only after the initial 33–34 peptide bond scission. No fragments from the β chain were detected. After a 30-min incubation in the presence of the inhibitor pepstatin, a profile indistinguishable from the zero time point was obtained.

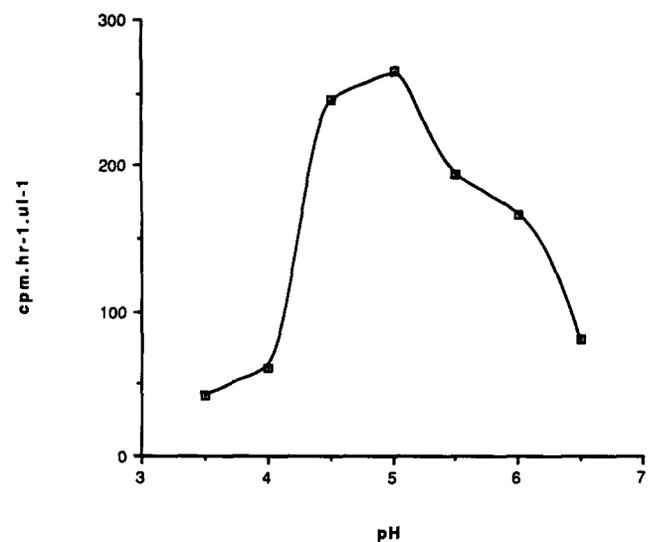The ability of the enzyme to cleave isolated α chains was

Figure 2. Influence of pH on activity of the purified protease. Enzyme activity was assayed using the standard Centricon 10 assay with citrate-phosphate buffer at varied pH. Assays were performed at a saturating substrate concentration of 7.5 μM.

Table 2. *NH₂-terminal Homology to Human Renin*

	Sequence	No. identical to renin
Renin	T T S S V I L T N Y M D T Q Y Y G E I G I G	
Cathespin D	G P I P E V L K N Y M D A Q Y Y G E I G I G	14/22
Cathepsin E	Q S A K E P L I N Y L D M E Y F G T I S I G	9/22
Pepsin	L V D E Q P L E N Y L D M E Y F G T I G I G	9/22
Gastricsin	S V T Y E P M A Y M D A A Y F G E I S I G	9/21
HIV	P Q I T L W Q R P L V T I K I G	3/16
RSV	L A M T M E H K D R P L V R V I L T N T G	1/21
Penicillopepsin	G V A T N T P T A N D E E Y I T P V T I G	4/21
Endothiapepsin	G S A T T T P I D S L D D A Y I T P V Q I G	4/22
Rhizopuspepsin	G T V P M T D Y G N D V E Y Y G Q V T I G	7/21
Yeast proteinase A	G G M D V P L T N Y L N A Q Y Y T D I T L G	9/22
Aspartic hemoglobinase	A G D S V T L N D V A N V M Y Y G E A Q I G	9/22

Malaria aspartic hemoglobinase was isolated by SDS-PAGE, transferred to a PVDF membrane, excised, and sequenced as described in experimental procedures. All other sequences are as referenced (46–48).
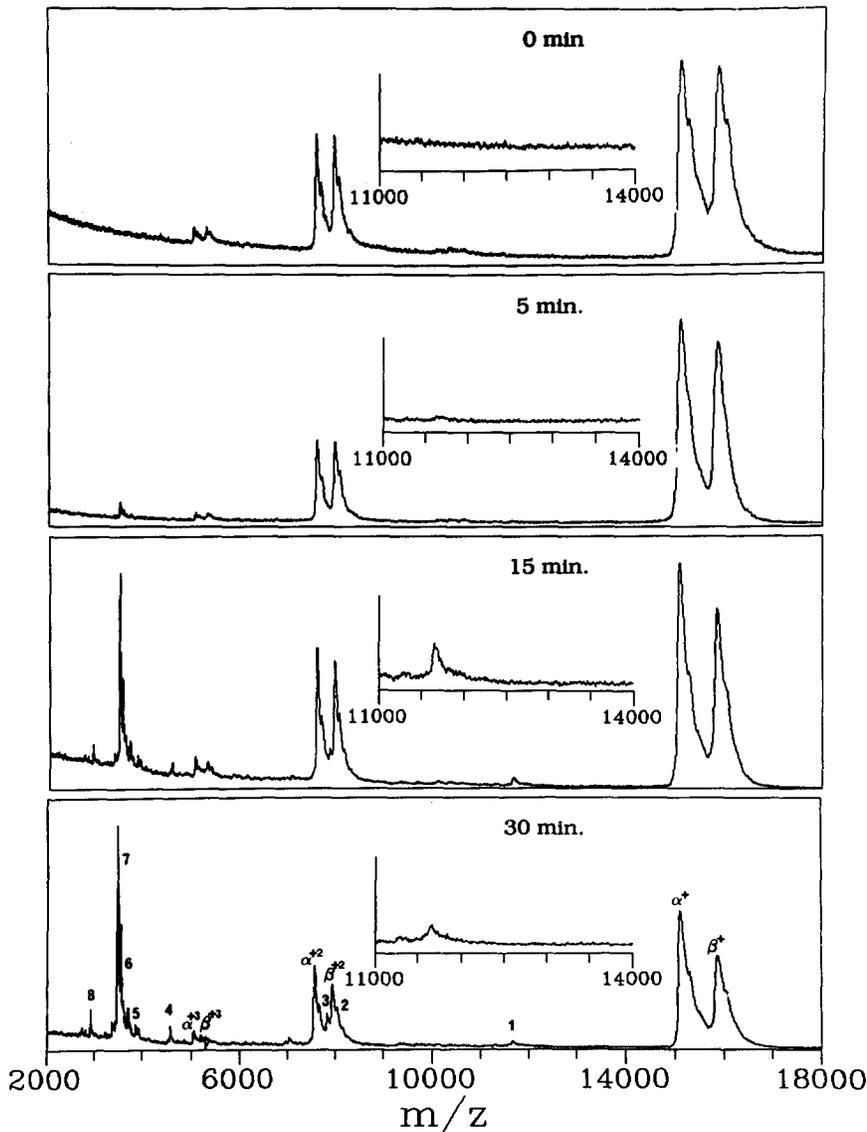
Figure 3. Time course of hemoglobin degradation measured by laser desorption mass spectrometry. Purified malaria aspartic protease (activity, 0.12 pmol/min) was incubated with 4 μM human hemoglobin at pH 5.0, for the times indicated. (a) 0 time (similar to incubation with 10 μM pepstatin present); (b) 5 min; (c) 15 min; (d) 30 min. Axes are signal intensity vs. mass/charge (m/z) ratio. Peaks in the bottom panel are numbered as assigned in Table 2. α and β denote peaks corresponding to the molecular ions of the globin chains and their double and triple charged species. Inset: ×4 enlargement of the 11,000–14,000 region.

Table 3. *Assignment of Mass Spectrometry Peaks*

Peak	Mass (measured)	Assignment	Mass (predicted)
	daltons		*daltons*
1	11,670	34–141	11,670
2	8,144	34–108	8,144
3	7,832	34–105	7,832
4	4,564	64–105	4,563
5	3,855	106–141	3,855
6	3,542	109–141	3,543
7	3,473	1–33	3,474
8	2,910	1–29	2,911

Predicted masses were calculated by summation of amino acid residues in the assigned peptides.

determined in similar fashion (Fig. 4). The enzyme was substantially more active with this substrate. By 5 min of incubation, most of the starting material was degraded. Again, prominent peptides resulting from cleavage between amino acids 33 and 34 were seen. Interestingly, a peak of m/z =

11,307 daltons, corresponding to α1–105 was detected. The enzyme can therefore make an initial cleavage at the 105–106 bond in an isolated α chain, but can only cleave there after the initial 33–34 scission in native hemoglobin. After 30 min, the peak corresponding to α1–33 was the only fragment remaining. All other pieces were degraded to a size <2 kD, the lower limit of detection of this method.

To confirm the assignment of fragments above, the reaction products were fractionated by SDS-PAGE (Fig. 5), transferred to a PVDF membrane, and stained with Coomassie blue. Product bands indicated with arrows were excised from the membrane, and NH₂-terminal sequencing of the first 10 amino acids of each was performed. Product 1 yielded a sequence of L-S-F-P-T-T-K-T-Y-F, while product 2 had the sequence V-L-S-P-A-D-K-T-N-V. These are the predicted NH₂-terminal analyses for fragments α34–141 and 1–33, respectively.

Mass spectrometry of the mammalian lysosomal protease cathepsin D incubated with hemoglobin under similar conditions was also performed. 27 substantial peaks, corresponding to a variety of cleavage sites, were apparent. There was a minor peak corresponding to α1–33, though no 34–141 signal. β chain fragments were also present (data not shown).

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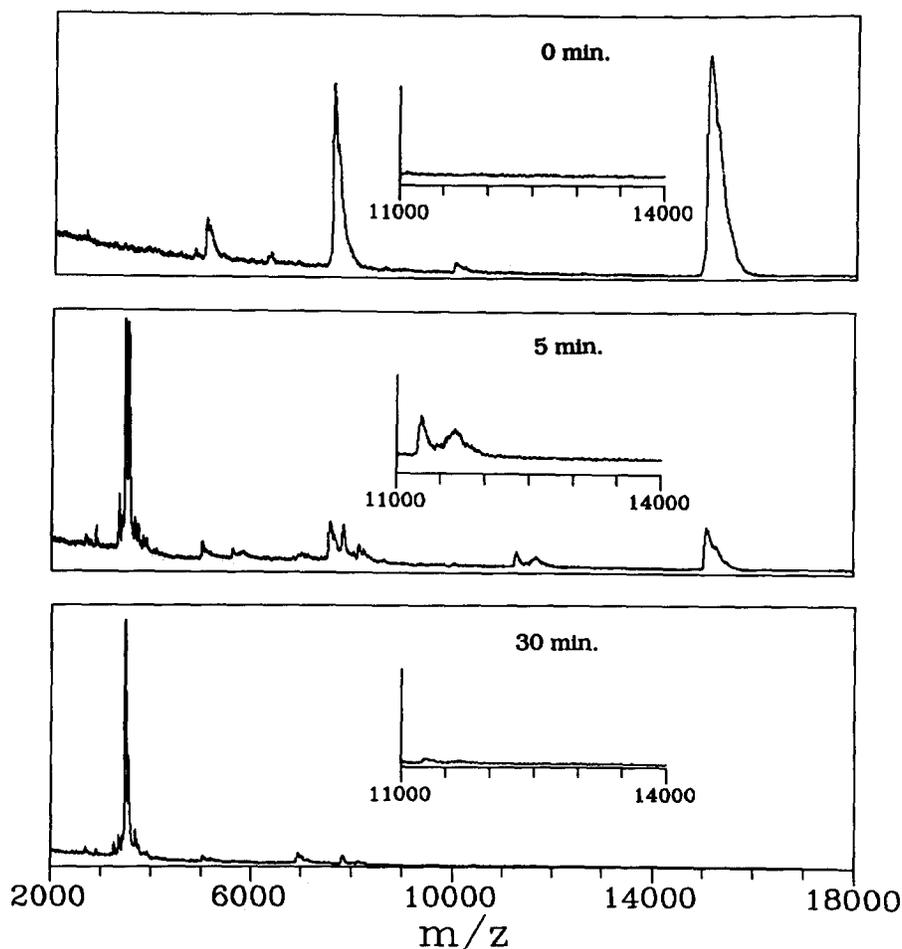
Figure 4. α globin degradation measured by laser desorption mass spectrometry. Conditions were similar to those described in Fig. 3. (*a*) 0 time (similar to 30-min incubation with 10 μM pepstatin present); (*b*) 5 min; (*c*) 30 min. There is an extra peak at m/z = 11,307, not seen in the incubations with native hemoglobin. Inset: ×2.5 enlargement of the 11,000–14,000 region.

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Figure 4. α globin degradation measured by laser desorption mass spectrometry. Conditions were similar to those described in Fig. 3. (*a*) 0 time (similar to 30-min incubation with 10 μM pepstatin present); (*b*) 5 min; (*c*) 30 min. There is an extra peak at m/z = 11,307, not seen in the incubations with native hemoglobin. Inset: ×2.5 enlargement of the 11,000–14,000 region.
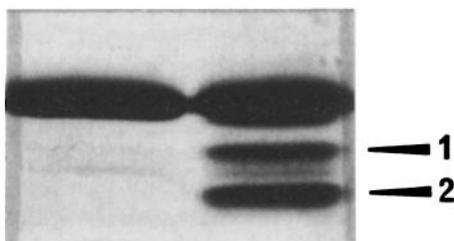
Figure 5. SDS-PAGE of proteolysis products. Right lane: Hemoglobin was incubated with malaria aspartic protease for 30 min as in Fig. 3. An 8–25% gradient gel was run under denaturing, reducing conditions on a Pharmacia Phastgel system (Pharmacia Fine Chemicals). The gel was developed by silver staining. Arrows 1 and 2 mark the two peptide fragments that were transferred to PVDF from a similar but larger gel, excised, and sequenced. (*Left lane*) A similar incubation carried out in the presence of 10 μM pepstatin.

vacuoles, vacuoles were purified by differential centrifugation and density gradient fractionation, as previously described (16). The vacuoles were permeabilized by freeze-thaw and incubated with exogenous hemoglobin. After 30 min, the incubation products were analyzed by mass spectrometry (Fig. 6). Fragments corresponding to peptides α1–33 and 34–141 were the predominant species seen. The hydrolysis was abolished by inclusion of pepstatin in the incubation mixture (data not shown).

Discussion

Our previous studies have shown that hemoglobin proteolysis in the digestive vacuoles of malaria parasites is an or-

dered process. An aspartic protease activity is required to act on intact hemoglobin before further degradation by other proteases can occur (16). Aspartic protease activities have previously been partially purified from *P. lophurae* (23) and *P. falciparum* (38), although the function and intracellular location of these enzymes has not been defined. In this paper, we describe an analysis of the cellular function of the malaria aspartic hemoglobinase that initiates hemoglobin degradation. The purified enzyme differs from the previously described activities in terms of molecular weight and pH optimum, and as discussed below has an interesting specificity dependent on the tertiary structure of its substrate. The assignment of this protein to the aspartic protease family is a tentative one, and is based on inhibition by pepstatin, lack of inhibition by other classes of protease inhibitors, and NH₂-terminal homology with known aspartic proteases. The 40% homology to renin in a poorly conserved region of the aspartic protease molecule is quite good, and suggests that the malaria enzyme is more closely related to its mammalian host enzymes than to other microorganisms, which have minimal homology to the malaria hemoglobinase in the region at issue. Further data pertaining to the evolutionary relationships of this protease, and confirming its aspartic mechanism, will come from cloning and expression studies currently in progress.

Laser desorption mass spectrometry was used to identify the peptide fragments of hemoglobin generated by the aspartic hemoglobinase. This new method is a powerful tool for defining the proteolytic fragments of a protein of known primary structure. It is more sensitive than NH₂-terminal sequencing, much more rapid, and does not depend on prior separation of the peptides generated. It is accurate to ± 0.01%,
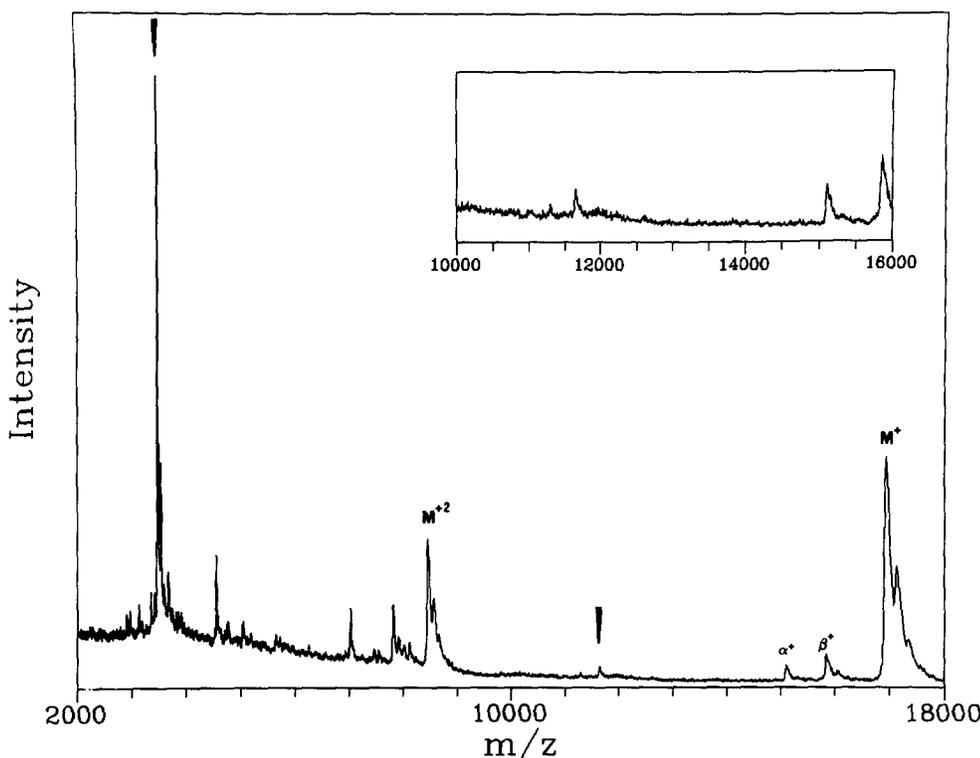

Figure 6. Vacuolar hemoglobin degradation. Permeabilized vacuoles were incubated with human hemoglobin for 30 min at pH 5.0. Incubation products were analyzed by laser desorption mass spectrometry as detailed in Materials and Methods. Arrows mark the peaks at m/z = 3473 and 11,670, corresponding to peptides α1–33 and α34–141. Peaks corresponding to the molecular ions of myoglobin (*M*) used as a calibrant, as well as α and β globin, are labeled. Inset: ×2.5 enlargement of the 10,000–16,000 region to show the peak at 11,670 more clearly.

i.e., to within one mass unit in the molecular mass range up to ∼10 kD (34). This allows precise assignment of peptide sequence using a simple algorithm to sum the molecular weights of consecutive amino acid residues of the protein sequence. One other useful property of the analysis is that it is exquisitely sensitive to the presence of contaminating proteases in the incubation mixture. Our partially purified enzyme preparations were capable of making similar hemoglobin cleavages as the purified preparation, but additionally cleaved residues 1 and 2 from the α and β globin subunits, and residue 33 from the α1–33 peptide. These extraneous hydrolyses were due to pepstatin-insensitive exopeptidases. Further purification removed these minor contaminants (data not shown).

The hemoglobinase makes an initial attack between residues α33Phe and 34Leu, and then can make subsequent cleavages at several other sites. The 33–34 bond is located in the hinge region of hemoglobin. When oxygen binds to a hemoglobin tetramer, one α/β dimer rotates ∼15 degrees around the other (39). Extensive salt bridges between subunits are broken, but in the hinge region, intermolecular contacts are maintained to keep the tetramer together (40). It is likely that a cleavage in this area would cause unraveling of the molecule, exposing other sites for proteolytic attack by the aspartic and other proteases, leading to further degradation. This region of hemoglobin is highly conserved between vertebrate species (41), and no human variants with a homozygous mutation in this region have been described (42). It appears that the malaria parasite has evolved to take full advantage of the structural features of hemoglobin.

The difference in specificity of the aspartic hemoglobinase against native compared with fragmented or less folded hemoglobin is striking. In the intact molecule the α33–34 site is kinetically favored. No fragment extending from the NH₂ terminus (residue 1) to a point beyond residue 33 is seen. Once peptides 1–33 and 34–141 are generated, the protease can go further and will cleave 34–141 into several fragments. When isolated α chains, which have much less tertiary structure than native hemoglobin (43), are used as substrate, the same 33–34 cleavage is made, but other breaks appear at the same time. Fragment 1–105 is prominent early on. This peptide arises from cleavage at 105–106 without previous cleavage at 33–34. The aspartic protease can therefore recognize the 105–106 peptide bond as an initial attack site in the loosely folded α chain, but not in native hemoglobin. As might be expected from this, degradation is much more rapid with the isolated α chain.

No cleavage of the β chain was detected with hemoglobin as substrate for the purified protease. This suggests the existence of a β chain hemoglobinase in the *Plasmodium* digestive vacuole. Alternatively, the α chain–degrading enzyme may function much more slowly on the β chain or require different conditions than those in our incubations. Finally, it is possible that the β chain does not get degraded by the parasite. It may be noted in Fig. 3 that the ratios of α to β chain seem to change somewhat during the incubation. This is within the variability of the technique used, and was not reproducible in other experiments. During the incubation, 2% of the substrate was cleaved (quantitated by centricon assay). Loss of substrate is therefore not quantifiable by the laser desorption mass spectroscopy technique. We cannot fully rule out some degradation of the β chain to fragments too small to detect.

That the enzyme characterized here is the aspartic protease responsible for the key first step of hemoglobinolysis in the vacuole is suggested by several observations. Most importantly, an extract of the purified vacuoles is able to degrade hemoglobin, generating primary fragments identical to those seen with the isolated aspartic hemoglobinase, in a pepstatin-inhibitable reaction. Secondly, the pH of the digestive vacuole, which has been measured to be about pH 5 (44, 45), is optimal to support activity of the aspartic hemoglobinase. Lastly, SDS-PAGE of the purified vacuoles reveals a protein band at M_r 40,000 (data not shown), though we cannot be confident at this point that this band is the aspartic protease. Confirmation of the vacuolar location of this enzyme will be achieved once immunolocalization can be performed.

Previously, we proposed that hemoglobin degradation in the malaria parasite digestive vacuole is a highly ordered process (16). This paper describes the characterization of an enzyme that appears to play a key initial role in this catabolic pathway. We have studied the protease's specificity with its natural substrate. The enzyme is highly site selective when confronted with folded protein, and not nearly as selective when given unfolded or fragmented protein. The data provide insight into the biochemical adaptation of the malaria organisms for survival in the host erythrocyte. Further study of the malaria aspartic hemoglobinase should afford a better understanding of protease-substrate interactions, and may allow rational design of a new class of antimalarial agents.

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