The malaria parasite *Plasmodium falciparum* degrades host cell hemoglobin within an acidic food vacuole. The metalloprotease falcilysin has previously been identified as an important component of this catabolic process. Using random peptide substrate analysis, we confirm that recombinant falcilysin is highly active at acidic pH, consistent with its role in hemoglobin degradation. Unexpectedly, the enzyme is also robustly active at neutral pH, but with a substantially different substrate specificity. Imaging studies confirm the location of falcilysin in the food vacuole and reveal association with vesicular structures elsewhere within the parasite. These data suggest that falcilysin may have an expanded role beyond globin catabolism and may function as two different proteases in its two locations.

*Plasmodium falciparum* is the causative agent of the deadliest form of human malaria. Each year 500 million infections and up to 2 million deaths are attributed to *Plasmodium* parasites (1). This disease is a major public health issue in many areas of the world where the increasing prevalence of drug-resistant strains underscores the need to identify new drug targets. Examination of the parasite's unique metabolic pathways, such as hemoglobin degradation, provides numerous attractive candidates for chemotherapeutic development.

*Plasmodium* infects and develops within human erythrocytes where hemoglobin is degraded in a semi-ordered cascade of proteases to provide energy and nutrients to the growing parasite (2). Hemoglobin degradation occurs in the unique acidic environment of the food vacuole. A family of aspartic proteases, the plasmepsins, mediates the initial cleavage of the globin terminus (3, 4). The denatured globin is further degraded by plasmepsins in concert with a family of cysteine proteases, falcipain-2 and -3 (5, 6–7). The resulting globin peptides serve as substrates for acidic and neutral pH, consistent with the acidic environment of the food vacuole (9, 10). The temporal expression pattern of FLN is similar to that of other globinases yet differs by the absence of proteolytic processing during biosynthesis (11). FLN is a member of the M16 family of the clan ME zinc metalloproteases. This family is characterized by an inverted active site motif of HXXHEH (12), where the two histidines and a glutamic acid residue located 75–84 residues C-terminal to this motif are involved in coordinating the catalytic zinc ion. The M16 family is divided into subfamilies A and B. Subfamily B includes the mitochondrial processing peptidase (MPP) and the chloroplast stromal processing peptidase; both enzymes are involved in removing N-terminal targeting peptides (13–15). Falcilysin belongs to subfamily A whose members are oligoendopeptidases involved in processing biologically important peptides such as yeast α factor (16, 17), insulin (18), and transforming growth factor α (19). All of the subfamily A members are large enzymes (greater than 100 kDa) with the catalytic machinery located in the N terminus. The function of the C terminus remains unknown.

In the current study we explore the substrate preferences of FLN. These studies reveal a bimodal pH optimum with starkly different substrate specificities for acidic and neutral pH conditions. Immunolocalization studies reveal a second location for FLN, outside of the acidic food vacuole. These data point to the possibility of an expanded role for this protease in *Plasmodium falciparum* biology.

**MATERIALS AND METHODS**

**Culture of Parasites**

*P. falciparum* clone HB3 (Honduras, gift of W. Trager, Rockefeller University) was cultured using the methods of Trager and Jensen (20) in serum-free Roswell Park Memorial Institute media supplemented with 0.5% Albumax (Invitrogen, Grand Island, NY). Synchrony was maintained with sorbitol treatment (21).

**Antibody Production and Specificity**

The affinity-purified A1219T antibody was generated as previously described (11). For generation of 1624T, polyclonal rabbit antisera was generated using a synthetic eight-branch multiple antigenic peptide (MAP) peptide containing residues 603–612 of FLN (KKRIENFNEQ) (Invitrogen, Carlsbad, CA).

**Immunofluorescence Microscopy**

Infected RBCs at the trophozoite stage were prepared as previously described (22). Samples were immunolabeled with rabbit anti-FLN (A1219T, 1:10, or 1624T, 1:1000), mouse anti-PM IV (13.9.2 1:10), rat anti-Bip (1:1000, MR4, J. H. Adams). Secondary antibodies used for detection included fluorescein isothiocyanate-conjugated goat anti-mouse IgG, Alexa 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), Alexa 488 anti-rat IgG, and Alexa 488 goat anti-mouse IgG. Coverslips were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and viewed using a Zeiss axioskope microscope at 1000× magnification. Both αFLN antibodies gave similar results. There was no difference in FLN distribution in trophozoites or schizonts.

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The abbreviations used are: FLN, falcilysin; MPP, mitochondrial processing peptidase; RBC, red blood cell; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; EM, electron microscopy; MMP, metalloprotease.

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**Membrane Extraction**

Synchronized mid-stage trophozoite culture (~5 × 10⁴ parasites) was labeled with 150 μCi/ml [[³⁵S]methionine/cysteine (PerkinElmer Life Sciences, Boston, MA). After a 4-h incubation at 37 °C, parasites were harvested in a 10 mM phosphate buffer (pH 7.1) with protease inhibitors. Parasites were disrupted via sonication and divided into equal portions, which were centrifuged for 1 h at 214,000 × g at 4 °C. The resulting pellets were resuspended in 0.5 ml in one of several solutions: PBS, 0.5 mM NaCl in PBS, 100 mM Na₂CO₃ (pH 11), or 1% Triton X-100 in PBS. Extraction proceeded for 1 h on ice followed by a second centrifugation for 1 h at 4 °C. The resulting supernatant and pellet fractions were retained. The pellets were solubilized with 1% SDS. Immunoprecipitations with antisera A1219T (23) and autoradiography followed.

**Peptide Library Synthesis**

An N-terminal acetylated and C-terminal biotinylated random 10-mer peptide with an isokinetic mixture of 19 amino acids (except cysteine) was synthesized by Dr. Chistoph Turk (University of California, San Francisco). This library was used to determine the P motifs. Second generation libraries with fixed P positions and five random residues in the P5 positions were generated at the Tufts University protein core facility (Boston, MA). These libraries had free N termini and biotinylated C termini. The second generation libraries were used to predict preferred residues in the unprimed positions. The predicted optimal substrates were synthesized with DABCYL and EDANS modifications (24) by AnaSpec Inc. (San Jose, CA) for use in kinetic studies.

**Peptide Library Cleavage Assays**

To determine the P motive—50 pmol of purified recombinant FLN (11) was incubated with 100 nmol of N-terminally acetylated random 10-mer substrate in 100 mM NaOAc, pH 5.2 (acidic conditions), or 100 mM bis-Tris, pH 7.2 (neutral conditions), in 400 μl. Cleavage reactions were incubated at 37 °C for 4 h; samples were then heated to 100 °C for 3 min. Cleavage products were analyzed by Edman degradation (Midwest Analytical, St. Louis, MO). Control pH titrations with the buffers used in these experiments showed insignificant buffer effects on enzyme activity.

Amino acid preference for a single position was determined by subtracting a residue’s raw abundance in the cleavage reaction from the abundance of residue in a control enzyme-free reaction, which eliminates bias for amino acids eluting at positions with background interference. This corrected abundance was divided by the prevalence of that residue within the 10-mer library, to control for bias present within the library. These data were then normalized to an average amino acid abundance of 1. Similar results were obtained using incubations of 0.5 h and overnight. The data shown are the average of three incubations.

**To Determine the P motif**—Each experiment with a second generation library used substrate incubated with purified rFLN (1000:1 molar ratio), as well as no-enzyme reactions. Incubations proceeded in 100 mM NaOAc, pH 5.2 (acidic conditions), or 100 mM bis-Tris, pH 7.2 (neutral conditions), at 37 °C overnight; samples were then heated to 100 °C for 3 min. The sample with enzyme and substrate, along with a substrate-only sample, were incubated with an excess of avidin-Sepharose (0.6 ml) (Pierce, Rockford, IL) for 1 h at room temperature to remove the C-terminal portion of the cleavage product along with any uncleaved substrate prior to N-terminal sequence analysis.

The abundance of each amino acid was quantified for each position in the cleavage product by first subtracting the abundance of each residue from the background library (substrate only plus avidin). Next, the percent abundance of each corrected residue was divided by the abundance of the same corrected residue in the control, enzyme-free reaction without avidin pull-down. These data were also normalized to 1. There were three independent determinations of the unprimed motif. The data shown represents the average of these experiments.

**Single Substrate Kinetic Assays**—3.5 nm purified rFLN was incubated with the quenched fluorogenic peptides (final concentrations ranging from 0.031 μM to 30 μM) in the presence of 100 mM NaOAc, pH 5.2, or 100 mM bis-Tris, pH 7.2, at 37 °C. Each concentration was tested in duplicate or triplicate. A PerkinElmer Life Sciences LSS60 fluorimeter with an excitation wavelength of 338 nm (slit width 5) and emission detection at 490 nm (slit width 8) was used to monitor the generation of cleavage products in real-time at 37 °C. The cleavage reaction was followed for 50 s. Kₘ and Vₘₐₓ were determined by measuring the initial velocities of product appearance. Apparent kₐ values were calculated (kₐ = Vₘₐₓ/[E]) using the purified enzyme protein concentration (BCA, Pierce) as an estimate of active enzyme. Kinetic analyses were also performed with the optimized substrates over a range of pH conditions with overlapping buffers: pH 3.6–5, NaOAc; pH 5–7, bis-Tris; pH 7–10, Tris. Optimized substrates were compared with fluorogenic α74–75 (HVD) [DFPN] (8), a known FLN globin peptide substrate. rFLN exhibited poor activity (kₐ/Kₘ several fold lower than for α74–75) against a decameric version of this globin peptide.

**RESULTS**

**Determination of FLN optimal P Motifs—Random peptide libraries provide a facile tool to probe enzyme function and selectivity.** Previous data indicated that FLN displayed good activity with globin peptide substrates at pH 5.2 but fared poorly with the same substrates at neutral pH (8). Moreover, falcisycin globin substrates lack a strong consensus sequence (8). We therefore pursued a strategy similar to that of Turk et al. (25) to refine FLN cleavage specificity at acidic pH and to investigate the possibility of activity and specificity at neutral pH. Experiments were carried out at pH 5.2 to mimic the pH of the food vacuole and at pH 7.2 to reflect conditions in the rest of the parasite. A random 10-mer library was utilized to determine the primed (P⁺) side motif of substrates, and a second generation library with fixed P positions was used to identify the unprimed (P⁻) motif.

As described under “Materials and Methods,” an N-terminal-acetylated, C-terminal-biotinylated random 10-mer library was used to identify residues preferred in the P⁻ positions. As shown in Fig. 1A, FLN strongly favors bulky hydrophobic amino acids in the P¹ position at both acidic and neutral pH. At P2, FLN prefers hydrophobic residues at pH 5.2, at pH 7.2, these same residues are abundant but Arg is more strongly preferred. Hydrophobic residues, especially Met, predominate in the P³ position at both pH conditions. At P4’ and P5’, there is a strong selection of acidic residues at pH 5.2. At neutral pH, the enzyme is much less selective at these positions. An optimal motif for FLN under acidic conditions is FFMEME and under neutral pH conditions is FRMRG.

**Second Generation Libraries Reveal the FLN P Motif—To determine a motif for the unprimed positions, a second generation C-terminal-biotinylated library was constructed using the P⁻ motifs and randomized amino acids in the P⁺ positions.** As shown in Fig. 1B, FLN strongly favors Ser in the P1 position under both acidic and neutral conditions. At the P2 position, Met and His were the most abundant amino acids generated at acidic pH, whereas Asn and His were preferred at neutral pH. At the P3 position, acidic conditions yielded Glu and His; neutral conditions favored Arg and Gly. Asn was highly preferred at the P4 position at pH 5.2. In contrast, neutral pH conditions favored several residues, most prominently Lys. Tyr was the most abundant residue in the P5 position under acidic conditions while Met was highly preferred with neutral conditions. These experiments predict an optimal motif for acidic pH of YNEHS↓FFMEME. A substrate with the sequence MKRHS↓FRMRG should provide optimal cleavage at neutral pH.

**Kinetic Analysis of Optimized Substrates—Peptide library predictions can be evaluated by measuring the kinetic capabilities of optimized substrates.** Our standard of comparison is the peptide α74–75, which is a sequence in the hemoglobin alpha chain recognized by FLN for cleavage (8). To assess the contribution and overall importance of both the P⁻ and P⁺ portions of the optimized substrates, we synthesized substrates with a...
combinatorial approach. Our hypothesis, based on the published experience with other metalloproteases, was that the P motif would be the most important in determining overall catalytic efficiency. Furthermore, we predicted that a sequence optimized under one pH condition would not be as efficient at the opposite pH. The use of quenched fluorogenic substrates enabled us to kinetically assess each substrate by measuring the cleavage in real-time (Table I).

Analysis of α74–75-based substrates optimized in the P’ positions revealed that manipulation of these C-terminal sequences alone can confer a significant increase in the overall catalytic efficiency. Optimization with the P’ acid sequence (74–75/P’ acid) led to a 5.5-fold increase in $k_{cat}/K_m$ at pH 5.2. The P’ neutral sequence (74–75/P’ neut) bestowed a striking 24-fold increase in catalytic efficiency at pH 7.2. These increases arose mostly from a lower $K_m$, because $k_{cat}$ values remained within the same range. When each substrate was tested at the opposite pH from which it was optimized, there

![Fig. 1. FLN P’ and P motifs differ under acidic and neutral pH. Data for each primed (P1’ to P5’) and unprimed (P1 to P5) position, where recombinant enzyme was incubated with the random 10-mer peptide library under acidic (pH 5.2) or neutral (pH 7.2) conditions. Cleavage products were analyzed and quantified by Edman degradation. Data were normalized to 1, which represents the average amount of amino acid released in a given position. Amino acid abundance is depicted on the y-axis, and the one-letter amino acid symbols are on the x-axis. Acidic pH data are shown in yellow; neutral pH data are shown in blue. The red line demarcates an abundance of 1; residues at this level are neither preferred nor disfavored.](image)
was a decrease in $k_{cat}/K_m$ as compared with the preferred pH (a 4.6-fold decrease for P′acid and an 11-fold decrease with P′neutral). Again, this was mostly attributed to differences in substrate binding. The 74–75/P′acid substrate at the disfavored pH 7.2 was still, however, much more efficient than a74–75 ($k_{cat}/K_m$ of 970 versus 194). But a74–75 remained a better substrate than 74–75/P′neut at pH 5.2 ($k_{cat}/K_m$ of 928 as compared with 457). These data suggest that the P′ region of FLN substrates contributes substantively to catalytic efficiency.

Optimization in the P positions of 74–75-based substrates, however, argues that these residues are the greatest determinants of catalytic efficiency (Table I). Pacid/74–75 showed a 25-fold increase in $k_{cat}/K_m$ at pH 5.2, as compared with a74–75. We observed a similar pattern with Pneut/74–75, which produced a 320-fold increase in $k_{cat}/K_m$ at pH 7.2, as compared with a74–75. When each substrate was tested at the “disfavored” pH, we again observed decreases in catalytic efficiency. At pH 7.2, Pacid/74–75 produced an 11-fold decrease in $k_{cat}/K_m$ but remained a better substrate than a74–75 at the same pH. The $k_{cat}/K_m$ for Pneut/74–75 dropped 52-fold when the substrate was placed at pH 5.2. Interestingly, when compared with a74–75 at pH 5.2, Pneut/74–75 had a comparable efficiency ($k_{cat}/K_m$ of 928 and 1200, respectively). The changes in $k_{cat}/K_m$ for these substrates derive both from tighter binding and faster catalysis.

Complete optimization in both the P and P′ positions for acidic pH yielded an 180-fold increase in catalytic efficiency compared with a74–75. This was the result of a lower $K_m$ and a higher $k_{cat}$. As expected, this substrate performed less efficiently at neutral pH ($k_{cat}/K_m$ of 80,277 versus 147,949). This neutral value was still a 414-fold increase in $k_{cat}/K_m$ compared with a74–75 at pH 7.2. The Pneut/P′neut peptide yielded a 2,755-fold increase in $k_{cat}/K_m$ compared with a74–75 at neutral pH. This improvement was a result of tight substrate binding and faster catalysis. At pH 5.2, Pneut/P′neut yielded a 36-fold increase in catalytic efficiency compared with a74–75, a 158-fold decrease from the optimal performance of the substrate at pH 7.2. Fig. 2 shows the catalytic efficiencies of Pacid/P′acid and Pneut/P′neut from pH 3.6 to 10. Pacid/P′acid had two peaks of catalytic efficiency, one at pH 5 and the other at pH 7. Pneut/P′neut was dramatically different, exhibiting one broad peak of catalytic efficiency from pH 7 to 8.5.

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**Table I**

| Kinetic summary of optimized peptides |
|--------------------------------------|
| pH | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (μM⁻¹ s⁻¹) |
|----|------------|----------------|--------------------------|
| a74–75 | 5.2 | 11.5 ± 1.37 | 9.5 × 10⁻³ | 824 |
| Pacid/74–75 | 5.2 | 0.51 ± 0.05 | 2.31 × 10⁻³ | 4,529 |
| YNEHS | 7.2 | 1.7 ± 0.26 | 3.3 × 10⁻³ | 216 |
| AMHVD | 7.2 | 4.1 ± 0.8 | 1.66 × 10⁻³ | 970 |
| AMHVD/FMRRG | 7.2 | 0.81 ± 0.11 | 4.23 × 10⁻³ | 5,222 |
| Pneut/74–75 | 5.2 | 0.787 ± 0.1 | 1.67 × 10⁻² | 20,234 |
| MKRHS | 7.2 | 2.09 ± 0.32 | 3.98 × 10⁻³ | 1,906 |
| Pacid/P′acid | 5.2 | 0.317 ± 0.05 | 4.69 × 10⁻² | 147,950 |
| YNEHS/FMRRG | 7.2 | 0.169 ± 0.02 | 1.36 × 10⁻² | 80,277 |
| Pacid/P′neut | 5.2 | 6.27 ± 1 | 2.7 × 10⁻¹ | 43,242 |
| YNEHS/FMRRG | 7.2 | 0.972 ± 0.1 | 3.01 × 10⁻¹ | 309,414 |
| Pneut/P′neut | 5.2 | 12.1 ± 2 | 4.1 × 10⁻² | 3,371 |
| MKRHS/FMRRG | 7.2 | 0.405 ± 0.04 | 2.16 × 10⁻¹ | 534,532 |
| Pneut/P′acid | 5.2 | 0.5811 ± 0.08 | 4.1 × 10⁻² | 70,258 |
| MKRHS/FMRRG | 7.2 | 0.8 ± 0.08 | 1.61 × 10⁻² | 202,150 |

**Fig. 2.** Optimized substrates behave differently over a range of pH conditions. A, catalytic efficiency of Pacid/P′acid was measured under a variety of pH conditions. For pH 3.6–5 NaOAc was used, for pH 5–7 bis-Tris was used, and for pH 7–10 Tris was used. Initial velocities were measured in triplicate at multiple substrate concentrations to determine catalytic efficiency. B, a similar curve for Pneut/P′neut.

When the Pacid or P′acid motif was combined with the P′neut or Pneut motif, respectively, catalytic efficiency was lower than the fully optimized substrates, as expected (Table I). Optimized substrates with an amino acid substitution were also tested, because sometimes there was more than one preferred residue for a given position (Table II). For the pH 5.2-optimized sequence, we synthesized substrates with a change at the P2 (H to N) and P3 (E to H) positions. For the pH 7.2-optimized...
and exhibited no change in localization of FLN partners well with the dual localization data. Two independently anti-peptide polyclonal antibodies were used to probe the subcellular distribution of falcilysin.

An indirect immunofluorescence signal from the anti-FLN antibody could be detected in the food vacuoles of acetone-fixed cells (Fig. 3A), overlapping with the distribution of the food vacuole marker, plasmepsin IV. In addition, an FLN signal was found in vesicular structures within the parasite. Similar vesicular staining was seen with formaldehyde fixation, which would not permeabilize the food vacuole (Fig. 3B). The vesicular signal appeared to be specific, because Fig. 3B reveals that as little as 10 μg/ml of immunizing peptide was sufficient to block FLN staining. Staining with a second αFLN antiserum, 1624T, gave a pattern similar to that of A1219T. Pre-immune 1624 serum did not detect a signal by immunofluorescence (data not shown). Both antisera were monospecific by immunoblotting (data not shown). These data suggest that falcilysin resides in two locations within the parasite.

Co-localization studies were carried out with BiP, a marker of the endoplasmic reticulum (ER). The distribution of FLN was largely distinct from that of BiP (Fig. 3C) and remained so even after treatment with Brefeldin A (data not shown). Furthermore, FLN was confined to the parasite body, because signal from LWL-1, a marker of the parasitophorous vacuolar membrane, surrounded the FLN staining (Fig. 3D). Cryo-immunoelectron microscopy (EM) corroborated the immunofluorescence data; by EM, FLN localized to the food vacuole (Fig. 4A) and other vesicular structures within the parasite (Fig. 4B and C). Again, FLN and BiP had minimal overlap.

**FLN Is a Peripheral Membrane Protein—** Membrane extraction experiments were performed using radiolabeled trophozoite sonicates (Fig. 5). With PBS and 0.5 M NaCl extractions, FLN remained associated with the pellet. However, extraction in pH 11 carbonate resulted in a shift of FLN into the soluble, or supernatant fraction. FLN was detected in the supernatant fraction after 1% Triton X-100 detergent lysis (data not shown). This stands in contrast to mature plasmepsin I, which in these studies (not shown) and previous work (23) was detected in the supernatant fractions with all extraction conditions. These data indicate that FLN is peripherally associated with membranes.

**Immunofluorescence Suggests Multiple Locations for FLN—** FLN was originally purified from food vacuoles. Yet, in vitro, falcilysin displayed robust activity at neutral pH. This raised the question of whether FLN might be located in structures outside the acidic food vacuole. Two independent anti-peptide polyclonal antibodies were used to probe the subcellular distribution of falcilysin.

**The Dual Specificity of Falcilysin**

**Table II**

| pH  | $K_m$ μM | $k_{cat}$ s⁻¹ | $k_{cat}/K_m$ μM⁻¹ s⁻¹ |
|-----|----------|----------------|------------------------|
| 5.2 | 0.317 ± 0.05 | 4.69 × 10⁻² | 147,950 |
| 7.2 | 0.169 ± 0.02 | 1.36 × 10⁻² | 80,277 |
| 5.2 | 0.253 ± 0.02 | 3.26 × 10⁻² | 128,587 |
| 7.2 | 0.239 ± 0.03 | 1.6 × 10⁻² | 66,911 |
| 5.2 | 0.255 ± 0.04 | 1.87 × 10⁻² | 73,194 |
| 7.2 | 0.09 ± 0.01 | 1.52 × 10⁻² | 169,593 |
| 5.2 | 12.1 ± 2 | 4.1 × 10⁻² | 3,371 |
| 7.2 | 0.405 ± 0.04 | 2.16 × 10⁻¹ | 534,532 |
| 5.2 | 14.81 ± 1.7 | 2.99 × 10⁻² | 2,025 |
| 7.2 | 1.28 ± 0.17 | 3.86 × 10⁻¹ | 901,752 |

**Fig. 3.** FLN localizes to the food vacuole and other vesicular structures. A, indirect immunofluorescence using parasites fixed in acetone. FLN (A1219T) staining is red (left panel); PMIV staining is green (center). The merged image (right panel) has additional nuclear staining (DAPI) in blue. B, Left, αFLN (A1219T) staining of formaldehyde-fixed parasites; middle, αFLN Ab (A1219T) was preincubated with 10 μg/ml immunizing peptide; right, αFLN (1624T) staining of formaldehyde-fixed parasites. C, immunofluorescence with antibodies directed against FLN (red, A1219T) and BiP (green) after formaldehyde fixation. Merged image is shown on the right. D, immunofluorescence with antibodies directed against FLN (red, A1219T) and LWL-1 (green) after formaldehyde fixation. Merged image is shown on the right.

**DISCUSSION**

Prior to our current studies, all of the available data (8, 11) pointed to falcilysin as a food vacuole enzyme involved exclusively in hemoglobin degradation. However, the dual specificity of FLN partners well with the dual localization data. Two independent and specific αFLN antisera, under a variety of fixation conditions, document falcilysin localization to membranous structures within the parasite, in addition to the food vacuole. We cannot be sure of the identity of these membranes; there are morphologic similarities to endoplasmic reticulum, yet we observed minimal overlap in the distribution of the ER marker, BiP, and FLN. FLN staining was confined to the parasite body, because it is internal to the signal of LWL-1, a resident protein of the parasitophorous vacuolar membrane that delimits the parasite. It is possible that FLN, which lacks a signal peptide (11) and exhibits no change in localization after Brefeldin A treatment, is located in or traffics through the
vesicles associated with the alternative secretory pathway characterized by Brefeldin A-independent secretion (26–28). Previous work analyzing the biosynthesis of falcilysin indicates that the enzyme does not require proteolytic processing to be active (11). Therefore, these data and the specificity studies suggest that FLN may be active along its targeting route or may have a resident function in these vesicles.

The random peptide libraries document that FLN is indeed active at both pH 5.2 and 7.2. This is not unprecedented for proteases. The striking finding is the pH-dependent substrate selectivity of FLN. The consensus specificity at acidic pH ((Y/A)N(E/H)(X)(N/H)#(Y/F/L)(M/E/D)(E/D)) has stretches of neutral polar, bulky hydrophobic, and acidic residues. At neutral pH (MK/R/G)(H/A)(S/R)#(Y/F/L)(R/M/R) positively charged residues dominate. The pH-dependent switch in substrate specificity presumably is related to protonation changes within the active site, although distal changes leading to conformational differences are also possible. A crystal structure of FLN will likely be necessary to determine the substrate-switch mechanism. The mitochondrial processing peptidase (MPP) is the only M16 metalloprotease with structural information available (29), and it provides few clues for falcilysin. MPP and FLN share a low degree of homology (8), and belong to different M16 subfamilies. Furthermore, MPP differs in that is a α/β heterodimer with the active site lying at the subunit interface (29).

Kinetic analyses of the optimized substrates validated the peptide library predictions, because catalytic efficiencies were dramatically improved compared with the standard substrate α47–75. The α47–75 peptide was originally chosen for assay development, because it was a site in globin recognized by food vacuole lysate and not characteristic for the specificity of other known globin-degrading enzymes. This peptide is cleaved successfully by FLN, but the optimized substrates determined by the peptide library analysis are three orders of magnitude better. Pacid/P’acid was recognized optimally by FLN at pH 5, but also had a smaller activity peak at pH 7. In contrast, Pneut/P’neut was a poor substrate at pH 5; it was optimal from pH 7 to 8. Thus, using some substrates one would conclude that this is an acid protease and with other substrates one would think FLN is a neutral protease. Some change in substrate preference with pH has been seen with other proteases (30), but a bold switch like that of falcilysin has not been reported. FLN is virtually one protease at acidic pH and another at neutral/basic pH.

Whether pH-dependent specificity is a general feature of metalloproteases remains to be seen. However, falcilysin has specificity similarities to other metalloproteases. Mitochondrial processing peptidase is the falcilysin family member most extensively characterized. MPP favors aromatic residues in the P1’ position (31), as does FLN. Additionally, MPP substrates often carry an Arg in the P2 or P3 position (32, 33). The FLN motif for pH 7.2 contains an Arg at P3 and His at P2. Non-related metalloproteases such as MMP-7 have a strong preference for Ser in the P1 position (25). Ser is the clearly favored residue in P1 for falcilysin in both acidic and neutral pH. In the metalloproteases examined by Turk et al. (25), also via random peptide libraries, there is very little preference similarity elsewhere in the unprimed positions, but the primed positions do exhibit modest agreement with the FLN cleavage motifs. Leu is found in P1’ in every one of the six metalloproteases they analyzed (MMP-7, MMP1, MMP-2, MMP-9, MMP-3, and MMP-14). Additionally, Arg is present in each of the proteases at the P2’ position, which concurs with the motif for FLN at pH 7.2. Finally, Met is found for three of the six metalloproteases in the P3’ position. Both FLN motifs carry a Met in this same position. Comparing the FLN cleavage residue abundance values to those determined by Turk et al. (25) reveals that, with the exception of P1, FLN exhibits a stronger, more robust preference for residues in all positions. Turk and colleagues used a similar library approach to determine their cleavage motifs and yet the majority of the preferred residues have abundance values less than 2.0. The bulk of the preferred residues of falcilysin have abundance values above 3 and ranging as high as 6 to 11, even at distal residues P5 and P5’. In other words, falcilysin appears to be a more specific enzyme.

In summary, we present biochemical evidence that falcilysin is active at neutral pH and is found in non-food vacuole locations. This raises the possibility of an expanded role for FLN beyond globin catabolism. This notion is further supported by data that identify FLN as part of the Plasmodium sporozoite transcriptome (34) and proteome (35). Hemoglobin degradation does not occur in sporozoites, and falcilysin likely plays a more
fundamental role in parasite biology. We are currently using our neutral pH specificity data to aid in the identification of non-globin FLN substrates. Biochemical and mutational analyses are also underway to dissect the mechanism of the specificity switch in this unusual protease.

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