Identification and Characterization of Falcilysin, a Metallopeptidase Involved in Hemoglobin Catabolism within the Malaria Parasite *Plasmodium falciparum* (Received for publication, May 25, 1999, and in revised form, August 24, 1999)

Kathleen Kolakovitch Eggleson, Kevin L. Duffin‡, and Daniel E. Goldberg§

From the Howard Hughes Medical Institute, Washington University, Departments of Molecular Microbiology and Medicine, St. Louis, Missouri 63110 and §Monsanto Corporate Research, Monsanto Company, St. Louis, Missouri 63198

The malaria parasite *Plasmodium falciparum* degrades hemoglobin in its acidic food vacuole for use as a major nutrient source. A novel metallopeptidase activity, falcilysin, was purified from food vacuoles and characterized. Falcilysin appears to function downstream of the aspartic proteases plasmeplins I and II and the cysteine protease falcipain in the hemoglobin proteolytic pathway. It is unable to cleave hemoglobin or denatured globin but readily destroys peptide fragments of hemoglobin. Falcilysin cleavage sites along the alpha and beta chains of hemoglobin are polar in character, with charged residues located in the P1 and/or P4 positions. In contrast, plasmeplins I and II and falcipain prefer hydrophobic residues around the scissile bond. The gene encoding falcilysin has been cloned. Its coding sequence exhibits primary structural features of metallopeptidases, including an “inverted” HXXEH active site motif. Falcilysin shares primary structural features with M16 family members such as insulysin, mitochondrial processing peptidase, nardilysin, and pirlitilysin as well as with data base hypothetical proteins that are potential M16 family members. The characterization of falcilysin increases our understanding of hemoglobin catabolism in *P. falciparum* and the unusual M16 family of metallopeptidases.

*Plasmodium falciparum* is a protozoan parasite that causes the most lethal form of human malaria. Upon invasion of a human erythrocyte, the parasite grows and matures surrounded by cytosol consisting predominantly of a single protein, hemoglobin. Amino acids derived from the proteolysis of hemoglobin are incorporated into parasite proteins and parasites require supplementation with only a few amino acids that are absent or deficient in hemoglobin for normal growth in culture (1, 2). Hemoglobin proteolysis occurs within an acidic organelle, the food vacuole. This compartment has a pH estimated at 5.0–5.4 (3, 4).

Nonproteolytic acid hydrolases could not be detected in food vacuoles isolated from *P. falciparum* (5). Thus, it appears that the food vacuole of *P. falciparum* does not function in degradation and recycling of macromolecules in general. The catabolic capability of this organelle is focused on hemoglobin. Disruption of hemoglobin catabolism causes parasite death in an animal model and in culture (2, 6, 7). The vital and specialized process of hemoglobin degradation within the food vacuole provides promising targets for the development of novel antimalarial drugs, greatly needed in the face of increasing resistance to existing chemotherapeutic agents (8).

Multiple proteases within the food vacuole facilitate the degradation of hemoglobin to peptide fragments. Three aspartic proteases have been identified, purified from food vacuoles, and characterized (2, 9–14). Two aspartic proteases, plasmeplisin I and plasmeplisin II, can cleave native hemoglobin. A cysteine protease, falcipain, is able to cleave denatured globin but not native hemoglobin (10, 15). Exopeptidase activity capable of generating individual amino acids from peptide fragments is absent from the food vacuole (16). However, peptides may traverse the food vacuole membrane and could be converted to amino acids by exopeptidase activity in the parasite cytoplasm. An aminopeptidase that functions at neutral pH has been purified from parasites and characterized (17–19).

When hemoglobin was incubated with food vacuole lysate at acidic pH, a series of discrete peptide fragments was generated (16). Cleavage sites along the hemoglobin alpha and beta chains were located an average of eight amino acids apart. Many cleavage sites corresponded to the peptide bonds previously identified as sites for the known vacuolar proteases. Twenty-four cleavage sites that could not be attributed to the known proteases of the vacuole were identified. Unlike the preference of plasmeplisin I, plasmeplisin II, and falcipain for hydrophobic residues, many of the novel cleavage sites contained polar residues at the P1 and/or P1’ positions. These results suggested that one or more vacuolar endopeptidases had remained unidentified (16). In this report, we describe the discovery of a novel proteolytic activity, purification of the enzyme responsible for this activity, characterization of the purified enzyme, and the corresponding molecular sequence data. Furthermore, we provide evidence that this enzyme, falcilysin, has a distinct, downstream role in the semiordered hemoglobin degradation pathway of *P. falciparum*.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human hemoglobin, human globin, pepstatin, E64, EDTA, dipicolinic acid, amastatin, bestatin, bacitracin, 1,10-phenanthroline, cobalt sulfate, magnesium sulfate, manganese sulfate, zinc}

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* This work was supported in part by National Institutes of Health Grant AI-31615. Preliminary sequence data for *P. falciparum* chromosome 14 was obtained from The Institute for Genomic Research website. Sequencing of chromosome 14 was part of the International Malaria Genome Sequencing Project and was supported by awards from the Burroughs Wellcome Fund and the U. S. Department of Defense. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a Burroughs Wellcome Fund Scholar Award in Molecular Parasitology. To whom correspondence should be addressed: Washington University School of Medicine, Box 8230, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-1514; Fax: 314-362-1232; E-mail: goldberg@boricim.wustl.edu.
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sulfate, BisTris, KlenTaq LA, and Triton X-100 were obtained from Sigma. Endoproteinase Lys-C and PMSF were from Roche Molecular Biochemicals (Indianapolis, IN). N-Ethylmaleimide was from Fluka (Buchs, Switzerland). Coomassie Brilliant Blue, gel filtration standard vials, high range SDS-PAGE standards, and Tween 20 were from Bio-Rad Laboratories. Silver nitrate was from Fisher Scientific; Eastman Kodak (Rochester, NY). The Ultraphase C18 HPLC column was from P. J. Cobert Associates, Inc. (St. Louis, MO). The Microbore C18 column was from The Separations Group (Hesperia, CA). Mono S PC 1.6/5, Superose 12 HR 10/30, DEAE-Sepharose, and CM-Sepharose were from Amersham Pharmacia Biotech, MES and acetonitrile (CH3CN) were from Fisher (Pittsburgh, PA). Millipore UV polycarbonate filters (0.45-μm) were from Millipore (Bedford, MA). Synthetic oligonucleotides were from Integrated DNA Technologies (Coralville, IA). The quenched fluorescence substrates were from AnaSpec (San Jose, CA).

Culture—P. falciparum clones HB3 (gift of W. Traeger, Rockefeller University) and 3D7 (gift of P. Rathod, Catholic University) and 3D7 (gift of P. Rathod, Catholic University) were grown in the Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. For mass spectrometry, parasites were from the London Malaria Reference Laboratory (London, United Kingdom). Mass scans from 125 to 2000 m/z were obtained on a quadrupole TOF mass spectrometer (Micromass, Manchester, United Kingdom) at a flow rate of 100 μl/min. 0.5-min fractions were collected and the peptides were detected at 215 nm on a Hewlett-Packard 1090 HPLC system (Hewlett-Packard, Palo Alto, CA). Peptides were sequenced on a PROCISE 492 protein sequencer (PE Biosystems, Foster City, CA).

Metal Requirement—The apoenzyme form of falcilysin was generated by incubation of 15 units of purified falcilysin with 207 pmol (3.2 μg) of hemoglobin or acid-denatured globin overnight at 37 °C in 100 mM sodium acetate buffer, pH 5.2. Recombinant plasminogen II was prepared as described previously (25). 0.04 units of recombinant plasminogen II measured using a quenched fluorescence peptide substrate for plasminogen II activity (25) was also incubated with 207 pmol of hemoglobin or acid-denatured globin under the same conditions. As controls, similar incubations without enzyme were performed. 10 μl of each 40-μl sample was subjected to 15% SDS-PAGE.

In order to determine whether falcilysin could cleave fragments of hemoglobin, 162 μg of human hemoglobin was incubated overnight at 37 °C with 41 units of recombinant plasminogen II in 150 mM sodium acetate buffer, pH 5.2. More than 90% of the 162 μg of hemoglobin was degraded as monitored by the loss of absorbance at 410 nm relative to hemoglobin incubated separately. The sample was treated with pepstatin to inhibit plasminogen II. This substrate material was split into two 26 units of purified falcilysin in 150 mM sodium acetate buffer, pH 5.2. Each sample was applied to an Ultraphase C18 column for reverse phase chromatography with a linear gradient of 0–60% acetonitrile at 1 ml/min over 50 min and peptide elution was monitored at 214 nm.

Determination of Falcilysin Cleavage Sites—Hemoglobin fragments were generated by incubation of 560 μg of human hemoglobin with 176 units of recombinant plasminogen II as above. Pepstatin was added to block further plasminogen II action. This material served as substrate for the next reaction. Half was incubated overnight at 37 °C as above with 123 units of purified falcilysin and half was incubated separately from the falcilysin only control. The overnight reaction was stopped by adding Tris-HCl, pH 8.5, to the sample with pepstatin. For mass spectrometry, 50 μg of aqueous 0.1% trifluoroacetic acid. Five microliters were injected onto a 0.32-mm inner diameter x 15-cm capillary column packed with Vydc C-18 (300 Å) stationary phase. Peptide fragments were eluted from the column with 0–80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 5 μl/min. Column eluate passed through a 100-μm inner diameter fused silica transfer line to the electrospray interface of a Micromass Q-TOF mass spectrometer (Manchester, United Kingdom). Mass scans from m/z 390–4000 were summed over 1 s to generate each mass spectrum in the LC/MS chromatogram. 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 the mass spectral data. The peptide sequences of the falcilysin activity were determined by multiplying the m/z value of the peptide by 12 C isotope by the charge state of the peptide. The abundance values were determined by summing the selected ion currents of the (M+H)+, (M+2H)+, (M+3H)+, etc. from the LC/MS chromatograms. The units are counts/s, and represent the ion current that the mass spectrometer detects for each ion. This value is consistent with the concentration of
peptide in the LC eluate.

Peptides of interest with respect to their abundance in the falcilysin digest versus control sample were selected for LC/MS/MS characterization after making a second LC injection. In this experiment, the quadrupole mass analyzer sequentially passed peptides of selected m/z values into the collision cell of the mass spectrometer, where the peptides underwent 50 eV collisions with nitrogen gas. Collision-induced dissociation fragments (product ions) of each of the peptides were then mass analyzed by the time-of-flight segment of the mass spectrometer over a 1-s period to generate a MS/MS spectrum of the peptide. Product ion masses present in the MS/MS spectra were programmed into MS-Tag software (Protein Prospector, University of California, San Francisco) (26, 27) to determine the identities of selected peptides.

Falcilysin Gene Identification and Sequencing—Preliminary sequence data for *P. falciparum* chromosome 14 was obtained from The Institute for Genomic Research website. Sequencing of chromosome 14 was part of the International Malaria Genome Sequencing Project. The entire coding region was amplified from strain 3D7 genomic DNA with synthetic oligonucleotides annealing 56 base pairs 5’ of the start codon (5’-TTATTTATTTATGTTTTATATTACACC-3’) and 63 base pairs 3’ of the stop codon (5’-ACAAGGGTATAATTGTAAGTGTGC-3’) using KlenTaq LA and was directly cloned into the pCR 2.1 vector using the TA cloning kit from Invitrogen (San Diego, CA). Plasmid DNA template was extracted from *Escherichia coli* and prepared for sequencing using the Nucleobond Plasmid Midi Kit from CLONTECH (Palo Alto, CA) or Qiagen (Valencia, CA) plasmid mini kit. Sequencing samples were generated using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit from the Applied Biosystems division of PerkinElmer (Foster City, CA). Automated sequencing was performed at the DNA Sequencing Facility of the Protein and Nucleic Acid Chemistry Laboratory at Washington University School of Medicine. Both strands of the falcilysin gene were sequenced once, new polymerase chain reaction products were generated and cloned into pCR 2.1, and then both strands of the falcilysin gene were sequenced a second time. Comparison of pairs of sequences was performed using the BESTFIT program of the CLUSTAL W (29).

RESULTS

Purification of Falcilysin from Food Vacuoles—In our previous work the peptide products of human hemoglobin digestion by food vacuole lysate were identified as discrete fragments of the α and β chains (16). Analysis of food vacuole enzyme cleavage sites indicated the existence of one or more new vacuolar endopeptidases. A prominent cleavage site between two adjacent aspartic acid residues at positions 74 and 75 on the human α chain was found (16); therefore, a quenched fluorescence octapeptide substrate corresponding to α71–78, Dabcyl-Gaba-AHVDMPN-Edans, was synthesized. This substrate was cleaved by food vacuole lysate. Cleavage was not blocked by inhibitors of aspartic, cysteine, or serine proteases but potent inhibition was observed with metal chelators (data not shown). Prior to this experiment, aspartic and cysteine proteases were the only proteolytic enzymes known to function in the food vacuole. The α71–78 substrate was used to detect a novel metallopeptidase, which we call falcilysin, during purification from food vacuoles.

Falcilysin was purified from the *P. falciparum* food vacuoles by sequential anion and cation exchange chromatography. Yields of up to 11% were obtained. Purified falcilysin could be stabilized for storage at −70 °C using final concentrations of 1 mg/ml bovine serum albumin, 0.01% Tween 20, or 50 mM sodium acetate, pH 5.2.

Properties of Falcilysin—When cation exchange fractions were applied to a Superose 12 column, a peak of activity against the α71–78 quenched fluorescence substrate eluted with an estimated mass of 125 kDa (Fig. 1). The activity peak has a slight asymmetry. The reason for this is not known. However, it could be due to a minor proteolytic fragment of falcilysin or an interaction of the enzyme with the column. A silver-stained SDS-PAGE gel showed that falcilysin migrates as a single band at 125 kDa (Fig. 1, inset).

The apoenzyme form of purified falcilysin was generated. No activity against the α71–78 substrate could be detected using the apoenzyme. Divalent metal cations were added to the apoenzyme at varying final concentrations and tested for their ability to reconstitute activity against the α71–78 substrate (Fig. 2). Magnesium restored very low levels of activity in a range of concentrations spanning 5 orders of magnitude. Magnesium was ineffective at low concentrations but reconstituted a small amount of activity at 10 mM. Cobalt at 100 μM generated the highest level of activity observed. Zinc restored high levels of activity at 1 μM and greater concentrations were inhibitory, typical of zinc metalloenzymes (30).

A variety of inhibitors were tested for their abilities to block cleavage of the α71–78 substrate by falcilysin. As expected for a metalloendopeptidase, high levels of activity remained in the presence of the aminopeptidase inhibitors amastatin and bestatin, the aspartic protease inhibitor pepstatin, the cysteine protease inhibitor E64, and the serine protease inhibitor PMSF. Potent inhibition was observed with the chelators EDTA, dipicolinic acid, and 1,10-phenanthroline. The alkylating agent N-ethylmaleimide also inhibited falcilysin activity by 80%.

The fluorogenic assay was performed with sodium acetate buffer at different pH values using saturating substrate. The pH optimum of falcilysin was determined to be approximately 5.2 (data not shown). Optimal function at acidic pH is expected of vacuolar enzymes, since the pH of the food vacuole is between 5.0 and 5.4 (3, 4).

Falcilysin Cleaves Fragments of Hemoglobin—Plasmepsin I, plasmepsin II, and falcipain readily cleave hemoglobin and/or acid-denatured globin at vacuolar pH (10). We wished to determine whether falcilysin also participates in the destruction of hemoglobin or globin in the food vacuole. Hemoglobin or acid-denatured globin were incubated overnight with 0.04 units of plasmepsin II or 15 units of falcilysin (where 1 unit of activity equals 1 pmol of quenched fluorescence substrate cleaved/min). No cleavage of either hemoglobin or acid-denatured globin by falcilysin could be detected by silver stained SDS-PAGE. In contrast, recombinant plasmepsin II was able to cleave both substrates. The falcilysin activity used was sufficient to cleave a quantity of its quenched fluorescence substrate 68-fold greater than the molar amount of hemoglobin/globin present in the reaction.

Since hemoglobin and globin were not cleaved by falcilysin,
Falcilysin, a Vacuolar Metallopeptidase from *P. falciparum*

![Graph: Activity of falcilysin apoenzyme restored by metals]

**Fig. 2.** Activity of falcilysin apoenzyme is restored by metals. The apoenzyme form of falcilysin exhibited no activity in the fluorogenic assay for falcilysin. Cobalt sulfate (*open circle*), magnesium sulfate (*filled circle*), manganese sulfate (*open square*), or zinc sulfate (*filled square*) were added to the apoenzyme at various concentrations and tested for their ability to restore activity against the α71–78 quenched fluorescence substrate.

We wished to investigate whether falcilysin had a role in the hemoglobin catabolism process subsequent to the fragmentation of globin. Fragments of hemoglobin generated with recombinant plasmsarin II were incubated with or without purified falcilysin, and subjected to reverse phase HPLC (Fig. 3). In the falcilysin-treated sample, the plasmsarin II-generated fragments were efficiently destroyed and several peaks representing peptide products appeared at lower retention times.

**Fig. 3.** Falcilysin cleaves hemoglobin fragments. Hemoglobin was digested with recombinant plasmsarin II, the resulting material was split into two equal portions, and each portion was incubated with (lower panel) or without (upper panel) purified falcilysin. Each sample was applied to an UltraspHERE C18 column for reverse phase chromatography with a linear gradient of 0–60% CH₃CN.

Nucleotide Sequence and Deduced Amino Acid Sequence of the Falcilysin Gene—Purified falcilysin was digested with endoproteinase Lys-C and amino acid sequences were determined for the resulting peptides. Eight sequences were obtained, and these were used to search for the falcilysin gene in the data bases of The International Malaria Genome Sequencing Consortium for the *P. falciparum* genome project. A match was obtained with preliminary shotgun data for chromosome 14 from The Institute for Genomic Research. This information allowed the falcilysin gene to be amplified from strain 3D7. BLAST searches (31) conducted with the falcilysin sequence revealed homology with other metallopeptidases (Fig. 6). While only one sequence is shown for each type of enzyme in this analysis, it should be noted that many of these enzymes have been identified in a wide variety of species. Fig. 6 includes only the β subunit of heterodimeric mitochondrial processing peptidase because the β subunit has been shown to be responsible for catalytic activity (32, 33) while the α subunit is thought to function in substrate recognition (34). All of these enzymes, including falcilysin, are classified in the family M16 of metallopeptidase clan ME (35). Clan ME metallopeptidases are defined by an active site motif, HXXEH. This sequence is inverted compared with the HEXHX motif found in the MA and MB clans. The inversion observed in metallopeptidase clan ME in fact extends beyond the HXXEH motif, as a hydrophobic residue found two residues COOH-terminal to the HEXHX motif of clans MA and MB is located two residues NH₂-terminal to the HXXEH motif of clan ME metallopeptidases (36). The M16 family has some common sequence features. Three residues function as zinc ligands, the two histidines of the HXXEH motif and a glutamic acid residue located 75–84 residues COOH-terminal to the HXXEH motif (37). Two other glutamic acid residues are part of the active site and are important for activity. The glutamic acid within the HXXEX motif is essential for activity but is not required for zinc binding (36, 38, 39). When the glutamic acid residue seven residues NH₂-terminal to the zinc ligand glutamic acid was mutated to glutamine in pitrilysin, the resulting enzyme retained only one-third of wild type activity and one-half the wild type amount of zinc (37). An asparagine located 23–28 residues COOH-terminal to the HXXEH motif is strictly conserved among all M16 family members known to be proteolytically active (35). A tyrosine located 129–163 residues COOH-terminal from the HXXEH motif is conserved among all members known to be active except AXL1 (35).

**DISCUSSION**

Our previous work suggested that there may be an additional unknown enzyme(s) in the food vacuole that participates in hemoglobin degradation (16). This paper describes the identification of a novel metallopeptidase activity in *P. falciparum*. Falcilysin was purified from food vacuoles and characterized. It is an oligoendopeptidase of the M16 family which contains two subfamilies. Subfamily B contains consists of the mitochondrial processing peptidases and chloroplast stromal processing peptidases that cleave NH₂-terminal signal peptides from proteins (35). Subfamily A of family M16 consists of large proteins (approximately 100 kDa or more) that function as oligoendopeptidases. Substrates for these enzymes include peptides such as α-factor (40, 41), insulin (42), glucagon (43), atrial natriuretic peptide (44), transforming growth factor α (45),

![Graph: Metal Concentration vs. Activity of falcilysin apoenzyme restored by metals]

![Graph: Activity of falcilysin apoenzyme restored by metals]
TABLE I

Peptides identified from samples of hemoglobin fragments incubated with falcilysin and in an untreated control

| Peptide mass | Peptide identity | +Falcilysin abundance | Control abundance | Role in reaction |
|--------------|------------------|-----------------------|-------------------|-----------------|
| 1877.9       | β33–47           | 31                    | 612               | Preferred substrate |
| 1471.9       | β33–45           | 49                    | 412               | Preferred substrate |
| 1906.0       | α34–45           | 55                    | 322               | Preferred substrate |
| 1701.0       | α34–47           | 94                    | 928               | Preferred substrate |
| 2096.4       | β13–32           | 100                   | 210               | Substrate |
| 2102.4       | α83–100          | 208                   | 345               | Substrate |
| 1200.9       | α91–100          | 2270                  | Background        | Abundant product |
| 803.3        | Unknown          | 711                   | Background        | Abundant product |
| 894.6        | α34–41           | 596                   | Background        | Abundant product |
| 1048.7       | Unknown          | 554                   | Background        | Abundant product |
| 544.4        | Unknown          | 549                   | Background        | Abundant product |
| 588.3        | Unknown          | 543                   | Background        | Abundant product |
| 509.3        | β129–132         | 505                   | Background        | Abundant product |
| 825.4        | α42–47           | 489                   | Background        | Abundant product |
| 465.3        | Unknown          | 447                   | Background        | Abundant product |
| 421.2        | Unknown          | 323                   | Background        | Product |
| 547.2        | α75–79a          | 291                   | Background        | Product |
| 548.4        | β41–47           | 272                   | Background        | Product |
| 558.4        | Unknown          | 248                   | Background        | Product |
| 510.8        | β74–78           | 120                   | Background        | Product |

* Indicates assignment by LC/MS only. Some masses could have been derived from more than one possible fragment and their identity is therefore marked as unknown.

Fig. 4. Falcilysin cleavage sites along the α and β chains of human hemoglobin. Peptide products of falcilysin digestion could be assigned to specific segments of the α and β chains of human hemoglobin by LC/MS or MS/MS (Table I). Deduced falcilysin cleavage sites are listed. A relative consensus sequence is displayed below. * + indicates that a charged residue is preferred.

The importance of zinc to intraerythrocytic *P. falciparum* has been demonstrated. Levels of zinc increase in parallel with parasite maturation within the red blood cell (50). Treatment of cultures with dipicolinic acid does not prevent schizont rupture or reinvasion of host erythrocytes but blocks parasite maturation from the ring to the trophozoite stage, coincident with the onset of hemoglobin degradation (50, 51). Inhibition of hemoglobin catabolism is lethal to intraerythrocytic *P. falciparum* (2, 7). The specific role of falcilysin in the essential process of hemoglobin degradation may have encouraging implications for the development of new chemotherapeutic agents. Plasmins I and II as well as falcipain prefer to cleave the α and β chains of hemoglobin at sites with hydrophobic residues at the P1 and/or P1’ positions (10). In contrast, falcilysin sites are polar in character, with charged residues at the P1 and/or P4’ positions. Internal cleavage of the large peptides generated by the plasmins and falcipain at distinct sites is likely to be critical for production of peptides small enough to cross the food vacuole membrane for cytosolic amino acid production. Falcilysin appears to play an integral role in this process.

- β-amyloid protein (46), somatostatin 28 (47), β-galactosidase (48), and now, peptide fragments of hemoglobin (this work). Inhibition by alkylating agents has been observed in inulosyn (42), nardilysin (47), mitochondrial processing peptidase of *Neurospora crassa* (49), and falcilysin (this work). Nardilysin is inhibited by the aminopeptidase inhibitors amastatin and bestatin (47) but this was not observed in falcilysin.

The M16 family of metallopeptidases is growing. Besides the addition of falcilysin presented in this work, BLAST searches revealed sequence characteristics common to M16 family members in several uncharacterized protein sequences from different organisms (Fig. 7). In fact, many potential M16 family members share greater sequence identity with falcilysin than the known members. Future investigations of these sequences will reveal which of these proteins function as metallopeptidases and whether one or more new types of enzymes with distinct biological functions are among them.

As expected for a metallopeptidase of this class, falcilysin is unable to cleave polypeptides such as the α and β chains of hemoglobin (141 and 146 amino acids in length) but is able to cleave within peptides generated by the other proteases of the food vacuole. In this study falcilysin cleaved peptides up to 20 amino acids and preferred those 11–15 residues in length (Table I). It thus functions downstream of other vacuolar proteases, providing strong evidence for order in the hemoglobin degradation process. Our current understanding of the sequential nature of the proteolytic events is that the initial cleavages of the native hemoglobin molecule are made by the plasmins. Denatured or fragmented globin is susceptible to cleavage by falcipain as well as the plasmepsins (15). Falcilysin cannot cleave hemoglobin or globin, but is able to cleave hemoglobin fragments. It is likely that many peptide intermediates are susceptible to cleavage by multiple enzymes. The distinct but overlapping roles of the proteolytic enzymes of the food vacuole and the location of exopeptidase activity across the food vacuole membrane in the parasite cytoplasm suggest that hemoglobin catabolism within *P. falciparum* is a semiordered pathway.
FIG. 5. The falcilysin gene encodes an 1193 amino acid protein containing internal peptide sequences obtained from the native enzyme. Residues are numbered at the end of each row. Underlined sequences represent all eight internal peptide sequences obtained by endoprotease Lys-C digestion of purified falcilysin.

FIG. 6. Sequence alignment of known members of metallopeptidase clan ME family M16. An alignment of amino acid residues 233–416 of nardilysin from Rattus norvegicus (Swiss-Prot P47245, 1161 amino acids, 17.9% identical to falcilysin), 87–280 of insulysin from Homo sapiens (Swiss-Prot P14735, 1019 amino acids, 18.6% identical to falcilysin), 56–245 of sporozoite developmental protein from Eimeria bovis (Swiss-Prot P42789, 596 amino acids, 19.1% identical to falcilysin), 67–257 of pitrilysin from E. coli (Swiss-Prot P05458, 962 amino acids, 20.6% identical to falcilysin), 47–247 of AXL1 from Saccharomyces cerevisiae (Swiss-Prot P40851, 1208 amino acids, 18.8% identical to falcilysin), 217–403 of chloroplast stromal processing peptidase from Pisum sativum (GenBank U25111, 1259 amino acids, 14.7% identical to falcilysin), 62–248 of mitochondrial processing peptidase b subunit from N. crassa (Swiss-Prot P11913, 476 amino acids, 21.2% identical to falcilysin), and 112–327 of falcilysin from P. falciparum (GenBank AF123458) is shown. Zinc ligands (*), active site residues (∧), and conserved residues (1) are labeled above the aligned sequences.

FIG. 7. Sequence alignment of potential members of metallopeptidase clan ME family M16 with falcilysin. An alignment of amino acid residues 42–239 of nardilysin from Rattus norvegicus (Swiss-Prot P47245, 1161 amino acids, 17.9% identical to falcilysin), 87–280 of insulysin from Homo sapiens (Swiss-Prot P14735, 1019 amino acids, 18.6% identical to falcilysin), 56–245 of sporozoite developmental protein from Eimeria bovis (Swiss-Prot P42789, 596 amino acids, 19.1% identical to falcilysin), 67–257 of pitrilysin from E. coli (Swiss-Prot P05458, 962 amino acids, 20.6% identical to falcilysin), 47–247 of AXL1 from Saccharomyces cerevisiae (Swiss-Prot P40851, 1208 amino acids, 18.8% identical to falcilysin), 42–238 of mitochondrial processing peptidase b subunit from N. crassa (Swiss-Prot P11913, 476 amino acids, 21.2% identical to falcilysin), and 112–327 of falcilysin from P. falciparum (GenBank AF123458) is shown. Zinc ligands (*), active site residues (∧), and conserved residues (1) are labeled above the aligned sequences.
Acknowledgments—We thank Anna Oksman for assistance with culturing parasites, Ilya Gluzman for providing recombinant plasmodisin II, Choukri Mamoun for genomic DNA and assistance with computer analyses, and Christoph Turek for determining internal peptide sequences.

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