The *Plasmodium falciparum* cysteine protease falcipain-2 captures its substrate, hemoglobin, via a unique motif

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Falcipain-2 (FP2) is a papain family cysteine protease and important hemoglobinase of erythrocytic *Plasmodium falciparum* parasites. Inhibitors of FP2 block hemoglobin hydrolysis and parasite development, suggesting that this enzyme is a promising target for antimalarial chemotherapy. FP2 and related plasmoidal cysteine proteases have an unusual 14-aa motif near the C terminus of the catalytic domain. Recent solution of the structure of FP2 showed this motif to form a β-hairpin that is distant from the enzyme active site and protrudes out from the protein. To evaluate the function of this motif, we compared the activity of the wild-type enzyme with that of a mutant lacking 10 aa of the motif (Δ10FP2). Δ10FP2 had nearly identical activity to that of the wild-type enzyme against peptide substrates and the protein substrates casein and gelatin. However, Δ10FP2 demonstrated negligible activity against hemoglobin or globin. FP2 that was inhibited with trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (FP2 E-64) formed a complex with hemoglobin, but Δ10FP2 E-64 did not, indicating that the motif mediates binding to hemoglobin independent of the active site. A peptide encoding the motif blocked hemoglobin hydrolysis, but not the hydrolysis of casein. Kinetics for the inhibition of Δ10FP2 were very similar to those for FP2 with peptidyl and protein inhibitors, but Δ10FP2 was poorly inhibited by the inhibitory prodomain of FP2. Our results indicate that FP2 utilizes an unusual motif for two specific functions, interaction with hemoglobin, its natural substrate, and interaction with the prodomain, its natural inhibitor.

macromolecular interaction | malaria | drug discovery | antimalarial chemotherapy

Malaria is one of the most important infectious diseases in the world. *Plasmodium falciparum*, the most virulent human malaria parasite, is believed to cause hundreds of millions of illnesses and over a million deaths each year (1). The control of malaria has been hindered by increasing resistance of malaria parasites to available drugs (2). New antimalarial drugs, ideally directed against new targets, are urgently needed (3). Among potential new targets for antimalarial therapy are proteases that hydrolyze hemoglobin. Intraerythrocytic malaria parasites break down hemoglobin in an acidic food vacuole to supply amino acids for parasite protein synthesis and to maintain osmotic stability (4, 5). Multiple proteases appear to participate in hemoglobin processing (6). Cysteine protease inhibitors block hemoglobin hydrolysis, indicating that cysteine proteases play a key role in this process (7). Falcipain-2 (FP2) and falcipain-3 (FP3) are papain-family cysteine proteases of erythrocytrophic stages of *P. falciparum* that localize to the food vacuole and readily hydrolyze hemoglobin (8, 9). Disruption of the FP2 gene led to the accumulation of undegraded hemoglobin in trophozoites, confirming a critical role for this enzyme in hemoglobin hydrolysis (10). Inhibition of FP2 and related proteases led to a block in parasite development (11, 12) and cured mice with murine malaria (13, 14), and efforts to develop falcipain inhibitors as new antimalarial agents are underway. These efforts will be facilitated by a detailed understanding of the biochemical properties of FP2.

The four falcipains are classical papain-family (family C1A) cysteine proteases. FP2, a nearly identical copy, FP2', and FP3 are similar in sequence (68% identity between the catalytic domains of FP2 and FP3) and encoded within a 12-kb stretch of chromosome 11. These enzymes comprise the FP2 subfamily, which is represented in all other evaluated species of *Plasmodium* (15). Falcipain-1 (FP1) is much less similar in sequence (38% identity between the catalytic domains of FP1 and FP2), encoded by chromosome 14, and, as determined by recent gene disruption studies, not essential for erythrocytrophic parasites (16, 17). Other putative *P. falciparum* family C1 proteases (15, 18) are three dipeptidyl peptidases, one of which (dipeptidyl peptidase I) was recently characterized as an exopeptidase (19), and nine serine repeat antigens (SERAs), one of which (SERA-5, which has replacement of the catalytic Cys by Ser) was recently shown to have serine protease activity (20).

FP2 subfamily proteases have two features that are unusual for family C1A proteases. First, they encode short (~20 aa) N-terminal extensions of the catalytic domain that, uniquely among described papain-family proteases, permit folding of the mature proteins to active enzymes without the participation of the prodomain (21, 22). The N-terminal extensions of different FP2 subfamily enzymes have rather limited (~20–45%) sequence identity, but are functionally conserved (22). Second, FP2 subfamily proteases contain an unusual motif near the C terminus, between highly conserved active site histidine and asparagine residues (Fig. 1A). A motif of identical size (14 aa) is found in all studied proteases of this subfamily, although sequence identity is modest. Inspection of 607 family C1A cysteine protease sequences on the Merops database (http://merops.sanger.ac.uk; updated December 30, 2004) identified 40 sequences with insertions encoding >12 aa at this location. Notably, 18 of these sequences were those of falcipains and homologues from other plasmodial species. In addition, smaller motifs are present in the sequences encoding *P. falciparum* serine repeat antigens (10 aa) and dipeptidyl peptidase I (8 aa), and larger (>20 aa) motifs are present in two other putative *P. falciparum* dipeptidyl peptidase genes (15, 18).

To evaluate the function of the unusual C-terminal motif, we compared the biochemical properties of FP2 constructs with and without this structure. Our results indicate that the motif mediates specific interactions with hemoglobin and with the FP2 prodomain.

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Abbreviations: Fn, falcipain n; AMC, 7-amino-4-methyl coumarin.

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Experimental Methods

Expression, Purification, and Refolding of FP2 and Mutant Enzymes. FP2 was expressed and purified as described (23). To generate the Δ10FP2 mutant (with deletion of 15 amino acids: Gly-182 to Lys-196), we amplified two fragments from the pTOP-FP2 plasmid encoding FP2, using the primers 4TyrFP2 forward and FP2RH reverse primers. The DNA and dC2FP2 reverse primers were purified and refolded exactly as described for FP2 (23).

Appropriately digested plasmids (pQE-30; Qiagen), and used to transform E. coli (Qiagen). Proteins were confirmed by standard methods. Purified DNA fragments from the overlap extension reactions were cloned into the 4TyrFP2 forward and FP2RH reverse primers (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) and FP2RH reverse (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) in one reaction and dCFP2 forward (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) and FP2RH reverse (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) in the other. Products from the two reactions were then combined and used as templates in an overlap PCR extension (24) using 4TyrFP2 forward and FP2RH reverse primers. Similarly, to generate the Δ10FP2 mutant (with deletion of 10 amino acids: Glu-185 to Gly-194), we amplified fragments with the primers dC2FP2 forward (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) and FP2RH reverse (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) and FP2RH reverse (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) in one reaction and dCFP2 forward (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) and FP2RH reverse (5′-GGTTATTATTATATAATTAAGAACTCATGGGGA-3′) in the other. Products from the two reactions were then cloned into the pGEM-T vector (Promega). The sequences of recombinant genes were confirmed by standard methods. Purified DNA fragments were digested with BamHI and HindIII, ligated into appropriately digested plasmids (pQE-30; Qiagen), and used to transform M15 (pREP4) Escherichia coli (Qiagen). Proteins were purified and refolded exactly as described for FP2 (23).

Enzyme Kinetics. The concentrations of Δ10FP2 and Δ10FP2 were determined by titration with morpholine urea–leucine–homophenylalanine–fluoromethyl ketone (Mu-Leu-Hph-CH2F). Activity and inhibition of FP2 and mutant enzymes were studied as described (8, 21). Briefly, peptide 7-amino-4-methyl coumarin (AMC) substrates (50 μM) were added to 1 mM enzyme in 100 mM sodium acetate/8 mM DTT, pH 5.5, and release of the AMC group was monitored continuously for 20 min at room temperature with a spectrophotometer. The kinetic constants Km, Vmax, and kcat were determined by using PRISM (GraphPad, San Diego).

Hydrolisis of Protein Substrates. To assess gelatin hydrolysis, equal quantities (200 nM) of FP2 and Δ10FP2 were incubated with or without trans-epoxysuccinyl-1-leucylamido-(4-guanidino)butanate (E-64; 1 μM) in 20 mM Bis-Tris, pH 5.8 for 10 min at room temperature and then resolved by SDS/PAGE using nonreducing gels copolymerized with 0.1% gelatin (Sigma). The gels were then washed in 2% Triton X-100 to remove SDS, incubated overnight at 37°C in 100 mM sodium acetate, pH 5.5/8 mM DTT, and stained with Coomassie blue. To assess hydrolysis of casein (Sigma), FP2 and Δ10FP2 were incubated with 3 μg of casein in the presence or absence of E-64 (1 μM) for 2 h at 37°C in 100 mM sodium acetate, pH 5.5/8 mM DTT, and reaction products were resolved by SDS/PAGE and staining with Coomassie blue. As a second measure of casein hydrolysis, FITC-casein (Sigma; 10 μg) was incubated with different quantities of proteases (2–14 μg per 350 μl) at room temperature for 10 min and fluorescence resulting from hydrolysis was measured (excitation 485 nm, emission 527 nm).

For evaluation of hydrolysis of native human hemoglobin and human globin (Sigma), the proteins (3 μg) were incubated with 200 nM FP2 or Δ10FP2 in a 30-μl reaction for 2 h at 37°C in 100 mM sodium acetate/8 mM DTT, pH 5.5, and reaction products were resolved by SDS/PAGE and staining with Coomassie blue. As a second assay of hemoglobin hydrolysis, hemoglobin (30 μg) was incubated with 200 nM enzyme in 100 mM sodium acetate, pH 5.5/2 mM reduced glutathione at 37°C for 2 h, absorbance at 410 nm was monitored at 37°C with a spectrophotometer, and the absorbance of each sample, expressed as percentage of the absorbance of hemoglobin with buffer only, was subtracted from 100 to yield percent hydrolysis.

Interaction Between FP2 and Hemoglobin. Equal quantities of FP2 or Δ10FP2 (400 nM) were incubated with E-64 (1 μM) for 10 min to block their active sites; a lack of activity against Z-Leu-Arg-AMC after this incubation was confirmed with the spectrophotometric assay described above. The enzymes were then incubated with 3 μg of native hemoglobin in 20 mM Bis-Tris, pH 5.8 for 30 min at room temperature. The proteins were then mixed with native gel (without SDS) loading buffer (50 mM Tris, pH 6.8/0.1% bromophenol blue/10% glycerol), resolved by 5% native PAGE, and visualized with Coomassie blue staining.

Plasmon resonance based biomolecular interaction analysis was done by using a BIACore 1000 analytical system at room temperature (25). A carboxymethylated dextran matrix CM 5 sensor chip was preconditioned following the manufacturer’s instructions. A total of 10 μl of 250 μg/ml hemoglobin was immobilized on the chip by using 20 mM phosphate, pH 7.3 at a rate of 5 μl/min. Unreacted N-hydroxysuccinimide ester groups were inactivated by using 1 M ethanolamine-HCl. The hemoglobin-coupled flow cell was equilibrated in 20 mM Bis-Tris, pH 5.8. FP2E-64 and Δ10FP2E-64 were then injected over the
sensor chip at 25 μl/min, and signals, representing binding to immobilized hemoglobin, were recorded. The background signal was measured by injecting the same volume of running buffer over the coupled flow cell, and regeneration of the flow cell was achieved by sequential salt washes using 1 M NaCl and 4 M MgCl2. All buffers and samples were filtered (0.2 μm) before analyses.

Determination of the Complex Ratio of FP2 and Hemoglobin. A Superose 6 column (Amersham Pharmacia) was precalibrated (flow rate, 0.5 ml/min) with blue dextran and standard proteins (thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B12; Amersham Pharmacia) using 20 mM Bis-Tris, pH 5.8 at 4°C. A total of 200 μg of FP2, hemoglobin, and the FP2E-64, hemoglobin complex were loaded by using the same buffer and flow rate at 4°C, and masses were determined based on comparison with standards.

Dynamic light scattering (26) experiments were carried out with a Zetasizer Nano ZS (Malvern Instruments) using a helium–neon gas laser (wavelength, 633 nm; power, 5 mW). Samples were analyzed in the above-mentioned buffer at a concentration range of 0.5–2 mg/ml, after filtration with a 0.2-μm filter. All measurements were at 25°C with a 173° scattering angle. Population distributions were fitted to the experimental correlation curve by using the CONTIN program provided by the manufacturer.

Effect of the Motif Peptide on Hydrolysis of Casein and Hemoglobin by FP2. Hydrolysis of FITC-casein and hemoglobin by FP2 was assayed with and without a peptide corresponding to 15-aa spanning the motif, using the same buffers described above for measurements of FP2 activities against these substrates. The peptide was preincubated with 10 μg of FITC-casein or 30 μg of native hemoglobin for 20 min at room temperature before addition of 200 nM FP2. For FITC-casein, fluorescence resulting from hydrolysis was measured as described above. For hemoglobin, hydrolysis was assessed spectrophotometrically, as described above.

Inhibitor Studies. Leupeptin, E-64, and chicken egg white cystatin were from Sigma, and the FP2 prodomain was obtained as described earlier (21). To produce chagasin, the gene encoding this inhibitor was cloned into the pQE-30 vector (Qiagen) and transformed into M15 (pREP4) E. coli (Qiagen). Soluble protein was coexpressed with GroEL (Qiagen) at 28°C before purification with a nickel-nitriolactiace acid column (Qiagen). For reversible inhibitors, different concentrations of compounds were incubated with 1 nM enzyme in the same buffer as for activity assays at room temperature, the substrate Z-Leu-Arg-AMC (4 μM) was added, product formation was monitored continuously for 20 min, and Kd, values were determined by nonlinear regression analysis using PRISM. To determine first-order inactivation rate constants (kobs) for the irreversible inhibitor E-64, progress curves (fluorescence versus time) were analyzed by nonlinear regression analysis. FP2 and Δ10FP2 (0.6 nM) were incubated with different inhibitor concentrations (0–600 nM) in the buffer described above with Z-Leu-Arg-AMC (25 μM). Product formation was continuously monitored for 10 min at room temperature. Plots of kobs versus the inhibitor concentration were used to determine kobs by the progress curve method (27) under pseudo-first-order conditions.

Results

FP2 and Δ10FP2 Have Similar Activities Against Peptide Substrates. We constructed two mutants of FP2, one lacking the full C-terminal motif (Δ10FP2) and the other lacking the 10 aa that make up the core of the motif (Δ10FP2) (Fig. 1). The mutants and FP2 were expressed in E. coli, purified by affinity chromatography, refolded in optimized refolding buffer, and further purified by ion-exchange chromatography (8). Δ10FP2 had limited activity against peptide and protein substrates (kcat/Km for Z-Leu-Arg-AMC = 580 M⁻¹s⁻¹), indicating that the larger mutation structurally compromised the enzyme, and this mutant was not studied further. In contrast, the activity of Δ10FP2 was nearly identical to that of FP2 against typical peptide substrates, indicating that the active site was not appreciably altered after removal of the motif (Table 1).

Loss of the FP2 C-Terminal Motif Ablates Hemoglobinase Activity, but Activity Against Other Protein Substrates Is Not Altered. We compared the hydrolysis of the protein substrates gelatin and casein by Δ10FP2 and FP2. Δ10FP2 and FP2 efficiently hydrolyzed both substrates with similar activity (Fig. 2). However, results were very different in a comparison of hydrolysis of the biologically relevant substrates hemoglobin and globin. FP2 readily hydrolyzed either substrate under conditions predicted for the P. falciparum food vacuole, as described in ref. 8, but Δ10FP2 had negligible activity against either hemoglobin or globin (Fig. 3). Thus, the motif is required for hemoglobin hydrolysis by FP2.

The Motif Mediates Interaction Between FP2 and Hemoglobin. To evaluate the interaction between FP2 and hemoglobin, we preincubated FP2 and Δ10FP2 with the covalent inhibitor E-64 to prevent proteolysis, and then incubated the proteases with hemoglobin. Evaluation of the proteins by native PAGE (lacking

<table>
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<tr>
<th>Substrate</th>
<th>FP2</th>
<th>Δ10FP2</th>
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<tbody>
<tr>
<td>Z-L-R-AMC</td>
<td>106,000 ± 2,180</td>
<td>103,000 ± 3,530</td>
</tr>
<tr>
<td>Z-V-R-AMC</td>
<td>5,440 ± 168</td>
<td>5,900 ± 250</td>
</tr>
<tr>
<td>Z-V-L-R-AMC</td>
<td>61,900 ± 5,503</td>
<td>64,500 ± 2,120</td>
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Results include standard deviations from two independent measurements, each performed in triplicate.
SDS) indicated formation of a complex between FP2E-64 and hemoglobin, but not \( ^{\text{10}}\text{FP2E-64} \) and hemoglobin (Fig. 4A). The interaction was further evaluated by using surface plasmon resonance based biomolecular interaction analysis. This analysis clearly indicated specific interaction between FP2E-64 and immobilized hemoglobin, but not between \( ^{\text{10}}\text{FP2E-64} \) and hemoglobin (Fig. 4B). An association constant could not be determined because of apparent unusual kinetics of FP2–hemoglobin interaction.

To assess the stoichiometry of the FP2–hemoglobin complex, we evaluated the rates of migration of FP2, hemoglobin, and the FP2E-64–hemoglobin complex by gel filtration chromatography. The comparative migration rates of free molecules and the complex indicated that four molecules of FP2 bind to each hemoglobin tetramer (Fig. 4C). A dynamic light scattering analysis also indicated a ratio of FP2 and hemoglobin in the complex of 4:1.

**Interaction Between FP2 and Hemoglobin Is Prevented by a Peptide Encoding the Motif.** We evaluated the effect of a peptide encoding the motif on hemoglobin hydrolysis by FP2. The peptide blocked hydrolysis of hemoglobin by FP2 in a dose-dependent manner (Fig. 5B). Interestingly, the peptide had no effect on hydrolysis of casein by FP2 (Fig. 5A). These results confirm that the FP2 motif mediates specific interactions with hemoglobin, but not with other protein substrates.

The FP2 Motif also Mediates Interaction with the Prodomain. We also evaluated the impact of removal of the motif on inhibition of FP2 by reversible and irreversible protease inhibitors. Among revers-
Table 2. Comparative inhibition of FP2 and Δ10FP2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FP2</th>
<th>Δ10FP2</th>
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<tbody>
<tr>
<td>Kᵣ, mM</td>
<td>0.18 ± 0.11</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>1.7 ± 0.53</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>Chagasin</td>
<td>6.5 ± 1.4</td>
<td>9.5 ± 2.3</td>
</tr>
<tr>
<td>Cystatin</td>
<td>0.33 ± 0.14</td>
<td>898 ± 71</td>
</tr>
<tr>
<td>Prodomain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kₛₑ, M⁻¹s⁻¹</td>
<td>12.28 (±1.40) × 10⁵</td>
<td>9.98 (±1.97) × 10³</td>
</tr>
</tbody>
</table>

Results include standard deviations from two independent measurements, each performed in triplicate.

Discussion

FP2 and closely related cysteine proteases of malaria parasites have an unusual motif near the C terminus. Insight into this motif came from solution of the structure of FP2 (S.X.W., K.C.P., J. Somoza, L. Brinen, P.S.S., P.J.R., and J.H.M., unpublished data). The 14-aa motif forms two extended β-strands that are connected by an abrupt turn and protrude from the enzyme surface. A deep cleft is found between the base of the motif and the protease core. This structure suggested a role for the unique FP2 motif in interacting with other proteins. Because FP2 plays a principal role in the hydrolysis of hemoglobin by erythrocytic parasites (10), a logical hypothesis is that the unusual motif facilitates this process. To test this hypothesis, we removed the motif and compared the biochemical properties of FP2 constructs with and without this structure. Our results show that the motif does, indeed, mediate hemoglobin hydrolysis by FP2. In addition, the motif mediates inhibition of FP2 by its prodomain.

Removal of the motif did not alter the ability of FP2 to hydrolyze a number of peptide or protein substrates or to be inhibited by peptidyl or protein inhibitors. Strikingly, however, removal of this motif left the protease unable to hydrolyze its natural substrate, hemoglobin, and relatively insensitive to inhibition by its natural inhibitor, the prodomain. Thus, the motif mediates the activity of FP2 that is most relevant biologically, the hydrolysis of hemoglobin, which is a central process of erythrocytic malaria parasites (4). Furthermore, it mediates control of this activity by its relevant inhibitor, the prodomain. FP2 may also have other functions in erythrocytic malaria parasites. It cleaves ankyrin and protein 4.1 at neutral pH, and the protease may hydrolyze erythrocyte cytoskeletal proteins to mediate egress of mature parasites from erythrocytes (28). The role of the motif in hydrolysis of other proteins of potential biological relevance has not yet been explored.

Of interest, genes encoding other plasmodial family C1 proteases also include motifs between conserved active site amino acids near the C terminus. For other members of the FP2 subfamily, it is likely that the encoded motifs share the functions of the FP2 motif, because a number of members of this subfamily have been shown to be hemoglobinases (15). Sequence conservation among the motifs of FP2 subfamily proteases is not high, but, as in the case of the N-terminal extension that mediates protein folding (22), the presence of these unusual motifs in all related proteases probably indicates a conserved function. For genes encoding more distantly related enzymes, including FP1, dipeptidyl peptidases, and serine repeat antigens, C-terminal motifs vary greatly in sequence and size, and specific functions of predicted motifs are undefined. Nonetheless, the presence of a motif at this location in genes encoding all plasmodial family C1 cysteine proteases, but few other family C1 proteases, is intriguing.

Our data indicate that FP2 captures hemoglobin via its C-terminal motif before subsequently cleaving the substrate at the protease active site. The structure of FP2 indicates a protruding configuration for the motif, surrounded by a predominant negative charge (Fig. 1B; S.X.W., K.C.P., J. Somoza, L. Brinen, P.S.S., P.J.R., and J.H.M., unpublished data). Nega-

charged residues adjacent to the motif, including Asp-154, Asp-155, GLu-167, and Asp-170, extend from the motif to the active site. We propose that hemoglobin, which has many charged surface residues, first binds at the motif, is brought closer to the active site through charge–charge interactions, and is then hydrolyzed by the standard mechanism for papain-family cysteine proteases.

The function of the motif in mediating catalysis despite its separation from the active site somewhat resembles that of the hemopexin domain of matrix metallopeptases. The hemopexin domain is required for both the efficient cleavage of collagen (29) and the formation of complexes with both inhibitors and proteases to mediate biological activities (30). Thus, as is the case for the FP2 motif, the hemopexin domain serves to facilitate biologically relevant protein–substrate and protein–inhibitor interactions. However, the hemopexin domain is ∼20 times larger than the FP2 motif and, unlike the motif, is separated from the catalytic domain by a hinge region. Therefore, it appears that FP2 and matrix metallopeptases use similar means of biological control, but the specific mechanisms by which the proteases mediate interactions with substrates and inhibitors are very different. Another papain-family protease, cathepsin K, forms a pentameric complex with chondroitin sulfate to facilitate hydrolysis of collagen (31). Analogous to our results with FP2 and hemoglobin, structural requirements for complex formation between cathepsin K and chondroitin sulfate are required for hydrolysis of the biological substrate (collagen), but not gelatin. The cathepsin K Y212C mutation specifically prevents collagen (but not gelatin) hydrolysis, and leads to the bone disease pycnodysostosis (32). Another parallel is the inhibition of serine proteases by the protein inhibitorhirudin. Hirudin inhibits thrombin via binding both at and distant from the active site of the protease (33). In an analogous fashion, for FP2 interaction at a location distant from the active site is necessary for potent inhibition by the prodomain.

Why does FP2 contain a unique motif that mediates both the hydrolysis of hemoglobin and inhibition by the prodomain? This mechanism did not evolve simply to facilitate hemoglobin hydrolysis, because other papain-family enzymes without C-terminal motifs, including those of organisms without hemoglobins (e.g., papain), can efficiently degrade hemoglobin. Papain-family proteomes are also potent inhibitors of their cognate proteases. It seems that the utilization of a specific motif at this region to mediate enzyme–substrate interaction is an unusual but conserved feature of plasmodial cysteine proteases. It is possible that an evolutionary bottleneck occurred, with the presence of an ancestral plasmodial cysteine
protease that was relatively inactive and required augmentation to mediate specific efficient proteolytic functions, such as the facilitation of hemoglobin hydrolysis by the FP2 motif. Other plasmodial proteases may use their motifs to mediate other functions, and it will be of interest to test this possibility once the functions of other proteases are elaborated. Our results demonstrate a unique feature of plasmodial proteases that might be exploited in drug design. In the case of FP2, a unique motif is required for hemoglobin hydrolysis, and disruption of motif-hemoglobin interaction should offer a novel means of inhibition of parasite development.

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