Independent Intramolecular Mediators of Folding, Activity, and Inhibition for the Plasmodium falciparum Cysteine Protease Falcipain-2*

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The Plasmodium falciparum cysteine protease falcipain-2 is a trophozoite hemoglobinase and potential antimalarial drug target. Unlike other studied papain family proteases, falcipain-2 does not require its prodomain for folding to active enzyme. Rather, folding is mediated by an amino-terminal extension of the mature protease. As in related enzymes, the prodomain is a potent inhibitor of falcipain-2. We now report further functional evaluation of the domains of falcipain-2 and related plasmodial proteases. The minimum requirement for folding of falcipain-2 and four related plasmodial cysteine proteases was inclusion of a 14–15-residue amino-terminal folding domain, beginning with a conserved Tyr. Chimeras of the falcipain-2 catalytic domain with extensions from six other plasmodial proteases folded normally and had kinetic parameters ($k_{cat}/K_m$ 124,000–195,000 M$^{-1}$ s$^{-1}$) similar to those of recombinant falcipain-2 ($k_{cat}/K_m$ 120,000 M$^{-1}$ s$^{-1}$), indicating that the folding domain is functionally conserved across the falcipain-2 subfamily. Correct folding also occurred when the catalytic domain was refolded with a separate prodomain-folding domain construct but not with an isolated folding domain peptide. Thus, the prodomain mediated interaction between the other two domains when they were not covalently bound. The prodomain-catalytic domain interaction was independent of the active site, because it was blocked by free inactive catalytic domain but not by the active site-binding peptide leupeptin. The folded catalytic domain retained activity after purification from the prodomain-folding domain construct ($k_{cat}/K_m$ 168,000 M$^{-1}$ s$^{-1}$), indicating that the folding domain is not required for activity once folding has been achieved. Activity was lost after nonreducing gelatin SDS-PAGE but not native gelatin PAGE, indicating that correct disulfide bonds are insufficient to direct appropriate folding. Our results identify unique features of the falcipain-2 subfamily with independent mediation of activity, folding, and inhibition.

Malaria is one of the most important infectious diseases in the world, infecting hundreds of millions of people and causing over one million deaths each year (1). Control of malaria is increasingly difficult, in large part because of the spread of drug resistance, particularly in Plasmodium falciparum, the most virulent human malaria parasite (2). There is thus an urgent need for the development of new antimalarial drugs (3). One approach to antimalarial drug discovery is the identification of unique and essential parasite metabolic processes and the design of inhibitors of enzymes that are active in these processes (4).

Intraerythrocytic malaria parasites hydrolyze hemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis and preserve osmotic stability (5–7). Cysteine protease inhibitors block hemoglobin hydrolysis, causing the food vacuole to fill with undegraded hemoglobin and indicating that cysteine proteases play a key role in this process (8–10). The principal cysteine protease hemoglobinases of P. falciparum are falcipain-2 and falcipain-3, two related papain family cysteine proteases (11, 12). Inhibitors of these proteases block hemoglobin hydrolysis, prevent parasite development, and cure mice infected with malaria parasites (5, 13, 14). Falcipain-2 and falcipain-3 are thus promising new chemotherapeutic targets, and their characterization, including the elucidation of properties that differ from those of host proteases, is an important goal.

Papain family proteases typically are produced as inactivezymogens and then fold to active enzymes in a process mediated by a fairly well conserved prodomain (15–17). Falcipain-2 shares prodomain homology with papain and related proteases, but unlike any other studied papain family enzymes, it did not require the prodomain for folding (18). Rather, expression of mature falcipain-2 as insoluble inclusion bodies, followed by solubilization and an in vitro refolding protocol, produced abundant active enzyme. Falcipain-2, falcipain-3, and related proteases from other plasmodial species have an unusual 17–23-residue amino-terminal extension of the mature protease between highly conserved catalytic domain and prodomain sequences (5). We recently showed that this amino-terminal extension is required for folding to active enzyme and that it can mediate this process either when covalently bound to the catalytic domain or when included in refolding buffer as part of a separate prodomain-folding domain construct (18). The small folding domain did not mediate folding of the catalytic domain when included as a free peptide in refolding buffer. Thus, when the folding domain was not bound to the catalytic domain, the prodomain was required to mediate interactions that led to folding.

Our prior studies identified a unique folding domain of falcipain-2 but left many unanswered questions. First, what is the minimum extension required for folding? Second, is the folding domain functionally conserved among the falcipain-2 subfamily of plasmodial proteases, the only enzymes so far identified...
with this domain? Third, is the amino-terminal extension with this domain? We now report a series of mutagenesis studies that have answered these questions and shown that enzyme activity, folding, and inhibition are each controlled by proteolytic activity.

**EXPERIMENTAL PROCEDURES**

**Amplification and Cloning of Expression Constructs—** Constructs were amplified using vent DNA polymerase (New England Biolabs) and the primers listed below. PCR templates were the pTOP-2FP plasmid for falcipain-2 (11), the pQF-FP3 plasmid for falcipain-3 (12), a previously constructed pQE-30 plasmid for behcapan-2, Plasmodium vivax DNA (kindly provided by John Barnwell) for vivapain-2, and Plasmoid vivax DNA (kindly provided by Alan W. Thomas) for Plasmodium knowlesi DNA (kindly provided by John Barnwell) for vivapain-2, and vivax DNA (kindly provided by Alan W. Thomas) for Plasmodium knowlesi control of proteolytic activity.

**Expression, Refolding, and Purification of Protease Constructs—** Expression was induced with isopropyl-β-D-thiogalactopyranoside, and the expressed proteins were purified by nickel-nitrotriacetic acid chromatography under denaturing conditions as previously described (19). Optimal refolding buffers for each construct were determined by a microtiter plate refolding assay, as previously described (18). In brief, the proteins were diluted to 10 μg/ml in 350 μl of ice-cold refolding buffer and incubated at 4 °C for 20 h, and the refolding efficiency was then evaluated by assaying 20-μl samples for hydrolysis of benzoyl-arginine-4-methyl coumarin (Z-Leu-Arg-AMC; Pep- tides International), as described below. The optimized buffers were: for falcipain-2 constructs, 100 mM Tris-Cl, pH 9.0, 1 mM EDTA, 50% glycerol, 1 mM GSH and 1 mM GSSG; for falcipain-3 constructs, 100 mM Tris, pH 9.0, 1 mM EDTA, 15% sucrose, 250 mM l-arginine, 1 mM GSH, 0.5 mM GSSG; for vivapain-2 and knockapain-2, 100 mM Tris, pH 9.0, 1 mM EDTA, 1 M KCl, 20% sucrose, 5 mM GSH, 1 mM GSSG; and for behcapan-2, 100 mM Tris, pH 8.5, 1 mM EDTA, 0.4 M l-arginine, 1 mM GSH, 0.5 mM GSSG. Large scale refolding, processing, and purification were performed as previously described (19) with the refolding buffers listed above. All of the refolding reactions were carried out for the same duration (20 h).

For purification of the falcipain-2 catalytic domain (19αSpFP2) from a 100 mM refolding-domain construct (Pro-FoldingFP2), the proteins were bound to a Q-Septo bonds (Amersham Biosciences) column in 10 mM Tris, pH 8.0; the column was washed with five column volumes of 200 mM NaCl in 10 mM Tris, pH 8.0, and the proteins were eluted with a gradient of 200 mM, 1 mM NaCl in 10 mM Tris, pH 8.0. Determination of protein concentrations was performed using the Bradford method (20).

**Measurement of Protease Activity and Kinetics—** Activity was measured as the hydrolysis of the fluorogenic substrate Z-Leu-Arg-AMC by 25 μl of each refolded reaction (in 350 μl of 100 mM sodium acetate, 50 mM Z-Leu-Arg-AMC, 8 mM dithiothreitol (DTT), pH 5.5). Fluorescence (excitation, 355 nm; emission, 460 nm) resulting from hydrolysis of substrate was continuously measured at room temperature. Enzyme kinetic studies were performed as previously described (12) except that the reductant and detergent were omitted. Kinetic assays were performed using the Bradford method (20). For kinetic assessments, the rate of hydrolysis of Z-Leu-Arg-AMC in the presence of constant enzyme concentration and varying substrate concentrations was determined at room temperature in 100 mM sodium acetate, pH 5.5, 8 mM DTT. The kinetic constants K_m and V_max were determined using Prism (GraphPad Software).

**PAGE—Reducing SDS-PAGE and gelatin substrate SDS-PAGE were performed as previously described (18). For native gelatin substrate PAGE, a 10% native gel (without SDS) was co-polymerized with 0.1% gelatin; the proteins were incubated with loading buffer (50 mM Tris, pH 6.8, 0.1% bromophenol blue, 10% glycerol) for 10 min at room temperature; and they were then resolved on acrylamide gels using a running buffer (25 mM Tris, pH 6.8, 50% glycerol). Native gelatin substrate gels were incubated overnight at 37 °C in 100 mM sodium acetate, pH 5.5, 8 mM DTT and visualized by Comassie Blue staining.

**Immunoblotting—** The proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were blocked with 2% bovine serum albumin overnight at 4 °C, and then incubated with phosphorylated (pS20) or nonphosphorylated (PBST) incubated with rat antiserum raised against the falcipain-2 heavy chain (1:1500 dilution) for 60 min at room temperature, washed with PBST, incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma) at 1:2000 dilution for 60 min at room temperature, washed with PBST, and developed with 5-bromo-4-chloro-3-indolyl
nated with an asterisk), falcipain-3 (P. vivax), and homologs from (FP2, FP3), berghepain-2, knowlepain-2, and vivapain-2 constructs were synthesized corresponding to mature falcipain-2 including the minimum extension required for folding (4TyrFP2) and to the catalytic domain (19AspFP2), based on homology with papain and other proteases. Recombinant proteins were expressed in bacteria, purified under denaturing conditions, and refolded in optimized refolding buffer, and soluble protein was then purified by ion exchange chromatography. Equal quantities of each protein were electrophoresed. Proteins, labeled based on the amino-terminal amino acid, were then resolved by reducing SDS-PAGE (A) and stained with Coomassie Blue. Active proteases are identified as clear bands by gelatin substrate SDS-PAGE. The positions of molecular mass standards are shown in kilodaltons.

**RESULTS**

Identification of the Minimum Amino-terminal Extension Required for Folding of Falcipain-2—We previously showed that, unlike the case with other studied papain family proteases, the mature domain of recombinant falcipain-2 was capable of folding to active enzyme without the prodomain. However, folding did require a 17-residue amino-terminal extension of the mature protease (18). Comparison of cysteine protease sequences shows similar extensions for closely related plasmodial enzymes but not other papain family cysteine proteases (Fig. 1). To determine the minimum folding determinant within the extension, we expressed and purified seven mutants containing deletions truncating the extension and compared activities of the mutants with that of mature falcipain-2 (1GlnFP2, which contains the full 17-amino acid extension) after refolding of equal quantities of protein (Fig. 2). Deletion of the three most amino-terminal residues yielded a construct (4TyrFP2) that refolded normally, with nearly the same activity as 1GlnFP2 containing the full 17-amino acid extension after refolding of equal quantities of protein (Fig. 2). Deletion of the three most amino-terminal residues yielded a construct (4TyrFP2) that refolded normally, with nearly the same activity as 1GlnFP2 (97.3%, based on the rate of hydrolysis of Z-Leu-Arg-AMC). In contrast, six constructs with shorter amino-terminal extensions, including deletion of a single additional residue (5GluFP2), were completely inactive (Fig. 2). These data indicate that the minimum domain necessary for the folding of mature falcipain-2 begins with a well conserved Tyr residue (Fig. 1).

The Amino-terminal Extension Is Functionally Conserved within the Falcipain-2 Subfamily—The falcipain-2 subfamily consists of falcipain-2 and falcipain-3 from P. falciparum and homologs from other plasmodial species (5). These proteases share a number of unusual features, including the amino-terminal extension, but sequence conservation between these extensions is modest (for the sequences shown, 20–46% identity with the extension of falcipain-2; Fig. 1). To determine whether the extensions are functionally conserved as folding domains, we expressed other plasmodial cysteine proteases phosphate/nitro blue tetrazolium tablets according to the manufacturer’s instructions (Sigma).

**Competitive Inhibition Studies—**To assess the interactions between recombinant prodomain and catalytic domain constructs, varied concentrations of the constructs and leupeptin (Sigma) were incubated in assay buffer (100 mM sodium acetate, pH 5.5, 8 mM DTT) for 15 min at room temperature before adding Z-Leu-Arg-AMC and measuring hydrolysis as described above.

**FIG. 1.** Alignment of amino-terminal extensions of falcipain-2 and related proteases. Extensions and flanking sequences of falcipain-2 (FP2), falcipain-3 (FP3), and homologs from P. vivax (VP2 and VP3), P. knowlesi (KP2 and KP3), Plasmodium berghei (BP2), and Plasmodium vinckeii (VP2) were aligned with those of papain (Pap) and cruzain (Cru) using the DNASTAR program (CLUSTAL method), with some adjustments based on visual comparison. The sequences were previously deposited in GenBank™ except for those of P. knowlesi, which are from the National Center for Biotechnology Information Plasmodium Genome Data base. The amino-terminal cleavage sites of the mature proteases, when known, are indicated by bold letters. Amino acids that are identical to those of falcipain-2 and falcipain-3 are shaded, and gaps required for alignment are indicated by dashes. The Tyr that is the most upstream residue required for folding activity is indicated with an arrow, and the active site Cys is indicated with an asterisk.

**FIG. 2.** Minimum amino-terminal extension required for folding of falcipain-2. Constructs including different portions of the amino-terminal extension and the full catalytic domain of falcipain-2 were expressed in E. coli, purified under denaturing conditions, and refolded in optimized refolding buffer, and soluble protein was then purified by ion exchange chromatography. Equal quantities of each protein were electrophoresed. Proteins, labeled based on the amino-terminal amino acid, were then resolved by reducing SDS-PAGE (A) and stained with Coomassie Blue. Active proteases are identified as clear bands by gelatin substrate SDS-PAGE. The positions of molecular mass standards are shown in kilodaltons.
each enzyme, the constructs including the folding domain (corresponding to 4TyrFP2) but not truncated constructs (corresponding to 19AspFP2) refolded to active proteases (Fig. 3). Therefore, all of the tested members of the falcipain-2 subfamily require a short amino-terminal extension starting with a conserved Tyr residue, but not the prodomain, to mediate folding to active enzymes.

To further assess functional conservation, we expressed, refolded, and purified chimeras with extensions from six other proteases (falcipain-3, vivapain-2, vivapain-3, knowlepain-2, knowlepain-3, and berghepain-2) fused to the catalytic domain of falcipain-2. Each chimera refolded into active protease, with similar refolding efficiency, activity, and kinetic properties to those of falcipain-2 (Fig. 4 and Table I).

The Amino-terminal Extension Is Required for Folding but Not for the Activity of Falcipain-2—We previously showed that the amino-terminal extension of falcipain-2 could mediate folding of the protease either when covalently attached to the catalytic domain or when provided in trans by co-incubating a prodomain extension construct with the catalytic domain (18). It was not clear from these studies, however, whether the amino-terminal extension is required only for folding or whether it also plays an essential role in mediating enzyme activity. To address this question, we expressed the falcipain-2 catalytic domain (19AspFP2) and placed it in refolding buffer either alone or with a separate recombinant prodomain-folding domain construct (Pro-FoldingFP2). Consistent with our previous results, when refolded alone, the catalytic domain yielded inactive protease (19AspFP2Inactive), but when refolded in the presence of Pro-FoldingFP2, it yielded active falcipain-2 (19AspFP2Active). To determine whether the folding domain was required for activity, we incubated Pro-FoldingFP2 and 19AspFP2 in refolding buffer and then separated them by anion exchange chromatography (Fig. 5). Effective separation of the two polypeptides was confirmed by immunoblot analysis. After purification, refolded 19AspFP2Active showed activity...
very similar to that of 4TyrFP2 (Table II). Thus, the amino-terminal extension is required for folding of falcipain-2, but once folding has been achieved, the extension is not required for enzyme activity.

**Maintenance of Disulfide Bonding Is Not Sufficient to Allow Refolding of the Falcipain-2 Catalytic Domain—Falcipain-2 and other papain family proteases demonstrate activity on substrate gels after nonreducing SDS-PAGE, removal of SDS, and incubation in a protease assay buffer. However, activity is not seen if the proteases are electrophoresed in a reducing buffer. Thus, these denatured mature proteases can fold to active enzymes provided that disulfide bonds have not been broken. To determine whether intact disulfide bonds were sufficient to allow refolding of 19AspFP2 after it was refolded to active enzyme (19AspFP2Active) and then denatured, equal amounts (1 μg) of refolded 4TyrFP2, 19AspFP2Active, and 19AspFP2Inactive were subjected to gelatin substrate SDS-PAGE and native gelatin substrate PAGE (Fig. 6). Refolded 4TyrFP2 was active in both gel systems, and 19AspFP2Inactive was inactive, as expected. 19AspFP2Active was active against Z-Leu-Arg-AMC (330 fluorescence units/min/μg) with and native gelatin substrate PAGE but not gelatin substrate SDS-PAGE. 19AspFP2Active and 19AspFP2Inactive had identical mobilities under either reducing or nonreducing conditions (Fig. 6C), suggesting that folding in the presence or absence of the folding domain did not impact on disulfide bonding. Therefore, maintenance of disulfide bonding with substrate SDS-PAGE was insufficient to allow refolding, whereas non-denaturing electrophoresis retained the active conformation of the protease. These results indicate that, even when disulfide bonding is preserved, correct folding of falcipain-2 requires mediation by the folding domain.

**Folding Interactions between the Pro- and Catalytic Domains Are Independent of the Active Site**—We showed previously that, as is the case with other papain family proteases, the prodomain is a potent inhibitor of falcipain-2 (18). The catalytic domain refolds to active enzyme in the presence of the prodomain-folding domain construct but not with the isolated folding domain peptide (18). Thus, prodomain-catalytic domain interaction is required for the folding activity of the extension when it is not covalently bound to the catalytic domain. To further assess prodomain-catalytic domain interactions, we measured the ability of the inactive catalytic domain (19AspFP2Inactive), to compete with the active catalytic domain (19AspFP2Active) for inhibition by folded prodomain (ProFP2). As measured with the substrate Z-Leu-Arg-AMC, ProFP2 inhibited 19AspFP2Active, and 19AspFP2Inactive blocked this inhibition in a dose-dependent manner (Fig. 7). This result suggests that 19AspFP2Active and 19AspFP2Inactive interacted similarly with the prodomain. In contrast, 19AspFP2Inactive did not block the inhibitory activity of the peptidyl cysteine protease inhibitor leupeptin. These results indicate that, despite structural differences that have a profound impact on activity, correctly or incorrectly folded catalytic domains share the structural features necessary for interaction with the prodomain. The finding that the inactive catalytic domain competed with the active catalytic domain but not with the active site ligand leupeptin argues that interactions between catalytic and prodomains of falcipain-2 require associations independent of the active site.

**DISCUSSION**

Falcipain-2 and related cysteine proteases of malaria parasites are worthy of detailed study, because these enzymes play critical roles in mediating hemoglobin hydrolysis by erythrocytic parasites and are thus promising chemotherapeutic targets (5). It is of particular interest to characterize features of the plasmodial proteases that differ from those of other papain family cysteine proteases. We previously showed that, unlike the case for other studied papain family proteases, folding of falcipain-2 was mediated by a short amino-terminal extension of the catalytic domain and not by the prodomain. We have now conducted a series of mutagenesis studies to better characterize the independent functions of different portions of falcipain-2. The short folding domain was essential to allow folding to active protease. However, although it is a part of mature falcipain-2, the folding domain was not required for activity after folding had taken place. Thus, the catalytic domain, which is homologous to the mature domains of other papain family enzymes, solely mediates activity. The prodomain exhibited the inhibitory activity seen for other papain family prodomains and also, although not required for folding, allowed productive interaction between the folding and catalytic domains. In summary, our results identify independent domains

![Fig. 5. The folding domain is not required for falcipain-2 activity.](image-url)
with nonoverlapping functions for the folding, inhibition, and catalytic activity of falcipain-2 and indicate that specific interactions between these domains are required for control of enzyme activity.

In addition to their roles as mediators of folding, the prodomains of many papain family enzymes are potent inhibitors of their cognate proteases (17, 21–25). However, it is generally unclear whether the folding and inhibitory functions of these domains are linked. Our results indicate that, for falcipain-2, independent portions of the enzymes mediate folding and inhibition.

We further explored the nature of the folding information imparted by the falcipain-2-folding domain. A feature of papain family enzymes is that they can be denatured in SDS under nonreducing conditions and then, when SDS is removed, refold to active enzymes. However, the correctly folded falcipain-2 catalytic domain could not refold after nonreducing denaturation. Thus, in addition to maintenance of disulfide bonding, the folding domain is required to allow correct folding. For other papain family enzymes, which lack any amino-terminal extension, this additional requirement for refolding is clearly not present.

The folding domain of falcipain-2 exerts equivalent activity when acting in cis (bound to the catalytic domain) or in trans (bound to a prodomain construct). Thus, the prodomain has two functions related to catalysis, mediation of folding domain-catalytic domain interaction and enzyme inhibition. To determine whether these two activities are due to the same molecular interactions, we compared effects on the inhibitory activity of the proform and the small active site inhibitor leupeptin in the presence of inactive and active catalytic domain. The inactive catalytic domain, but not leupeptin, competed with active catalytic domain for binding to the prodomain. These results lead to two interesting conclusions. First, the degree of folding of the isolated catalytic domain, although not adequate to allow enzyme activity, did allow interaction with the prodomain such that it competed stoichiometrically for binding with correctly folded falcipain-2. Thus, relevant folding occurs independent of the folding domain, and this domain appears to be particularly responsible for the control of enzyme activity, presumably by its impact on active site architecture. Second, the prodomain interaction necessary to mediate activity of the folding domain did not require a functional active site, because this interaction was not altered by the presence of leupeptin.

Our studies of the recombinant falcipain-2 catalytic domain showed that it refolded as a soluble protein, but that activity was seen only when it included a 14-amino acid amino-terminal extension, or when this extension was provided in trans bound to the prodomain. The limitation on activity of the catalytic domain was thus not solubility but rather enzyme structure. As noted above, our results suggest that the inactive refolded catalytic domain did attain an adequate degree of structure to allow functional interaction with the prodomain. This result suggests that the refolded catalytic domain may represent a stable folding intermediate. It is possible that, as in the case of a kinetically trapped folding intermediate of the bacterial serine protease α-lytic protease (26, 27), the isolated falcipain-2 catalytic domain may be stable but may require an additional domain, in this case the small folding domain (in cis or in trans

### Table II

| Enzyme          | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------------|--------|-----------|---------------|
| 4TyrFP2         | 7.82   | 0.95      | 120,000       |
| 19AspFP2Active  | 7.99   | 1.34      | 168,000       |

The table shows kinetic parameters for folded falcipain-2 (4TyrFP2) and the purified folded catalytic domain (19AspFP2Active).
bound to the prodomain), to cross a kinetic barrier to attain the active conformation. It will be interesting to investigate whether the refolded but inactive catalytic domain is indeed kinetically trapped.

The roles of different portions of prodomains of other papain family proteases have not been extensively characterized. Considering the mediation of folding, a 22-amino acid deletion in the carboxyl terminus of the prodomain of cathepsin L prevented folding (17), whereas expression of the protease with only the 38 carboxyl-terminal residues of the prodomain allowed folding to active enzyme, albeit with a low yield (17). Considering inhibition, amino-terminal, but not carboxyl-terminal deletions of the prodomain of cathepsin L caused the loss of inhibitory activity (25). Crystallographic studies with cathepsin L (28) and cathepsin K (29) and our results with falcipain-2 suggest that for papain family proteases, an upstream globular portion of the prodomain is largely responsible for inhibition, and a downstream portion is responsible for folding of the catalytic domain. Uniquely for falcipain-2 and related plasmodial proteases, the downstream folding domain is not cleaved from the catalytic domain in the mature protease. The portions of prodomains that appear to mediate inhibition are quite well conserved, including well characterized ERFVIN and GNFD domains that likely mediate prodomain-catalytic domain interaction (30). However, even among the plasmodial proteases, the folding domains show only modest sequence identity, although they are functionally conserved, with the mediation of folding of falcipain-2 by the domains of related enzymes. Comparing falcipains with other papain family proteases, it is interesting that, although the mature protease cleavage sites differ, papain and some other members of the family show some homology within their proforms with the falcipain-2-folding domain. Notably, the first five amino acids of the folding domain of falcipain-2 (YEEV) are homologous to a region of papain (YEEVL) that is just upstream of the mature protease cleavage site (Fig. 1). This observation is consistent with similar roles because folding domains for a carboxy-terminal portion of the papain prodomain and the amino-terminal portion of mature falcipain-2.

It is not clear why malaria parasites have evolved a unique mechanism for the intramolecular control of cysteine protease activity. This mechanism may offer a tighter degree of control than that for other papain family proteases, because for falcipain-2, in addition to the inhibitory control provided by the prodomain, an additional level of interaction is required between two domains of the mature protease before enzyme activity is achieved. Falcipain-2 and falcipain-3 are food vacuole hemoglobinases, but recent findings have suggested other roles for the proteases, including the hydrolysis of host cytoskeletal proteins ankyrin and protein 4.1 to facilitate erythrocyte rupture (31). If falcipains must act in multiple intracellular locations, the additional level of control of activity may afford additional protection against deleterious proteolysis and better control of falcipain activity within the host erythrocyte.

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