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# Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase

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**Intraerythrocytic malaria parasites rapidly degrade virtually all of the host cell hemoglobin. We have cloned the gene for an aspartic hemoglobinase that initiates the hemoglobin degradation pathway in *Plasmodium falciparum*. It encodes a protein with 35% homology to human renin and cathepsin D, but has an unusually long pro-piece that includes a putative membrane spanning anchor. Immunolocalization studies place the enzyme in the digestive vacuole and throughout the hemoglobin ingestion pathway, suggesting an unusual protein targeting route. A peptidomimetic inhibitor selectively blocks the aspartic hemoglobinase, prevents hemoglobin degradation and kills the organism. We conclude that *Plasmodium* hemoglobin catabolism is a prime target for antimalarial chemotherapy and have identified a lead compound towards this goal.**

*Key words:* aspartic hemoglobinase/*Plasmodium*/protease/targeting/vacuole

## Introduction

Malaria afflicts several hundred million people world-wide, killing almost 2 million per year, mostly children (Sturchler, 1989). Antimalarial prophylaxis and therapy are no longer straightforward because drug resistance has become rampant (Wellems, 1991). Our antimalarial armamentarium is in danger of becoming obsolete for the most deadly malaria species, *Plasmodium falciparum*. Thus we have a desperate need for new chemotherapeutic agents. Chloroquine, when it is not pumped out of the cell by resistant organisms, is a highly potent antimalarial (Krogstad *et al.*, 1987). Its mechanism of action is controversial, but very likely involves disrupting the metabolism of the acidic digestive vacuole in which it concentrates and in which hemoglobin degradation occurs (reviewed by Goldberg, 1993). It would be desirable to have a new antimalarial that also disrupts digestive vacuole function, through a different mode of action.

During the intraerythrocytic stage of infection the malaria parasite avidly consumes hemoglobin as a source of nutrients (Scheibel and Sherman, 1988). Host cell cytoplasm is ingested by the cytostome, a specialized invagination at the parasite surface, from which vesicles pinch off to be transported to an acidic proteolytic compartment, the digestive vacuole. Here hemoglobin is catabolized and the toxic heme moiety is sequestered in crystalline form as hemozoin. By the end of the trophozoite stage of the cycle, most of the host cell hemoglobin has been consumed (Ball *et al.*, 1948; Morrison and Jeskey, 1948). This is a substantial accomplishment since hemoglobin comprises 95% of the red blood cell's cytosolic protein and the trophozoite stage lasts only a few hours. It is likely that several enzymes participate in hemoglobin digestion. Indeed, over the past several years hemoglobinase activities have been ascribed to various members of the cysteine and aspartic protease families (Vander Jagt *et al.*, 1987, 1992; Rosenthal *et al.*, 1988; Goldberg, 1992; Rosenthal and Nelson, 1992).

A method for isolating digestive vacuoles has recently been developed that permits analysis of vacuolar enzymes with minimal contamination by extravacuolar proteases (Goldberg *et al.*, 1990). Cleavage of native hemoglobin was studied using a digestive vacuole extract in the presence of a variety of enzyme inhibitors to determine the classes of protease involved in hemoglobin catabolism. It was found, unexpectedly, that the aspartic protease inhibitor pepstatin blocked the initial events in hemoglobin digestion. A vacuolar aspartic hemoglobinase has been purified to homogeneity and characterized (Goldberg *et al.*, 1991). The enzyme recognizes hemoglobin, making a single initial cleavage in the $\alpha$ chain between Phe33 and Leu34. This site is in the hinge region of hemoglobin which is involved in maintaining the integrity of the molecule as it binds oxygen (Perutz, 1987). It is a highly conserved stretch of amino acids in all vertebrate hemoglobins (Dickerson and Geis, 1983). None of the hundreds of characterized human variant hemoglobins has a homozygous defect in this region (Beutler, 1990), consistent with the notion that the parasite has found a way to attack the hemoglobin molecule at a site that cannot be altered without deleterious consequences to the host (Goldberg *et al.*, 1991). Cleavage by the hemoglobinase in this hinge region appears to unravel the substrate, after which the enzyme can make several secondary cleavages. These results led to the suggestion that hemoglobin breakdown is an ordered process requiring the specific action of an aspartic protease to initiate hemoglobin degradation, thereby making it accessible to other digestive vacuole enzymes (Goldberg *et al.*, 1990). We have now cloned cDNA encoding this aspartic hemoglobinase and have characterized its unusual features. We report that inhibition of this enzyme blocks the essential pathway of hemoglobin degradation, leading to death of the organism.

## Results

### *Cloning and analysis of the aspartic hemoglobinase cDNA*

The catalytic site of all known aspartic proteases is formed by folding two separate domains together to yield active



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enzyme (Tang and Wong, 1987). The active site amino acid sequences are highly conserved. We generated best-guess oligonucleotides derived from first active site consensus sequence and isolated aspartic hemoglobinase N-terminal sequence (Goldberg *et al.*, 1991). The oligonucleotides were used in a polymerase chain reaction (PCR) with *P.falciparum* DNA as template. A PCR product of 105 bp, the predicted size, was generated. Its sequence matched the known N-terminal sequence from isolated hemoglobinase and provided new sequence for subsequent screening. A λZap II genomic library was constructed using DNA from *P.falciparum* and screened by anchored PCR using authentic aspartic hemoglobinase sequence and λZap vector sequence. A 607 bp product was obtained (clone AP1) that contained about two-thirds of the mature coding sequence expected of a typical aspartic protease, including both active sites.

AP1 was used to screen cDNA libraries derived from both ring and trophozoite stage parasites of *P.falciparum*. A single clone with a 2.6 kb insert was obtained from each library. Restriction mapping of the two clones suggested that they are very similar or identical (not shown). The nucleotide sequence and predicted amino acid sequence for the trophozoite clone is shown in Figure 1A. The cDNA contains an open reading frame of 1356 nucleotides as well as 1200 bases of 3'-untranslated DNA. Northern blot analysis of mRNA derived from asynchronous cultures shows a band of ~3.0 kb (Figure 1B) suggesting that the clones are full-length, or nearly so. The hemoglobinase cDNA has a short poly(A) tail with a putative polyadenylation signal 45 bp upstream. The initiator methionine was predicted to be the first ATG in the sequence. The region surrounding this methionine matches the consensus eukaryotic initiation sequence (Kozak, 1987) and other initiation sequences from *Plasmodium* (Saul and Battistutta, 1990; Robson and Jennings, 1991). In addition, this methionine marks a shift in AT content from 97% upstream to 72% downstream. Typically 5'-untranslated regions in *Plasmodium* are much more AT-rich than coding regions (Weber, 1988). The 5' end of the hemoglobinase cDNA starts with a stretch of 49 T residues, 114 bp from the initiation codon. An oligothymidylate tract thought to be involved in initiation of transcription has been identified in the gp195 gene of *P.falciparum* (Myler, 1990), and may serve a similar function here.

*Plasmodium falciparum* genomic DNA was digested with several restriction enzymes, blotted to nylon membrane and hybridized with radiolabeled probes corresponding to the mature protein N-terminus, first active site consensus sequence and AP1. The enzymes chosen do not cut within the probe sequences. A single band in each lane was recognized by probe to the N-terminus (Figure 1C). The same band was recognized by the conserved active site probe in addition to a second band for both the *Hae*III and *Eco*RI digests (Figure 1D), suggesting that *P.falciparum* has two aspartic protease genes. A similar pattern was observed using the 607 bp AP1 probe but an additional band was detected in the *Eco*RI digest (not shown), raising the possibility of a third aspartic protease gene.

### *A coding region with an unusual pro-piece and active site changes*

The cDNA encodes a protein of 453 amino acids with a molecular weight of 51 428 Daltons. The N-terminal sequence of the isolated mature protein (Goldberg *et al.*, 1991) is identical to the predicted amino acid sequence from the cDNA starting at amino acid 125. This indicates that a 124 amino acid pro-piece is cleaved at an alanine residue to yield a mature protein of 36 912 Daltons. This result contrasts with other known aspartic proteases that have pro-pieces of <50 residues (Tang and Wong, 1987). Interestingly, a typical signal sequence is absent from the hemoglobinase. Instead, 36 amino acids from the initiator methionine there is a stretch of 21 hydrophobic residues. The hydrophobicity plot of the pro-enzyme (Figure 2A) is characteristic of an integral membrane protein.

When the sequence of the mature hemoglobinase is aligned with those of mammalian aspartic proteases for which the protein structure has been established (Figure 2B), 30–35% identity between the hemoglobinase and each of the others is apparent. This is highest for the processing proteases renin and cathepsin D and lower for the digestive proteases represented here by pepsin. The two active site sequences bear extensive homology to those of other aspartic proteases. The second active site triad is DSG instead of the much more common DTG; this serine has been found in several other aspartic proteases such as Rous sarcoma virus (Rao *et al.*, 1991) and *Saccharomyces* BAR1 protease (MacKay *et al.*, 1988). The sequence of the flap (loop 2) that folds over the substrate pocket is quite homologous to renin and cathepsin D, while loop 4, which extends into the binding pocket to interact with the S3 substrate site (Metcalf and Fusek, 1993), has two extra amino acids not found in other aspartic proteases. In the middle of this sequence is a glutamate, which might well be in a position to stabilize the basic amino acid found at the S3 position of the hemoglobin cleavage sites of this enzyme (Goldberg *et al.*, 1991). Other unusual features of the hemoglobinase include a truncated loop 5 that is missing a pair of cysteines found in all mammalian aspartic proteases, and a KKKL extension at the C-terminus. Similar to pepsin, there is only one proline in place of the polyproline loop of renin and cathepsin D (Metcalf and Fusek, 1993).

### *Aspartic hemoglobinase is located in the digestive vacuole and along the hemoglobin ingestion pathway*

We prepared two affinity-purified polyclonal antibodies, one to a peptide consisting of the first 22 amino acids of the mature N-terminus of the aspartic hemoglobinase (Ab467) and the second to the first 224 amino acids of cloned mature hemoglobinase (Ab574). When whole trophozoite extract was analyzed by Western blot, a doublet just below 40 kDa was observed using Ab574 (Figure 3A, lane 1). Only the top band was recognized by Ab467 (lane 2). To define the proteins recognized by the two antibodies, we fractionated digestive vacuole extract by HPLC chromatography (see Materials and methods). Two aspartic protease activities were separated on a hydroxylapatite column (Figure 3B) and then purified further. N-terminal amino acid sequencing confirmed that peak 2 corresponds to the aspartic hemoglobinase previously described (Goldberg *et al.*, 1991). We refer to this enzyme as aspartic hemoglobinase I. The peak 1 N-terminal sequence matched predicted amino acid sequence from a recently cloned second *P.falciparum* aspartic protease gene (J.Dame, personal communication). We call this enzyme aspartic hemoglobinase II. Characterization of the purified aspartic proteases will be described elsewhere (I.Y.Gluzman, S.E.Francis, A.Oksman, C.E.Smith, K.L.Doffin and D.E.Goldberg, submitted). Ab467 recognized purified aspartic hemoglobinase I but not purified

**A**

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      tttttttttttttttttttttttttttttttttttttttttttttttttattt tattttatttattttattttattttatttttttttttcctttttgttttatttataaag  113
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 M  A  L  S  I  K  E  D  F  S  S  A  F  A  K  N  E  S  A  V   N  S  S  T  F  N  N  N  M  K  T  W  K  I  Q  K  R  F  Q  I    40

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 L  Y  V  F  F  F  L  L  I  T  G  A  L  F  Y  Y  L  I  D  N   V  L  F  P  K  N  K  K  I  N  E  I  M  N  T  S  K  H  V  I    80

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 I  G  F  S  I  E  N  S  H  D  R  I  M  K  T  V  K  Q  H  R   L  K  N  Y  I  K  E  S  L  K  F  F  K  T  G  L  T  Q  K  P   120

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 H  L  G  N  A  G  D  S  V  T  L  N  D  V  A  N  V  M  Y  Y   G  E  A  Q  I  G  D  N  K  Q  K  F  A  F  I  F  D  T  G  S   160

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 A  N  L  W  V  P  S  A  Q  C  N  T  I  G  C  K  T  K  N  L   Y  D  S  N  K  S  K  T  Y  E  K  D  G  T  K  V  E  M  N  Y   200

GTATCTGGAACAGTAAGCGGATTTTTTAGTAAAGATATAGTTACTATAGCAAATTTATCA TTTCCATATAAATTTATTGAAGTTACAGATACCAATGGTTTCGAACCAGCTTATACCTTA  833
 V  S  G  T  V  S  G  F  F  S  K  D  I  V  T  I  A  N  L  S   F  P  Y  K  F  I  E  V  T  D  T  N  G  F  E  P  A  Y  T  L   240

GGACAATTTGATGGTATAGTAGGTTTAGGATGGAAAGATTTGTCTATAGGTTCAGTAGAT CCTGTTGTTGTCGAATTAAAAAATCAAAATAAAATCGAACAAGCCGTTTTTACCTTTTAT  953
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 H  T  V  G  F  A  L  A  K  K  K  L OCH                                                                                       453

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XbaI HaeIII EcoRI XbaI HaeIII EcoRI

B

7.5 –
4.4 –
2.4 –
1.4 –

– 23.1 –
– 9.4 –
– 6.6 –
– 4.4 –
– 2.3 –
– 2.0 –
– 0.5 –

C D

**Fig. 1.** Nucleotide sequence, Northern and Southern analysis of aspartic hemoglobinase. (**A**) Nucleotide and predicted amino acid sequence of the trophozoite cDNA clone. The beginning of the mature sequence is in bold. The polyadenylation signal is underlined as are the positions of oligonucleotides DG1 and DG3 for the initial PCR reaction. The AP1 insert spans nucleotides 548–1154. (**B**) Northern blot analysis using total cellular RNA. RNA was isolated from an asynchronous culture of *P.falciparum* clone HB3. Total RNA (10 μg) was electrophoresed, blotted onto a nylon membrane and probed with radiolabeled AP1. (**C** and **D**) Southern analysis of *P.falciparum* genomic DNA. DNA (10 μg) was cut with *Xba*I, *Hae*III and *Eco*RI. Digested DNA was electrophoresed on a 0.7% agarose gel, blotted onto nylon membrane and probed with end-labeled oligonucleotide DG1, a best-guess oligonucleotide to the N-terminus of isolated aspartic hemoglobinase (C) or with DG3, a best-guess oligonucleotide to the consensus sequence for the first active site of known aspartic proteases (D).

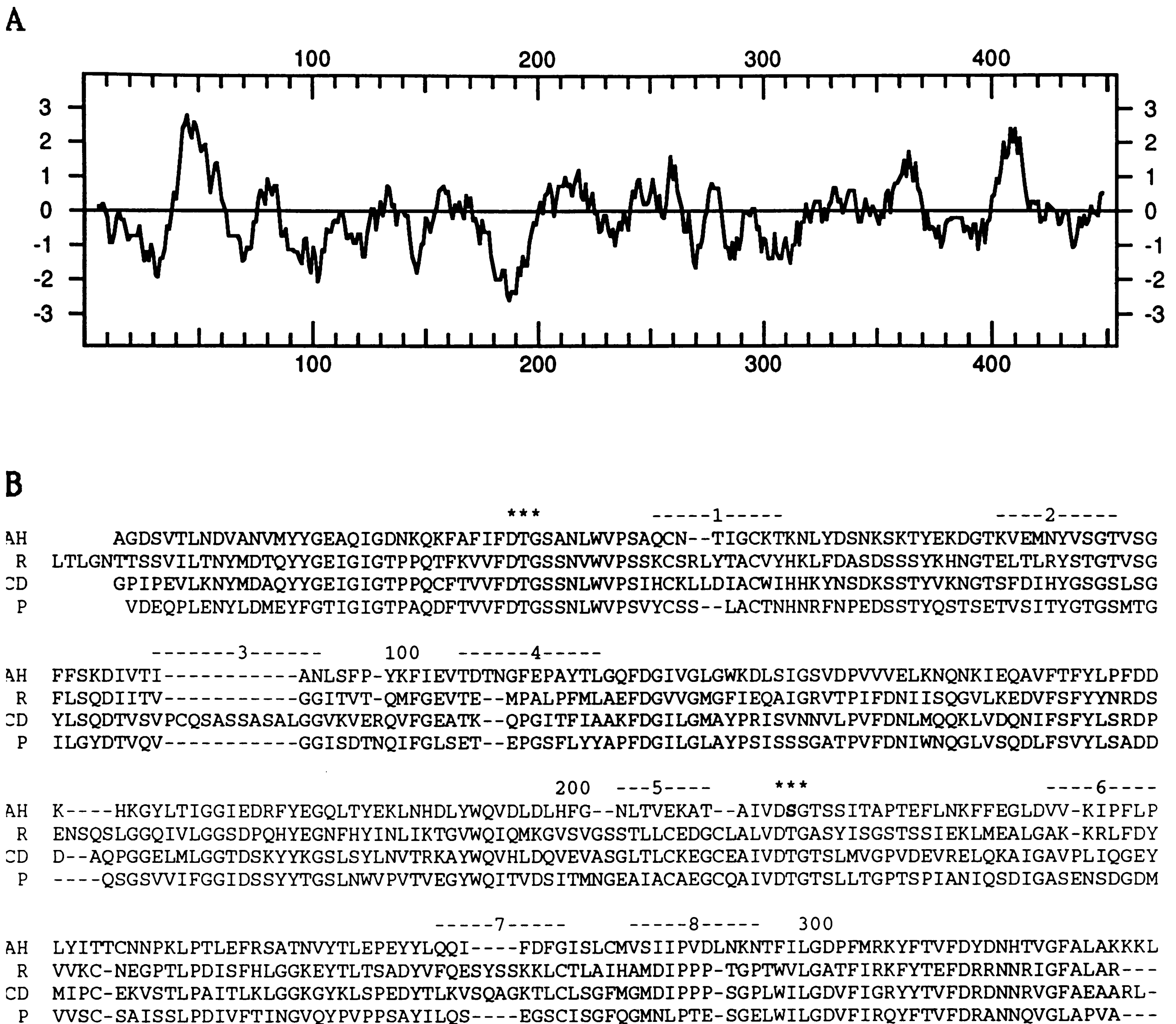


**Fig. 2.** Comparative analysis of aspartic hemoglobinase amino acid sequence. (**A**) Hydropathy plot of predicted amino acid sequence computed with Kyte–Doolittle algorithm (Kyte and Doolittle, 1982). (**B**) Amino acid sequence comparison between aspartic hemoglobinase I (AH), human cathepsin D (CD), human renin (R) and human pepsin (P). Sequences from Blundell *et al.* (1990). Only the mature protein sequences are shown. Regions corresponding to loops characteristic of aspartic proteases are demarcated. Active site triads are marked with asterisks.

aspartic hemoglobinase II, while Ab574 recognized both isolated enzymes (not shown). Aspartic hemoglobinase II has 73% identity to aspartic hemoglobinase I though only 40% homology at the mature protein N-terminus (J.Dame, personal communication).

In order to localize the aspartic proteases in the cell, immunofluorescence studies of *P.falciparum* infected erythrocytes were performed. Ab467 failed to recognize protein in fixed parasites. Using Ab574, intense signal was observed associated with the digestive vacuole of trophozoites (Figure 3D). This is consistent with detection of the aspartic proteases in purified digestive vacuoles and with the proposed role of the aspartic hemoglobinase in hemoglobin degradation. Signal is also associated with vesicles near the parasite surface. No cross-reactivity with numerous uninfected erythrocytes in the fields was seen.

Immunoelectron microscopy of intraerythrocytic trophozoites labelled with Ab574 (Figure 4) was in agreement with the immunofluorescence pattern. Although the level of labeling was low, several intracellular organelles labeled consistently and specifically with this antibody. Controls conducted with pre-immune rabbit serum were negative (not shown). The antibody labeled structures known to be involved in the internalization and processing of host hemoglobin including hemoglobin transport vesicles (A and E), the cytostome (C and D) and the digestive vacuole, where the greatest accumulation was in association with the hemozoin crystals (A and F). In addition, the antibody decorated small vesicular structures in the vicinity of the parasite surface membrane complex (C), as well as the membranes themselves (A and C). Aspartic protease was associated with the peripheral membranes of the parasite surface, but was mostly lumenal in hemoglobin transport vesicles and digestive vacuoles.

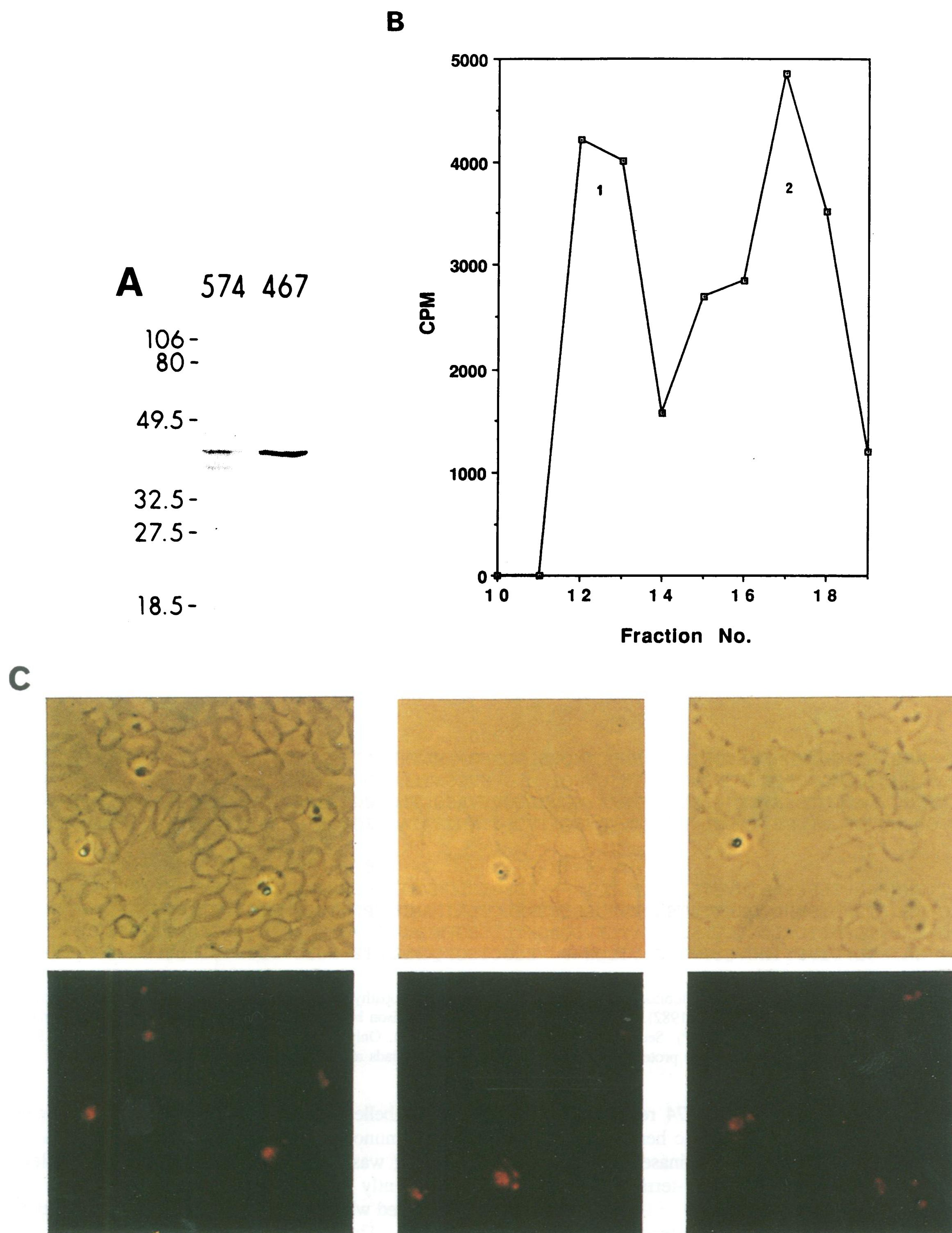


**Fig. 3.** Antibody characterization and immunolocalization. (**A**) Western blot of trophozoite extract probed with affinity purified anti-peptide 1–22 (Ab467), right and anti-expression fragment 1–224 (Ab574), left. (**B**) Separation of two vacuolar aspartic proteases by hydroxylapatite HPLC. Vacuole extract was partially purified by DEAE chromatography, fractionated on hydroxylapatite, and assayed for aspartic protease activity by the globin TCA assay. (**C**) Rhodamine immunofluoresence of trophozoite smears using Ab574. Shown are three separate fields. Top panels: phase-contrast. The digestive vacuole is clearly discerned by the presence of hemozoin pigment using phase-contrast optics; bottom panels: fluorescence. Control slides with pre-immune serum or secondary antibody alone were negative.

### *A peptidomimetic inhibits the aspartic hemoglobinase, blocks hemoglobin degradation and kills malaria parasites*

Non-hydrolyzable peptide-like agents have proven useful for *in vivo* inhibition of proteases such as the HIV aspartic protease (Ashorn *et al.*, 1990). Peptidomimetic compounds were screened for their ability to inhibit purified aspartic hemoglobinase I. The most potent inhibitor, SC-50083, was used for further studies. Its structure is shown in Figure 5A. It is an uncharged molecule with non-hydrolyzable amide

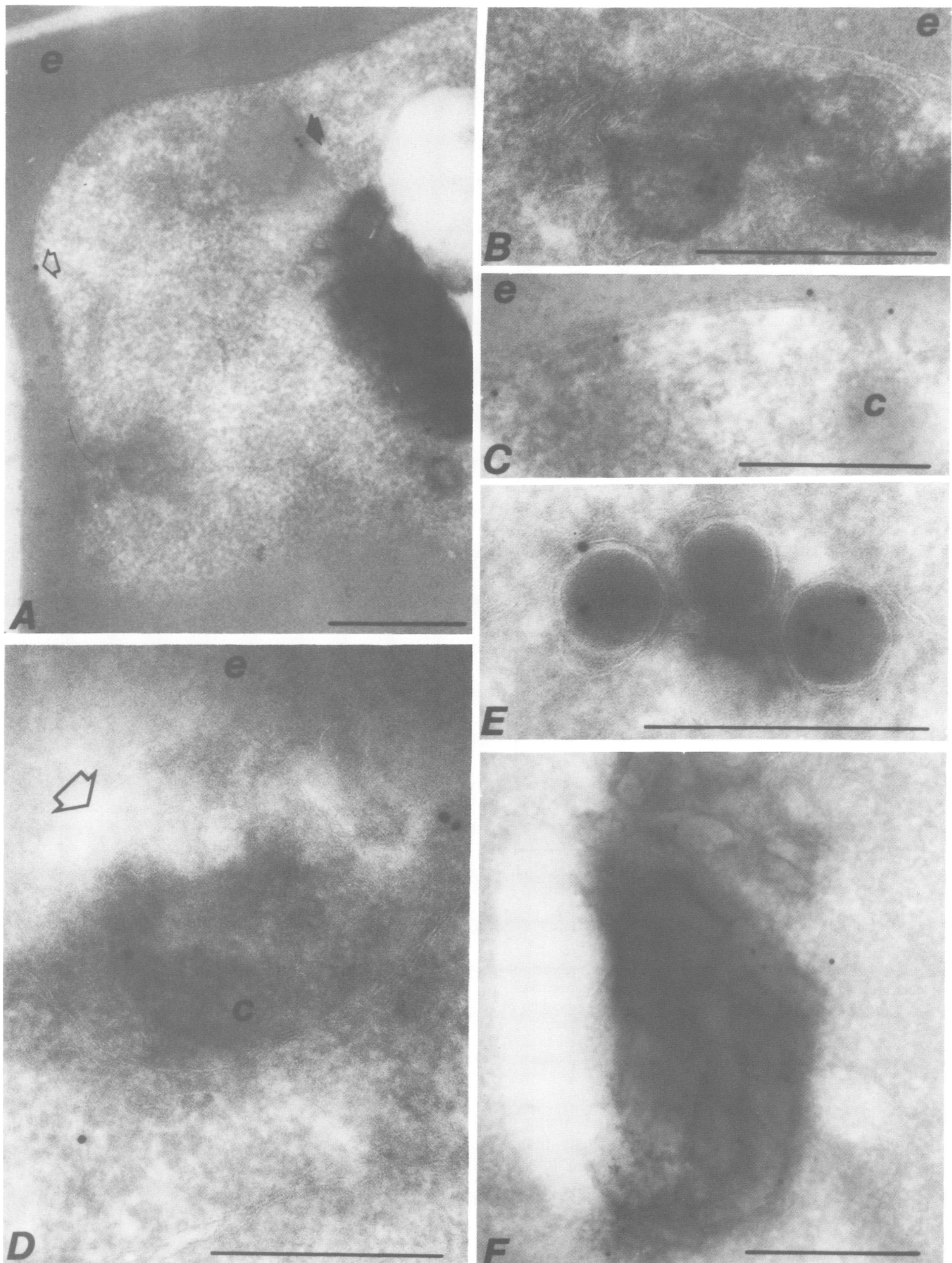


**Fig. 4.** Immunoelectron microscopy of *P.falciparum* infected trophozoites labelled with Ab574. In all micrographs 'e' signifies erythrocyte, 'c' signifies cytostome, and bars = 0.5 μm. (**A**) A general view of the cell showing label associated with a hemoglobin transport vacuole (closed arrow), the surface of the parasite (open arrow), and the condensed hemozoin in the food vacuole. (**B**) A micrograph showing label in the vesicular components that subtend the surface membrane complex of the parasite. (**C**) A micrograph demonstrating the labeling of the pellicular membranes. (**D**) A section through a cytostome of the parasite (open arrow). Membrane processes are present in the lumen of the cytostome. (**E**) The hemoglobin transport vesicles label strongly with antibody, predominantly in the lumen of the vesicle. (**F**) The antibody also reacts with the digestive vacuole itself, where it is associated with the hemozoin

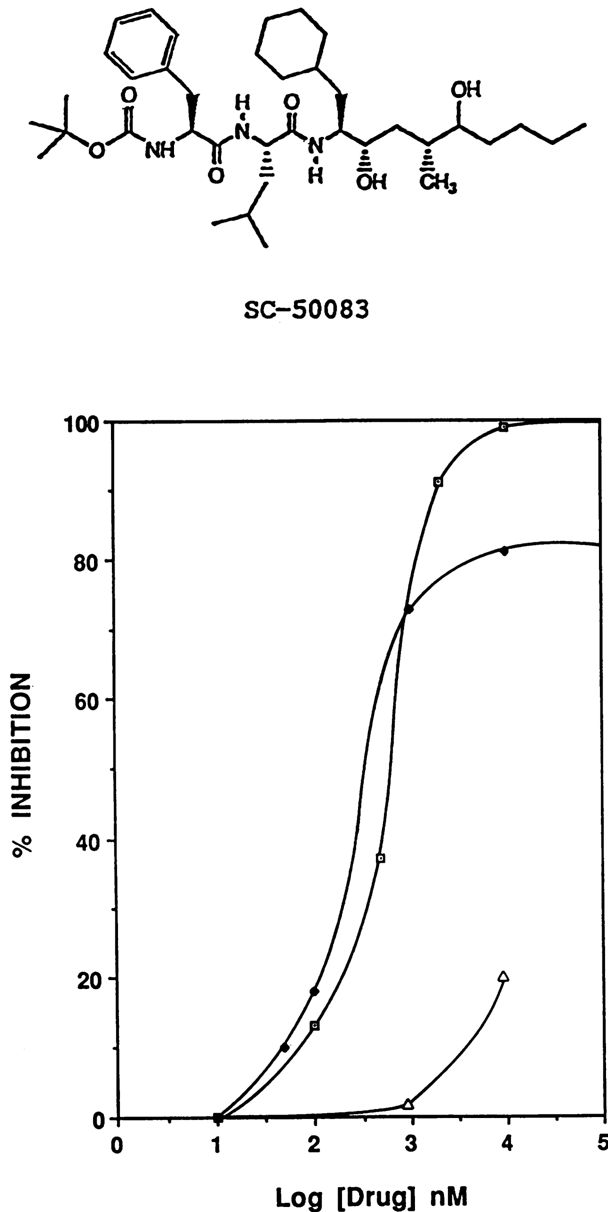


**Fig. 5.** Effect of inhibitor SC-50083 on hemoglobin proteolysis. (**A**) structure of SC-50083. (**B**) Quantitation of SC-50083 inhibition of hemoglobin proteolysis. Purified enzyme or pure vacuole extract was incubated with [$^{14}$C]globin in the presence of varying concentrations of inhibitor. Proteolysis was measured by the TCA assay. Data are averages of duplicates from a representative experiment. Open squares, vacuole extract; closed circles, aspartic hemoglobinase I; and open triangles, aspartic hemoglobinase II.

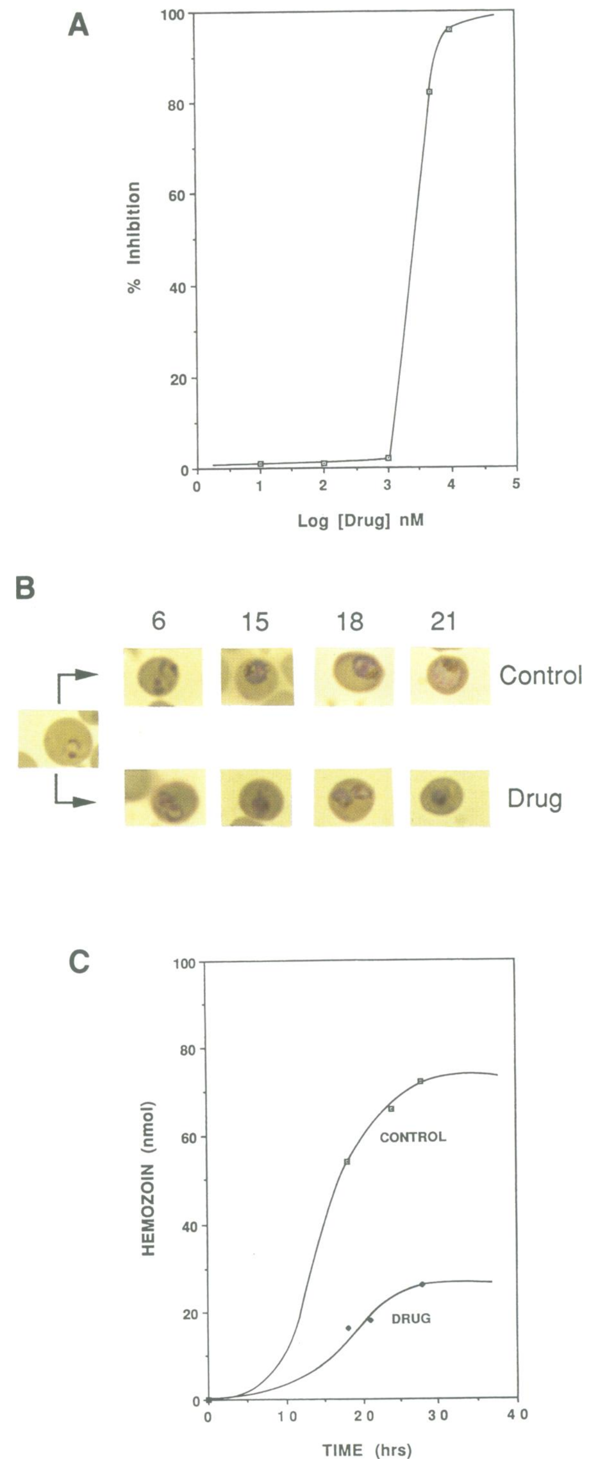


**Fig. 6.** Effect of SC-50083 on *P.falciparum* in culture. Ring-stage parasites (5% hematocrit, 10% parasitemia) were incubated with drug and analyzed. (**A**) Dose–response curve. After 24 h of incubation under our standard culture conditions, [$^{3}$H]hypoxanthine was added. 4 h later parasites were harvested onto filters and assayed for incorporated radioactivity. Percentage inhibition was calculated as the ratio of hypoxanthine incorporation in drug-treated cells to untreated cells. Data are averages of triplicates from a representative experiment. (**B**) Parasites were divided into two portions. Top series: parasites allowed to mature without drug treatment; bottom series; parasites incubated with 10 $\mu$M SC-50083. At the designated time points blood smears were made and stained with Giemsa. (**C**) Production of hemozoin during drug treatment. Cultures were incubated with or without 10 $\mu$M SC-50083 and hemozoin was quantitated at different time points. Results are averages of duplicates from a representative experiment.

bonds. When degradation was quantitated using a [$^{14}$C]globin assay (Figure 5B), purified aspartic hemoglobinase I was inhibited by SC-50083 with an $IC_{50}$ of $5-6 \times 10^{-7}$ M. When this assay was repeated using isolated aspartic hemoglobinase II, activity was inhibited by 2% at 1 $\mu$M drug and 22% at 10 $\mu$M, suggesting a dramatic difference in sensitivity of the two aspartic proteases to SC-50083. Using extract of purified digestive vacuoles, SC-50083 inhibited proteolysis with an $IC_{50}$ of $4 \times 10^{-7}$ M. Up to 80% of total vacuolar digestion could be inhibited at 10 $\mu$M. When native hemoglobin was used as substrate, SC-50083 also blocked the large majority of vacuolar proteolysis (assessed by SDS–PAGE, not shown). These data suggest that selective inhibition of the aspartic

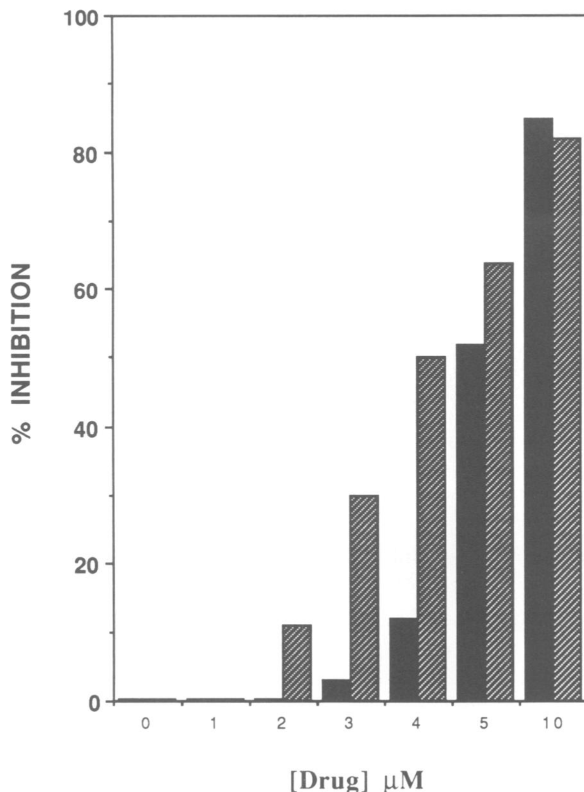


**Fig. 7.** Effects of SC-50083 on parasites cultured in minimal medium. Ring-stage parasites were cultured in normal RPMI medium or in RPMI depleted of all amino acids except isoleucine, cysteine, methionine, glutamine or glutamate. SC-50083 was added in various concentrations. After 24 h, [$^3$H]hypoxanthine was added and incorporation measured 4 h later. Shaded bars represent cultures grown in full RPMI; dark bars represent cultures grown in five amino acid medium. Inhibitor concentrations are micromolar. Results are averages of triplicate determinations from a representative experiment.

hemoglobinase dramatically impairs vacuolar hemoglobin degradation.

To assess the potency of this inhibitor against intra-erythrocytic parasites, cultures of *P.falciparum* were incubated with different concentrations of SC-50083. Parasite survival was measured by [$^3$H]hypoxanthine incorporation (Figure 6A). There was a sharp dose–response curve, and the $IC_{50}$ ranged from $2 \times 10^{-6}$ to $5 \times 10^{-6}$ M in different experiments. Cultured parasites were monitored microscopically over time to determine the effect of inhibitor (Figure 6B). A ring-stage culture was split into two parts, control and drug-treated. The control group developed normally. By 15 h the parasites had matured to trophozoites and vacuoles with hemozoin accumulation were visible. By 21 h they had matured to the early schizont stage, with the nuclear material starting to divide into multiple portions and a large hemozoin-laden vacuole obvious in each cell. Development of the inhibitor treated parasites appeared normal at 6 h. By 15 h the drug treated parasites looked condensed and had abnormal shapes. At 18 h vacuoles with small amounts of hemozoin could be detected in some cells. By 21 h parasites were scarce on the blood smears and the few survivors were pyknotic. Consistent with these data, the production of hemozoin was greatly diminished in the drug treated culture when quantitated by the pyridine–hemochrome method (Figure 6C). The drug appears to have an effect on the very stage when hemoglobin degradation is occurring. When drug was added to late trophozoites, the organisms matured normally and were not arrested until the beginning of the next trophozoite cycle (not shown). Parasite survivors of an $LD_{98}$ dose (10 μM) of SC-50083 were allowed to repopulate the culture in the absence of drug. The survivors had not developed resistance when tested. A chloroquine resistant strain (Indo-1) was also tested; the $IC_{50}$ for SC-50083 was 2 μM, comparable with that of chloroquine sensitive strain HB3.

*P.falciparum* is routinely cultured in a rich medium containing large amounts of all 20 standard amino acids. This does not reflect *in vivo* conditions. If SC-50083 acts in culture by blocking hemoglobin degradation, the parasites should be more susceptible to inhibitor when forced to rely solely on hemoglobin for most of their amino acids. Jensen and coworkers have shown that growth is impaired if methionine, cysteine, isoleucine, glutamine or glutamate is omitted from the medium (Divo *et al.*, 1985). Not coincidentally, most of these are present in low amounts or absent from human hemoglobin. We cultured parasites in medium containing only these five amino acids. Parasites grew normally under these conditions, but were more sensitive to inhibitor SC-50083 (Figure 7).

## Discussion

### *Biosynthesis and targeting implications*

We have cloned the gene for an enzyme critical to malaria parasite metabolism, and have shown that inhibiting this protease blocks hemoglobin degradation. The enzyme bears extensive homology to mammalian aspartic proteases, especially renin and cathepsin D (35% identical). There is considerably less homology to fungal aspartic proteases (16–27% identity, *Saccharomyces* proteinase A being the best). Mammalian and fungal aspartic proteases have homologous prepro-pieces of fairly uniform size, consisting of a leader peptide of ~20 amino acids followed by a propiece of ~40 amino acids (Tang and Wong, 1987). The *Plasmodium* hemoglobinase I is unusual in this regard. Its N-terminus does not have a significant hydrophobic region until amino acid 38, and the ensuing stretch of 21 amino acids is longer than reported leader hydrophobic sequences (von Heijne, 1985). Also, no signal sequence cleavage site (von Heijne, 1986) is present. Instead, the 21 amino acid hydrophobic stretch of the *Plasmodium* protease may constitute part of a signal anchor sequence; on the N-terminal side are three Lys and one Arg within eight amino acids of the hydrophobic sequence. This is suggestive of a type II membrane protein, with a cytoplasmic N-terminus (Lipp and Dobberstein, 1986; Lingappa, 1991; Parks and Lamb, 1991). *In vitro* translation and *in vivo* biosynthesis experiments will be required to demonstrate the topology of this protein.

By immunoelectron microscopy, antibody was visualized in proximity to the membranes at the parasite surface, in the cytostomal apparatus where hemoglobin ingestion initiates, and in the lumen of the hemoglobin laden transport vesicles, bringing the contents to the digestive vacuole. Analysis of these data is complicated by the fact that Ab574 recognizes both aspartic hemoglobinases. However, it seems likely that the two aspartic proteases share the same targeting route. Coupled with the presence of a putative signal anchor sequence in aspartic hemoglobinase I, our data lead us to postulate that nascent enzyme is delivered as an integral membrane protein to the parasite surface membrane

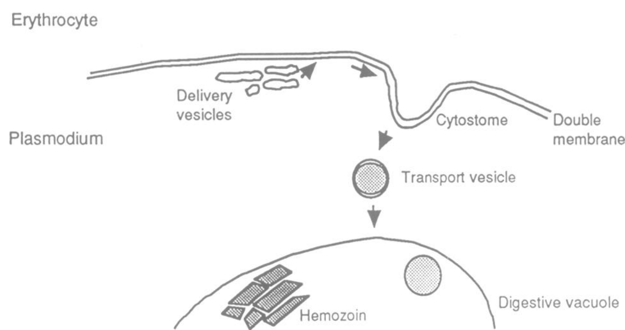


**Fig. 8.** Postulated route of aspartic protease trafficking. We hypothesize that nascent enzyme is delivered to the parasite surface via membranous structures, accumulates in the cytostome region, is internalized with ingested hemoglobin, and is delivered to the digestive vacuole in transport vesicles.

complex. It then accumulates in the cytostome region, is internalized during hemoglobin ingestion, is cleaved from the membrane during the ingestion process, and is delivered to the digestive vacuole (Figure 8), where it is found in soluble form (Goldberg *et al.*, 1991). At present, this remains a highly speculative model. Biosynthesis experiments and further immunolocalization studies using antibody that only recognizes one hemoglobinase will be necessary to solidify the hypothesis. A somewhat analogous route may exist in mammalian systems. Acid phosphatase has been proposed to be targeted via the plasma membrane to the lysosomes (Braun *et al.*, 1989; Peters *et al.*, 1990), where it too is soluble.

#### *SC-50083 is a highly selective inhibitor*

Our peptidomimetic compound is at least two orders of magnitude more potent against aspartic hemoglobinase I than against aspartic hemoglobinase II. The fact that selective inhibition of hemoglobinase I can substantially impair the whole process of hemoglobin degradation *in vitro* and in culture confirms the importance of the initial proteolytic role proposed for this enzyme. Aspartic hemoglobinase II appears incapable of sufficient hemoglobin catabolism to sustain the organism when hemoglobinase I is inactivated. It may function in the further degradation of fragments generated by aspartic hemoglobinase I. Along the same line, a cysteine protease proposed to play a role in hemoglobin breakdown (Rosenthal *et al.*, 1988, 1991) is capable of cleaving denatured globin but not native hemoglobin (I.Y.Gluzman, S.E.Francis, A.Oksman, C.E.Smith, K.L.Doffin and D.E.Goldberg, submitted).

#### *Mechanism of action of SC-50083*

The peptidomimetic compound works on purified enzyme, on isolated vacuoles and on whole parasites in culture. We suggest that these events are linked, and that inhibition of aspartic hemoglobinase I blocks hemoglobin degradation, resulting in death of the parasite. We cannot be sure that there are no secondary sites of action important for parasite killing. When forced to rely on hemoglobin as the sole source of most amino acids (Figure 7), the parasites proved more sensitive to low concentrations of inhibitor, providing further evidence for action of the drug on hemoglobin degradation. At higher drug concentrations parasites were killed effectively with or without amino acid supplementation, suggesting that the parasites still required hemoglobin catabolism for survival.

The $IC_{50}$ for parasite killing in culture is within one order of magnitude of the $IC_{50}$ for enzyme inhibition. This implies that the drug enters the parasites without difficulty. How is not clear; the task of crossing the erythrocytic membrane, parasitophorous vacuolar membrane, parasite plasma membrane and digestive vacuole membrane would seem to be daunting. It could be that the drug enters the erythrocyte and then is ingested by the parasite along with hemoglobin, using the parasite's own targeting route. A possible alternative means of drug uptake is the parasitophorous duct proposed to extend from the parasite to the cell surface (Pouvelle *et al.*, 1991), though the existence of this duct under physiological conditions has been questioned (Fujioka and Aikawa, 1993).

In contrast to the effects of SC-50083 on parasite morphology, chloroquine (Jacobs *et al.*, 1988) and the cysteine protease inhibitor leupeptin (Rosenthal *et al.*, 1988) cause vacuolar swelling to occur. The swelling seen with these drugs is similar to the effect of free heme on whole parasites (Orjih *et al.*, 1981). Possibly aspartic hemoglobinase inhibition prevents toxic heme release, while the other inhibitors allow toxic heme build-up with consequent membrane damage.

#### *Potential for antimalarial chemotherapy*

SC-50083 has some anti-renin and anti-cathepsin D activity. The renin inhibition is unlikely to be of physiological consequence, however, as >99% of renin activity must be blocked to see any effect on blood pressure. This agent has already been rejected as a potential renin inhibitor. A role for cathepsin D in antigen presentation has been proposed (van Noort and van der Drift, 1989) but the consequences of its short-term inhibition are unknown. Regardless, it will be desirable to improve aspartic hemoglobinase inhibition while diminishing mammalian protease interaction. Because the *Plasmodium* hemoglobinase recognizes a positive charge in the P3 substrate position (Goldberg *et al.*, 1991), where renin and cathepsin D prefer a bulky, hydrophobic amino acid, it may be possible to improve potency and selectivity at the same time. By using a group that is protonatable only at the low pH of the digestive vacuole, protonation trapping (similar to that responsible for vacuolar accumulation of chloroquine) should at the same time improve delivery.

Further characterization of the aspartic hemoglobinase is now possible. Expression of cloned hemoglobinase will permit production of enzyme in sufficient quantity for detailed specificity studies. Crystallization will permit refined analysis of protein–inhibitor interactions. These should allow simple modifications of our lead compound, which can be expected to generate a serious chemotherapeutic candidate. We have laid the groundwork here by demonstrating that *P.falciparum* hemoglobin degradation is an essential pathway that can be selectively inhibited, and that this pathway is mediated by intriguing enzymes whose study reveals much about the cellular biochemistry of this important organism.

## Materials and methods

#### *Culture*

*P.falciparum* clone HB3 (a kind gift of Dr W.Trager) was grown at 37°C under 3% oxygen/3% carbon dioxide in RPMI medium using 5% human

red blood cells (Trager and Jensen, 1976) supplemented with 10% human plasma (Hui and Palmer, 1984). Synchronization was attained by treatment with sorbitol (Lambros and Vanderberg, 1979).

### Preparation of genomic DNA

DNA was isolated from schizont stage parasites following 0.2% saponin lysis of parasitized erythrocytes (Goldberg *et al.*, 1991). The parasite pellet was suspended in DNA lysis buffer (100 mM Tris–HCl, pH 7.4, 200 mM NaCl, 50 mM EDTA, 1% SDS, 0.1% β-mercaptoethanol, 100 μg/ml proteinase K) and incubated for 1 h at 65°C. The DNA was extracted three times with phenol/chloroform (1:1), twice with chloroform/isoamyl alcohol (24:1) and recovered by ethanol precipitation.

### Genomic library construction

To make a *P.falciparum* genomic library, genomic DNA (15 μg) was digested overnight at 37°C with 10 U *Eco*RI. The digest (2.5 μg) was ligated overnight at 16°C with *Eco*RI-cut, phosphatased λZapII (Stratagene, La Jolla, CA). Ligations were packaged with Gigapack Gold (Stratagene) according to manufacturer's instructions. The resultant library contained about $2 \times 10^6$ primary recombinants, >95% of which contained inserts. Average insert size was ~5 kb. This material was subjected to one round of amplification prior to screening.

### Polymerase chain reactions

All PCRs were performed in a TC480 thermal cycler with either Taq polymerase or Ampli-Taq (Perkin-Elmer Cetus, Norwalk, CT) according to manufacturer's specifications. All reactions were preceded by a single 5 min, 95°C denaturation step and followed by a 5 min, 72°C final extension step.

Initial PCRs were performed with best guess oligonucleotides as primers and 10 ng *P.falciparum* genomic DNA as template. Oligo DG1 (5'-AATGATGTAGCAAATGTAATGTATTATGGAGAAGCACAAAT-3', sense 41-mer, 40/41 correct) is derived from N-terminal amino acid sequence of the mature protein (Goldberg *et al.*, 1991). Oligo DG3 (antisense 33-mer, 5'-TGGTACCCATAAATTACTACTTCCTGTATCAAA-3', 26/33 correct) was generated from the consensus aspartic protease active site (Tang and Wong, 1987). 25 cycles (94°C, 1.5 min; 30°C, 0.1 min; 72°C, 1 min) were performed, generating a 105 bp product that was subcloned into *Sma*I-cut pBSSKII+ (Stratagene) and subjected to sequencing. The 31 bp of authentic sequence so obtained were used to design oligo DG11 (sense, 5'-TGGAGATAATAAACAAAAGTTTGCTTTTATT-3').

PCR screening of the λZapII genomic library was performed with oligo DG1 (above) and the T3 oligo (5'-TAACCCTCACTAAAGGGA) recognizing vector sequence. After 30 cycles (94°C, 1 min; 40°C, 0.5 min; 72°C, 3 min), a band of ~650 bp was gel-purified by adsorbtion to glass slurry (Gene-clean, Bio101). The DNA was subjected to direct PCR sequencing, and found to encode amino acids with homology to mammalian aspartic proteases and identity to our known hemoglobinase N-terminus. The DNA sequence was used to design oligo DG12 (5'-AGTTGTGCCTATAGAACTAGTACCACT-3', sense 27-mer). Reaction (1 μl) from which the 650 bp band was purified was re-amplified with oligos DG11 and DG12 as primers (30 cycles, 94°C, 1 min; 40°C, 0.5 min; 72°C, 3 min). The resultant 607 bp band was subcloned (AP1) using a TA-Cloning Kit (Invitrogen) according to the manufacturer's instructions.

### Selection of cDNA clones

Approximately $10^5$ plaques each from poly(A)-primed cDNA libraries (generously provided by R.G.Nelson, UCSF) constructed in λZapII from *P.falciparum* trophozoite or ring-stage cDNA, were screened. Plaques were transferred to replicate filters and hybridized in 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS, 100 μg/ml salmon sperm DNA and 50% (v/v) formamide with $1 \times 10^6$ c.p.m./ml of radiolabeled AP1. One positive clone with a 2.6 kb insert was identified from each library, plaque-purified, and inserts were rescued with helper phage.

### DNA sequence analysis

DNA sequences were determined by dideoxy sequencing of both strands (Sanger *et al.*, 1977) using Sequenase (US Biochemical). PCR products were subjected to direct sequencing with a fmol DNA sequencing kit (Promega) according to the manufacturer's specifications with modifications (Sherman *et al.*, 1992).

### Southern analysis

Genomic DNA (10 μg) was digested with *Xba*I, *Hae*III and *Eco*RI (5–10 U per μg DNA) and subjected to 0.7% agarose gel electrophoresis. DNA was transferred to Magna NT filters (MSI) in 10 × SSC (1 × SSC contains 0.15 M NaCl and 0.15 M sodium citrate) and UV cross-linked at $1.2 \times 10^5$ μJ/cm². Filters were hybridized with radiolabeled probes in 2 × SSC, 5 × Denhardt's solution, 2 mM EDTA and 100 μg/ml sheared salmon sperm DNA for 14–16 h. Filters were washed to 0.5 × SSC at 50°C. Oligonucleotide probes DG1 and DG3 were end-labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Boehringer Mannheim).

### Northern analysis

Asynchronous HB3 cultures were harvested and freed from the red blood cell by saponin lysis (Goldberg *et al.*, 1991). Total RNA was isolated by guanidinium thiocyanate and phenol using RNAzol B (Tel-Test, Inc.) according to the manufacturer's specifications. RNA (10 μg per lane) was separated by 1.4% agarose formaldehyde gel electrophoresis and transferred to MagnaNT (MSI). Filters were UV cross-linked and hybridized with radiolabeled AP1 in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS, 100 μg salmon sperm DNA and 25% formamide for 16 h at 35°C. Blots were washed to 0.5 × SSC, 0.1% SDS at 55°C.

### Antibody production

A synthetic peptide was synthesized corresponding to amino acids 1–22 of mature hemoglobinase (Goldberg *et al.*, 1991), coupled to thyroglobulin (Harlow and Lane, 1988) and used for subcutaneous immunization of New Zealand white rabbits. A fusion protein was made by inserting a 672 bp fragment encoding amino acids 1–224 of mature hemoglobinase into expression vector pGEX-3X (Smith and Johnson, 1988). The gene was induced and overexpressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein which was gel purified. Gel slices were then used for rabbit immunization (Cowman *et al.*, 1991). Antipeptide (Ab467) and fusion protein (Ab574) antisera were affinity purified with SDS–PAGE fractionated, nitrocellulose-immobilized hemoglobinase from trophozoite extracts (Sambrook *et al.*, 1989).

### Western blots

Parasite extract or purified enzyme fractions were run on SDS–PAGE (Laemmli, 1970), transferred to nitrocellulose, incubated with primary antibody (1:50–1:100) followed by alkaline phosphatase-conjugated secondary antibody (1:1000), and developed as has been described (Harlow and Lane, 1988).

### Immunofluorescence

Blood smears of synchronized trophozoites were washed three times in phosphate-buffered saline (PBS), allowed to air dry, and fixed in acetone for 20 min at −20°C. After drying in air, they were incubated for 1 h at room temperature in pre-immune serum or Ab574 diluted 1:100 in PBS with 1% goat serum. Slides were washed three times for 5 min each in PBS and then incubated with rhodamine-conjugated anti-rabbit IgG (1:100 dilution). Slides were again washed three times for 5 min and visualized by Leitz fluorescence and phase microscopy. Under the conditions used, pre-immune serum gave no detectable signal.

### Immunoelectron microscopy

Infected cultures were fixed in 1% glutaraldehyde in HEPES–saline as described (Russell *et al.*, 1992). Fixed cells were embedded in gelatin and infiltrated overnight in 2.3 M sucrose in 20% PVP. Frozen sections were cut with an RMC MT7 ultramicrotome and CR31 cryobox. Sections were blocked with 5% goat serum and 10% fetal calf serum in PBS, and incubated in primary and secondary antibodies in the same blocking solution. Primary antibody was Ab574; secondary antibody was goat anti-rabbit IgG conjugated to 15 nm gold. Sections were washed, stained and embedded in 0.3% uranyl acetate in 2% polyvinyl alcohol.

### Aspartic protease purification

Aspartic hemoglobinase I was purified as previously described (Goldberg *et al.*, 1991) with the following modifications. (i) Digestive vacuoles isolated by sorbitol lysis/differential centrifugation (Goldberg *et al.*, 1990) omitting Percoll separation, were used as enzyme source. (ii) Hydroxylapatite chromatography was performed by HPLC (Vander Jagt *et al.*, 1992) instead of syringe column. (iii) The acid fractionation step was omitted. Contamination of purified enzyme was assessed with a panel of inhibitors of other classes of proteases (PMSF, *o*-phenanthroline, E-64) as described (Goldberg *et al.*, 1990) and no detectable contaminants were seen. Additionally, purified enzyme was completely inhibited by 10 μM pepstatin. N-terminal sequence analysis confirmed the identity of the purified hemoglobinase with that previously described (Goldberg *et al.*, 1991).

Aspartic hemoglobinase II was isolated as an earlier migrating activity peak on the hydroxylapatite chromatography described above. Further purification was achieved by repeating the DEAE chromatography at pH 7.5 instead of 7.0 on a Waters Protein-Pak DEAE 5PW HPLC column with

a gradient of NaCl from 0 to 0.5 M in Tris−HCl at a flow rate of 1 ml/min. Characterization of the purified enzyme will be described elsewhere (I.Y.Gluzman, S.E.Francis, A.Oksman, C.E.Smith, K.L.Doffin and D.E.Goldberg, submitted). N-terminal sequence analysis confirmed the identity of the purified protein to an aspartic protease whose gene has just been cloned (J.Dame, personal communication).

### Inhibitor SC-50083

Synthesis and analysis of this compound will be described elsewhere (R.Mueller, in preparation). The drug is soluble in DMSO and was stable in a 5 mM stock solution at −20°C for at least 3 months.

### Enzyme incubations

Purified digestive vacuoles were prepared and extract was made as previously described. For the trichloroacetic acid (TCA) assay, reaction mixtures contained 150 mM sodium acetate pH 5.0, 60 000 c.p.m. (6.25 μM) [$^{14}$C]methylated globin (Dottavio-Martin and Ravel, 1978), 10 μl vacuole extract or purified enzyme, and varying concentrations of SC-50083 in a 40 μl final volume. Reactions were stopped after 2 h by addition of TCA, centrifuged and the supernates assayed for radioactive proteolytic fragments as previously described (Goldberg *et al.*, 1991).

### Drug effects on P.falciparum culture

Late ring-stage cultures at 10% parasitemia were grown in the presence of various concentrations of SC-50083 (diluted in RPMI medium) for 16 h. At the end of this period, 1 μCi (17.2 Ci/mmol) of [$^{3}$H]hypoxanthine was added and the cultures were incubated for another 4 h. Parasites were harvested and [$^{3}$H]hypoxanthine incorporation measured as previously described (Desjardins *et al.*, 1979). As a control, the DMSO vehicle for the drug was diluted in RPMI to the same extent and added to a similar culture. This had no effect on parasite hypoxanthine incorporation. Parasitemia in the cultures paralleled hypoxanthine incorporation.

For the time course, ring-stage culture was incubated with or without addition of 10 μM SC-50083 for 6−21 h. At the designated times, aliquots of culture were removed and blood smears prepared using Giemsa stain.

### Hemozoin quantitation

Late ring-stage cultures (10% parasitemia) were allowed to mature in the presence or absence of 10 μM SC-50083. At various time points 24 ml aliquots of culture were harvested, brought to 1% Triton X-100, vortexed well, and centrifuged for 40 000 *g*-hours. The pellets were washed in water, then solubilized and heme content measured by the pyridine-hemochrome method (Slater and Cerami, 1992).

### Growth in five amino acid medium

The effect of different inhibitor concentrations was measured on cultures in complete RPMI 1640 or RPMI 1640 deficient in all amino acids except glutamine, glutamate, cysteine, methionine and isoleucine. Without inhibitor, hypoxanthine incorporation was comparable in full or selective medium. The 10% plasma used in these experiments could be either dialyzed or undialyzed; similar results were obtained in either case.

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## Note added in proof

The sequence of aspartic hemoglobinase I has been deposited in the EMBL sequence data library under the accession number X75787