Papain-family cysteine proteases of the malaria parasite *Plasmodium falciparum*, known as falcipains, are hemoglobinases and potential drug targets. Available data suggest that papain-family proteases require prodomains for correct folding into functional conformations. However, in prior studies of falcipain-2, an *Escherichia coli*-expressed construct containing only a small portion of the prodomain refolded efficiently, suggesting that this enzyme differs in this regard from other papain-family enzymes. To better characterize the determinants of folding for falcipain-2, we expressed multiple pro- and mature constructs of the enzyme in *E. coli* and assessed their abilities to refold. Mature falcipain-2 refolded into active protease with very similar properties to those of proteins resulting from the refolding of proenzyme constructs. Deletion of a 17-amino acid amino-terminal segment of the mature protease yielded a construct incapable of correct folding, but inclusion of this segment in *trans* allowed folding to active falcipain-2. The prodomain was a potent, competitive, and reversible inhibitor of mature falcipain-2 ($K_i, 10^{-10} \text{ M}$). Our results identify a chaperone-like function of an amino-terminal segment of mature falcipain-2 and suggest that protease inhibition, but not the mediation of folding, is a principal function of the falcipain-2 prodomain.

Malaria remains one of the most important infectious diseases in the world. *Plasmodium falciparum*, the most virulent human malaria parasite, is responsible for hundreds of millions of illnesses and more than one million deaths each year (1). Because available antimalarial agents are limited by drug resistance, toxicity, and cost, new drugs, ideally directed against new targets, are urgently needed (2). Among promising drug targets are proteases that hydrolyze hemoglobin during infection and thereby provide amino acids for parasite protein synthesis (3). Cysteine protease inhibitors blocked hemoglobinolytic activity in the erythrocytic stage of infection and thereby provided amino acids for parasite protein synthesis (3). Cysteine protease inhibitors blocked hemoglobinolytic activity in the erythrocytic stage of infection and thereby provided amino acids for parasite protein synthesis (3). Cysteine protease inhibitors blocked hemoglobinolytic activity in the erythrocytic stage of infection and thereby provided amino acids for parasite protein synthesis (3).

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Folding of the *Plasmodium falciparum* Cysteine Protease Falcipain-2 Is Mediated by a Chaperone-like Peptide and Not the Prodomain*

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The abbreviations used are: Z-Phe-Arg-AMC, benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl coumarin; Z-Leu-Arg-AMC, benzyloxycarbonyl-Leu-Arg-7-amino-4-methyl coumarin; Ni-NTA, nickel nitrilotriacetic acid; DTT, dithiothreitol.

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Both pSET-B and pQE-30 expression vectors add His-tag fusions at the amino terminus of the foreign proteins that allow one-step purification of recombinant proteins by nickel-nitrotriacetic acid (Ni-NTA) chromatography. Expression was induced with isopropyl β-D-thiogalactoside (IPTG), and expressed proteins were purified by Ni-NTA chromatography under denaturing conditions as described above. To purify further, Ni-NTA-purified proteins were bound to a SP-Sepharose column (Amersham Biosciences) and eluted with a step gradient of NaCl in 8 mM urea, 20 mM Tris-Cl, pH 8.0.

To determine the best refolding buffer for each of the constructs, 176 combinations of 11 different buffers were tested by a microtiter plate refolding assay (8). Refolding buffers for each protein were tested in 100 ml of each buffer, 50 mM sodium phosphate, pH 7.0, 50 mM EDTA, pH 9.0 (A) plus 500 mM l-arginine (B), 250 mM l-arginine plus 5% glycerol (C), 30% glycerol (D), 250 mM l-arginine plus 30% glycerol (E), 20% sucrose (F), 250 mM l-arginine plus 20% sucrose (G), 1 mM KCl (H), 1 mM KCl plus 20% glycerol (I), 1 x KCl plus 20% sucrose (J), and 16 different combinations of reduced (GSH) and oxidized (GSSG) glutathione as described above (8). For the refolding assay, 3 μl of denatured-reduced protein (150 pmol in 8 mM urea, 20 mM Tris-Cl, 200 mM imidazole, 10 mM dithiothreitol (DTT), pH 8.0) was added to 297 μl of one-icold refolding buffer and incubated at 4 °C for 20 h. Refolding was measured as the hydrolysis of Z-Leu-Arg-AMC by 25 μl of each refolding reaction in 350 μl of 100 mM sodium acetate, 50 μM Z-Leu-Arg-AMC, 10 mM DTT, pH 5.5 assayed fluorometrically, as previously described (11). For large scale refolding, equal amounts (100 nmol) of each denatured-reduced protein were diluted 100-fold in 200 ml of the optimal refolding buffer for each protein, containing 1 mM each GSH and GSSG (buffers B for −188FP2, C for −89FP2, and E for −35FP2 and FP2) and incubated at 4 °C for 20 h. Insoluble protein was removed using a 0.22-μm membrane. Each refolding reaction was concentrated to 20 ml, brought to pH 5.8 and 5 mM DTT at room temperature for 1.5 h (1 for −35FP2 and FP2), the pH was raised to 6.25, the protein was applied to a Q-Sepharose column, and active protease was eluted using a gradient of 1 M NaCl in 20 mM bis-Tris, pH 6.25. Elution fractions containing protease activity (indicated by Z-Leu-Arg-AMC hydrolysis) were combined, concentrated to 1–2 ml, and diluted with an equal volume of glycerol for storage. Quantities of unfolded expressed proteases were estimated by the Bradford assay (12). To estimate recovery of active protease, enzyme concentrations were determined by active-site titration with Z-Phe-Arg-FMK (13) before (−35FP2 and FP2) or after (−188FP2 and −89FP2) purification.

Amino-terminal Sequencing and Substrate SDS-PAGE—For amino-terminal sequencing, purified active products of −188FP2, −89FP2, −35FP2, and FP2 were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to an Immobilon-P SQ membrane (Millipore), stained with Coomassie Blue, excised, and sequenced at the Protein and Nucleic Acid Facility, Stanford University Medical Center. For substrate SDS-PAGE, protease samples were mixed with non-reducing SDS-PAGE sample buffer and electrophoresed in a 12% SDS-polyacrylamide gel. The gel was washed twice with 2.5% polyvinylpyrrolidone, and expressed proteins were purified by Ni-NTA chromatography under denaturing conditions as described above. To purify further, Ni-NTA-purified proteins were bound to a SP-Sepharose column (Amersham Biosciences) and eluted by a step gradient of NaCl in 8 mM urea, 20 mM Tris-Cl, pH 8.0.

Effect of the Prodomain on the Catalytic Properties of Falcipain-2—Protease concentrations were determined by active-site titration with Z-Phe-Arg-FMK. Constant amounts (1.0 μM with Z-Leu-Arg-AMC and 2.0 μM with Z-Phe-Arg-AMC) of enzyme were incubated with different concentrations of substrate, hydrolysis over time was assessed fluorometrically (excitation 395 nm, emission 480 nm) for 15 min (Z-Leu-Arg-AMC) or 30 min (Z-Phe-Arg-AMC) in 100 mM sodium acetate, 10 mM EDTA, 500 mM l-arginine-HCl, pH 7.5, incubated at 4 °C for 20 h, and concentrated (using a 10-kDa cut-off membrane, Millipore) to 10 μl. Insoluble protein was removed using a 0.22-μm syringe filter. Different concentrations of −188FP2 were preincubated with 0.96 μM falcipain-2 derived from 4 different constructs in 100 mM sodium acetate, 10 mM DTT, pH 5.5, for 10 min at room temperature. The substrate Z-Leu-Arg-AMC (4 μM, 2-fold below Ki) was added, and fluorescence was continuously measured for 10 min. Pseudo-first order conditions were assumed (substrate hydrolysis was shown to be <10%) (15). The results were plotted as (V/Vo) − 1 versus I, where Vo is the uninhibited rate, V the rate in the presence of the inhibitor, and I is the concentration of inhibitor. From this plot, the Ki,app was determined (slope = 1/Ki,app) and used to calculate the true Ki using the relationship Ki = Ki,app/1 + [S]/Ki,app, where [S] is the substrate concentration.

RESULTS

Unusual Features of Plasmodial Cysteine Proteases—Falcipain-2 and falcipain-3 are fairly typical papain-family proteases, but they share some unusual features, including large prodomains, a putative type II membrane-spanning region near the amino terminus, and some unique features of the mature protease sequence (6, 7). Most relevant for this study, the two plasmodial proteases lack the typical papain-family mature protease cleavage site, where cleavage occurs immediately upstream of the sequence XP, where X represents a nonpolar amino acid about 25 amino acids upstream of the catalytic cysteine (16) (Fig. 1). The conserved proline is seen in nearly all known papain-family proteases other than falcipain-2 and falcipain-3. Determination of the amino-terminal
sequences of native mature falcipain-2 and processed recombinant falcipain-2 and falcipain-3 showed that these two enzymes contain an amino-terminal extension, with an additional 17 amino acids in falcipain-2 and 26 amino acids in falcipain-3 relative to other papain-family proteases (Fig. 1). The sequences of the two plasmodial amino-terminal extensions are 47% identical and do not show significant identity with any other protein sequences in available data bases.

The Falcipain-2 Protease Domain Refolds without Its Prodomain—To investigate the role of the falcipain-2 prodomain, we expressed four constructs including mature falcipain-2 with different portions of the prodomain (\(-188\)FP2, \(-89\)FP2, and \(-35\)FP2) and mature falcipain-2 alone (FP2) in E. coli (Fig. 2, A and B). Each of the proteins was refolded at alkaline pH and underwent autohydrolysis at acid pH to a product of identical size (Fig. 2C). Amino-terminal sequencing of the active products revealed the native mature falcipain-2 terminus for the FP2 construct (QMNYEE) and microheterogeneity (DQMNYEE and MNYEE) for the three prodomain constructs. All four refolded proteins were enzymatically active, as demonstrated by hydrolysis of the substrates Z-Leu-Arg-AMC and gelatin (Fig. 2D). The refolding of each construct to active protein varied with the composition of refolding buffer and redox couple, as seen previously (8), but optimized recovery for each product was similar (Fig. 2A). The progress of refolding to active enzyme for each protein was very similar, with maximum activity achieved after \(-18\) h (Fig. 3). Thus, in contrast to all other described papain-family proteases, the prodomain of falcipain-2 is not required for folding of the active enzyme. Furthermore, including the prodomain in expression constructs does not improve the speed or efficiency of folding over that of the mature protease.

Prodomain Does Not Alter the Catalytic Behavior of Falcipain-2—It has recently been shown that alterations in prodomains can result in differential properties of mature proteases that are identical in sequence but have been processed from different proenzyme constructs (17). To determine whether this “memory” phenomenon was evident in falcipain-2, we compared catalytic properties of the four active proteases generated from different falcipain-2 constructs. The four proteases cleaved peptide substrates with very similar kinetics (Table I). Thus, the falcipain-2 prodomain does not appear to impart folding characteristics on the mature protease that impact on catalytic activity.
The Amino-terminal Extension of Mature Falcipain-2 Is Essential for Folding—Considering the lack of a requirement for the prodomain for folding of mature falcipain-2, we considered the role of the unusual amino-terminal extension of this mature protease. Three constructs from which the 17-amino acid extension was deleted were expressed in E. coli (Fig. 4, A and C), purified, and refolded. Proenzyme constructs from which the 17-amino acid extension was deleted (~188dFP2, ~89dFP2) and mature falcipain-2 lacking the 17-amino acid extension (dFP2) were all incapable of folding to active enzyme in any of the 176 tested buffers (Fig. 3). Thus, the short amino-terminal extension of falcipain-2 is required for correct folding of the mature protease.

The Amino-terminal Extension Can Mediate Folding in Trans—To determine whether the amino-terminal extension must be covalently linked to the remainder of the mature protease for folding, prodomain constructs with (~188P17, ~89P17) or without (~188P, ~89P) the amino-terminal extension were expressed (Fig. 4, B and D) and incubated with the mature protease lacking the extension (dFP2) in standard folding buffers (Fig. 5). The extension-deleted mature protease (dFP2) was successfully folded to active enzyme in combinations that included extended prodomain constructs (~188P17 or ~89P17) but not in those including prodomains without the extension (~188P, ~89P; Fig. 5). Thus, presentation of the amino-terminal extension of mature falcipain-2 in trans was adequate to mediate correct folding of truncated mature falcipain-2. When a synthetic peptide identical to the 17-amino acid extension was incubated with the extension-deleted mature protease (dFP2), folding to active enzyme was not seen (Fig. 5). Our results indicate that the short amino-terminal falcipain-2 extension does not need to be covalently bound to the mature protease to mediate folding. However, the free peptide could not mediate folding, suggesting that, when it is not bound to the remainder of the mature protease, the extension must be presented by the prodomain, presumably after this domain binds to the mature protease via structural features maintained by well conserved “ERFNNI” and other residues in many papain-family enzymes (Fig. 1; Refs. 18 and 19).

The Prodomain Is a Potent Inhibitor of Falcipain-2—In addition to their roles as mediators of folding, the prodomains of many papain-family (20, 21) and other (22, 23) proteases are potent inhibitors of their cognate proteases, but it is not clear if the folding and inhibitory functions of these domains are linked. It was of interest to determine whether the falcipain-2 prodomain retained an inhibitory function independent of folding activity. The inhibition of falcipain-2 by the prodomain was compared for the active products of four different constructs (~188FP2, ~89FP2, ~35FP2, and FP2; Fig. 2). The prodomain was a potent inhibitor of the mature protease, with similar inhibition constants for each active product (Table II). Inhibition of the protease was competitive and reversible, as indicated by very similar $V_{\text{MAX}}$ values but increasing $K_{m}$ values with increasing prodomain concentrations. For example, the $K_{m}$ for Z-Leu-Arg-AMC was 8.2 $\mu M$ ($V_{\text{MAX}}$, 2.7 $\times 10^{-7} \mu mol s^{-1}$) in the absence of the prodomain and 73.3 $\mu M$ ($V_{\text{MAX}}$, 2.7 $\times 10^{-7} \mu mol s^{-1}$) in the presence of 18 nM prodomain.

**DISCUSSION**

We have characterized folding determinants of the *P. falciparum* cysteine protease falcipain-2 and shown that, unlike other studied papain-family proteases, falcipain-2 does not require its prodomain for folding. Rather, falcipain-2 utilizes an unusual amino-terminal peptide to mediate folding. Although the falcipain-2 prodomain differs from other papain-family enzymes in this regard, it shares another function in that it is a potent inhibitor of the mature protease.

Molecular chaperones mediate folding of many proteins (24). A subset of chaperones, including the proforms of many serine (11, 25–27) and cysteine (9, 10) proteases, act intramolecularly by mediating folding of the cognate mature protein before processing and removal of the chaperone domain. Our results identify a novel intramolecular chaperone-like function for the amino-terminal extension of falcipain-2. It is described as chaperone-like, because unlike true chaperones, it remains bound to the mature protease.

A role in folding for small amino-terminal peptides has previously been hypothesized but not, to our knowledge, identified (28). It is likely that many other proteases utilize small intramolecular peptides to mediate folding, in particular proteins that lack prodomains and those, such as falcipain-2 and some other proteases (e.g. trypsin, chymotrypsin, and cathepsin D), that do not require their prodomains for folding to the active enzymes (29–31). Of note, the prodomain of falcipain-2 acts as a potent inhibitor of the mature protease, but it does not affect the rate or efficiency of protease folding or impact on the catalytic properties of the mature protease.

The unusual folding properties of falcipain-2 are probably shared by other plasmodial cysteine proteases. Falcipain-3 (but not falcipain-1) shares the presence of an unusual sequence at the amino terminus of the mature protease domain

**TABLE I**

| Parameter     | Substrate |
|---------------|-----------|
| $K_{m}$ ($\mu M$) | Z-Leu-Arg-AMC |
| $k_{\text{cat}}$ (s$^{-1}$) | Z-Leu-Arg-AMC |
| $k_{\text{cat}}/K_{m}$ ($M^{-1} s^{-1}$) | Z-Leu-Arg-AMC |
| $k_{\text{cat}}/K_{m}$ ($M^{-1} s^{-1}$) | Z-Phe-Arg-AMC |

| Active product of different constructs | $-188$FP2 | $-89$FP2 | $-35$FP2 | FP2 |
|--------------------------------------|----------|----------|----------|-------|
| Z-Leu-Arg-AMC                        | 7.6      | 7.5      | 8.9      | 8.8   |
| Z-Phe-Arg-AMC                        | 9.5      | 9.4      | 20.9     | 12.1  |
| Z-Leu-Arg-AMC                        | 1.6      | 1.2      | 1.5      | 1.9   |
| Z-Leu-Arg-AMC                        | 0.4      | 0.4      | 0.7      | 0.5   |
| Z-Leu-Arg-AMC                        | 202,600  | 155,400  | 162,400  | 214,300 |
| Z-Phe-Arg-AMC                        | 44,970   | 37,370   | 31,300   | 42,780 |

**FIG. 3. Progress of refolding.** Denatured-reduced recombinant proteins were diluted 100-fold in refolding buffer containing 1 mM each of reduced and oxidized glutathione (buffers B for ~188FP2 and ~89dFP2, C for ~89FP2 and ~89dFP2, and E for ~35FP2, FP2, and dFP2; see “Experimental Procedures” for description of buffers) and incubated at 4°C. Aliquots from each reaction were tested for Z-Leu-Arg-AMC hydrolysis as described under “Experimental Procedures,” and results are expressed as the percentage of maximum activity (fluorescence units/min) obtained with any protease.
In addition, a chimeric protein in which the amino-terminal extension of falcipain-2 was replaced by the corresponding region of falcipain-3 refolded with very similar efficiency to that of falcipain-2. \(^2\) Similar sequences are also seen in homologous proteases of murine malaria parasites \(^3\) but not in any other reported cysteine proteases. Thus, the unusual folding properties of a subset of the falcipains appear to be required due to a specific feature of the biology of malaria parasites.

Why has a plasmodial cysteine protease developed an unusual intramolecular control of protein folding? We hypothesize that this architecture may have been generated to allow the protease to perform two quite different cellular functions. First, falcipain-2 cleaves hemoglobin in the acidic \(P. falciparum\) food vacuole (4, 6). Second, the protease appears to be responsible for the hydrolysis of erythrocyte cytoskeletal proteins that is probably required to allow the egress of mature parasites from the erythrocyte. A role for falcipain-2 in the hydrolysis of cytoskeletal proteins is supported by the observation that cysteine protease inhibitors block the rupture of erythrocytes by mature malaria parasites (32, 33), the identification of \(P. falciparum\) cysteine protease activity that cleaves the erythrocyte cytoskeletal proteins ankyrin and protein 4.1 (34), and the

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\(^2\) P. S. Sijwali, unpublished data.

\(^3\) A. Singh, unpublished data.
demonstration that this activity is identical to that of recombinant falciopain-2 (35).

To access hemoglobin, falciopain-2 is likely transported to the parasite food vacuole by vesicular trafficking from the cytosol organelle after insertion into the parasite plasma membrane, as has also been proposed for aspartic plasmodial proteases (36). To access the erythrocyte cytoskeleton, we propose that falciopain-2 is cleaved from the plasma membrane by limited autohydrolysis or the action of exogenous proteases. This process likely occurs at the cytosome, the plasmodial organelle through which erythrocyte cytosol transits into the parasite, and where the parasite plasma membrane appears to be in direct apposition with erythrocyte cytosol (37). If this cleavage into erythrocyte cytosol liberates truncated, incompletely folded falciopain-2, the parasite may have evolved an intramolecular system for protease folding that is independent of the prodomain to allow the elaboration of hydrolytic activity against cytoskeletal targets. This model must take into account the need for control of falciopain-2 activity in the erythrocyte, presumably via inhibition by the free falciopain-2 prodomain or exogenous cysteine protease inhibitors such as cystatins. In any event, it explains the unique need in malaria parasites for a cysteine protease that can mediate its folding after cleavage of the prodomain.

More generally, our data show that inhibitory and folding functions can be independent in papain-family enzymes. This independent control of enzyme inhibition and folding may well be a common feature of proteases, including those that may utilize different portions of the prodomain for the two functions. Furthermore, our results identify the use of a small internal peptide to direct folding of a mature protease and suggest that many other proteins may incorporate similar systems in which internal sequences provide mediation of protein folding.

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