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Exploring the role of putative active site amino acids and pro-region motif of recombinant falcipain-2: a principal hemoglobinase of *Plasmodium falciparum*

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Abstract

Falcipain-2 is one of the principal hemoglobinases of *Plasmodium falciparum*, a human malaria parasite. It has a typical papain family cysteine protease structural organization, a large pro-domain, a mature domain with conserved active site amino acids. Pro-domain of *falcipain-2* also contains two important conserved motifs, "GNFD" and "ERFNIN." The "GNFD" motif has been shown to be responsible for correct folding and stability in case of many papain family proteases. In the present study, we carried out site-directed mutagenesis to assess the roles of active site residues and pro-domain residues for the activity of *falcipain-2*. Our results showed that substitutions of putative active site residues; Q36, C42, H174, and N204 resulted in complete loss of *falcipain-2* activity, while W206 and D155 mutants retained partial/complete activity in comparison to the wild type *falcipain-2*. Homology modeling data also corroborate the results of mutagenesis; Q36, C42, H174, N204, and W206 residues form the active site loop of the enzyme and D155 lie outside the active pocket. Substitutions in the pro-region did not affect the activity of *falcipain-2*. This implies that *falcipain-2* shares active site residues with other members of papain family, however pro-region of *falcipain-2* does not play any role in the activity of enzyme.

Keywords: *Falcipain-2*; Papain; Pro-region; Site-directed mutagenesis; Active site

Papain super-family is the largest family of cysteine proteases identified so far and its members include a wide range of enzymes in eukaryotes, prokaryotes, and viruses. Members of papain super-family perform diverse functions such as digestion, extra-cellular matrix turnover, antigenic presentation, and processing events. Parasitic cysteine proteases have been characterized to perform numerous indispensable roles in the biology of many species of parasites, i.e., in replication or growth, cell differentiation, signaling, host cell invasion, etc. From a pharmaceutical point of view, these enzymes represent viable drug targets for major diseases such as osteosclerosis, arthritis, immune-related disease, atherosclerosis, cancer, and for wide range of parasitic infections [1].

Structurally, all papain-like cysteine proteases are similar, i.e., they consist of a signal peptide, a pro-peptide, and a catalytic domain with the latter representing the mature proteolytically active enzyme [2]. Initially, all these proteases are synthesized as an inactive pro-enzyme because of the presence of an N-terminal pro-peptide extension. The pro-regions of cysteine proteases are more variable than the mature protease sequences. However, two regions of pro-domains ("GNFD" and "ERFNIN") are relatively well conserved among different cysteine proteases [3]. Site-directed mutagenesis studies have also shown that the "GNFD" domain plays an important role in processing and folding of papain family members [4]. The pro-domains of these enzymes have also been proposed to protect cells from the disastrous consequences of uncontrolled degradative activity of these enzymes [1,5,6].

Malaria remains a serious health problem in Africa, South America, and many parts of Asia [7]. One main

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reason for the persistence of malaria is the emergence of resistance to common anti-malarial drugs [8]. There is therefore a need to identify new targets and develop drugs aimed at these targets [9]. Among potential new targets for anti-malarial chemotherapy are proteases involved in hemoglobin degradation, an essential pathway in the malaria parasite survival [10]. Inhibitors of these proteases kill malaria parasite in culture and in animal models, suggesting that hemoglobin degradation pathway is crucial for parasite survival and is an important target for chemotherapy [11–13]. Hemoglobin degrading proteases in malaria parasite have been categorized in three different classes, aspartic proteases (Plasmepsins I–X) [14], cysteine proteases (Falcipains 1, 2, and 3) [15–17], and metallo-proteases (Falcilysin) [18]. These proteases have been proposed to act in a semi-ordered fashion [19,20]. Aspartic proteases act first, on hemoglobin [21,22], while cysteine and metallo-proteases act later in the degradation pathway [18,23,24]. Among three cysteine proteases, *falcipain-2* has been shown to play an important role in the initial cleavage as well as subsequent hydrolysis of hemoglobin [16]. RNA interference (RNAi) study using double stranded RNA corresponding to *falcipain-2* also showed that the blocking of its gene expression brings about severe morphological abnormalities, and substantial accumulation of hemoglobin in the parasite [25]. These findings suggest that inhibitors of this enzyme could serve as potential leads for malarial chemotherapy.

Falcipain-2 gene is of 1455-bp which codes for a protein of 484 amino acids. Analysis of parasite extract under non-reducing conditions showed that the protease migrates as a doublet at M_r 31,000 and 21,000, suggesting that the pro-region of protease is cleaved to generate the active mature protein [16]. Sequence alignment of *falcipain-2* with papain has shown that it has a typical structure of papain family protease, with a large pro-domain, a mature domain of 27 kDa, and conserved active site residues (Fig. 1B). At the same time, *falcipain-2* also shows certain unusual features for a papain family enzyme, i.e., lack of a typical mature protease-processing site, a large pro-domain, and an unusually large insert between highly conserved amino acid residues (i.e., H174 and N204 residues) near the C-terminus (Fig. 1B). Since *falcipain-2* shows many unusual features and a crystal structure for *falcipain-2* is yet to be resolved, it is important to know about the role of active site and conserved pro-domain amino acid residues for the activity of *falcipain-2*. Based on sequence alignment of *falcipain-2* with other members of papain family, we selected five probable active site amino acid residues (Q36, C42, H174, N204, and W 206), an amino acid away from active site but in mature region (D155), two amino acids (F-30 and D-28) residues of GNFD motif in the pro-region to carry out site-directed mutagenesis. All the amino acids of mature region were mutated to alanine while pro region amino acids were changed to glycine and glutamine, respectively. We report here expression,

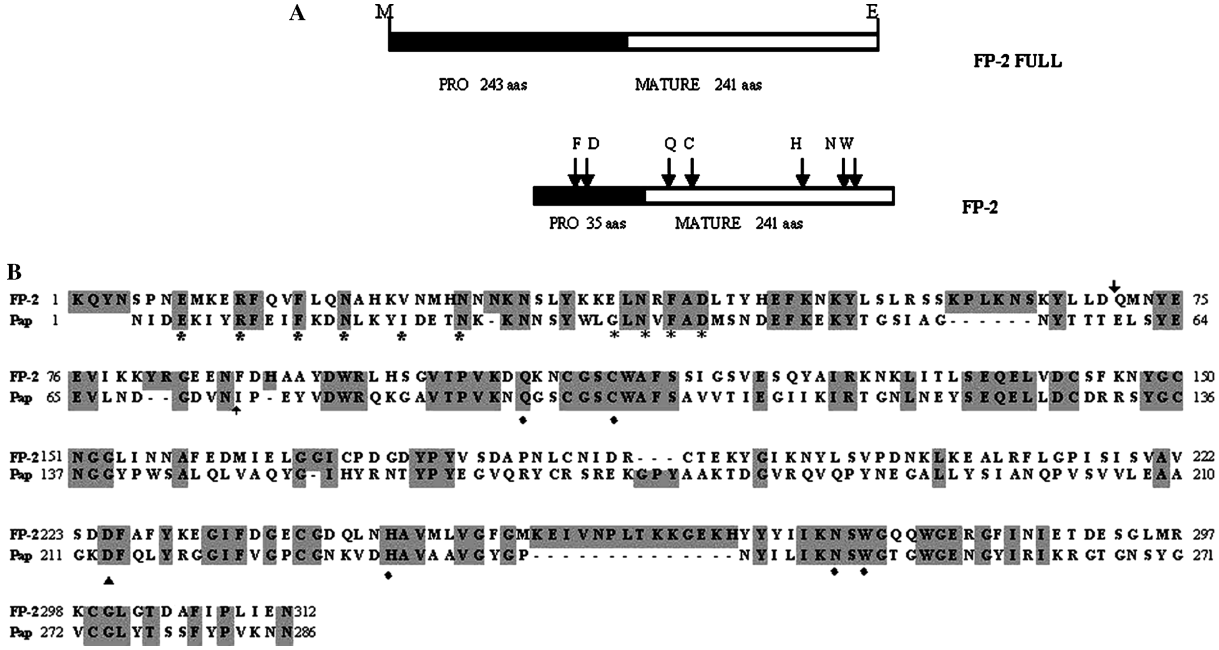

Fig. 1. (A) Domain organization of *Falcipain-2*. The schematic representation of full *falcipain-2* protein (FP-2 full) and the region of *falcipain-2* (FP-2) expressed in *E. coli*. The arrows indicate the position of point mutations in the pro- and the mature regions. (B) Sequence alignment of the *falcipain-2* (FP-2) with the corresponding region of papain (Pap) using the ClustalW method. The shaded portions represent the similarities between the two sequences. Pro region conserved motif is marked with (∗) and conserved active site residues are marked with (●). Mature protease start site is marked with (↑/↓). Conserved amino acid mutated away from active site is marked with (▲).

purification, and activity of mutant forms of enzymes in comparison to the wild type *falcipain-2*. Understanding the role of active site residues and effect of their substitutions on catalysis may have implications on the rationale drug design.

Materials and methods

Materials. All chemicals and bio-chemicals were purchased from Sigma Chemical. Restriction enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized from Sigma (Genosys). Vector pQE30 and M15/pREP4 were obtained from Qiagen Chemical. pGEM-T kit was purchased from Promega. Quickchange site-directed mutagenesis kit was purchased from Stratagene.

Cloning and expression. The wild type *falcipain-2* gene was amplified from genomic DNA of *Plasmodium falciparum* 3D7 strain, by PCR, using gene-specific primers and cloned into pGEM-T vector. Growth conditions, procedure for plasmid purification, and different enzymatic manipulations of DNA were performed according to the Sambrook et al. [26]. Cloning and expression were carried out as mentioned earlier [16]. Briefly FP-2 (35 amino acids of the pro-form plus full mature region) (Fig. 1A) fragment was amplified with primer pair;

Forward—5′-CCA TG**G GAT CC**A AAG AAT TAA ACA GAT TTG CC-3′

Reverse—5′-CCC **AAG CTT** TTC AAT TAA TGG AAT GAA TGC ATC-3′

and pGM-Fal-2 (pGEM-T clone with full-length *falcipain-2*) as a template, incorporating internal restriction endonuclease sites. This PCR product was cloned into pGEM-T vector to generate pGMwtFP2. This clone was digested with *Bam*HI and *Hind*III enzymes, gel purified, and ligated into pQE30 expression vector having N-terminal 6× His tag to produce expression construct pQwtFP2. M15 (pREP4) strain of *Escherichia coli* was transformed with pQwtFP2 and transformants were analyzed by PCR amplification and restriction digestion. Positive clones were further confirmed by sequence analysis. Bacteria containing pQwtFP2 insert were grown to mid log phase and induced with 1 mM IPTG for 5 h at 37 °C. Expression of protein was analyzed by SDS–PAGE.

Substitution of residues by site-directed mutagenesis. Mutants of *falcipain-2* were constructed using the Quick-change site-directed mutagenesis kit from Stratagene, following manufacturer's instructions. In brief, PCR was performed using *Pfu* turbo polymerase using wild type *falcipain-2* cloned in pQE30 as a template DNA (pQwtFP2) and corresponding primer pair was used to introduce mutation (Table 1). PCR was followed by the *Dpn*I enzyme treatment of the PCR mix at 37 °C for 1 h. This *DpnI*-treated PCR mix was transformed into XL1-Blue super competent cells. Five clones were picked for each mutant and then checked by restriction digestion analysis. Mutations were confirmed by sequencing, using an automated DNA sequencer (ABI prism). Clones having the mutation were transformed in M15 (pREP4) cells for expression analysis. Expression of the protein for each mutant was analyzed on SDS–PAGE.

Purification and refolding of (recombinant) wild type and mutant falcipain-2. Wild type and mutant proteins were purified and refolded as described previously [16]. After refolding, proteins were dialyzed in 100 mM sodium acetate containing 20% glycerol and stored at −20 °C before using it for any assay.

Characterization of wild type and mutant falcipain-2. Substrate gel analysis was performed as previously described [27] using SDS–PAGE

Table 1
Oligonucleotide primers used for mutagenesis of *falcipain-2* protein

Primer	Fal-2 mutant isolated	Codon change
Q MUT F 5′-CACCTGTAAAGGAT(**GCA**)AAAAATTGTGGATC-3′	Q36A	CAA → GCA Q → A
Q MUT R 5′-GATCCACAATTTTT(**TGC**)ATCCTTTACAGGTG-3′		
C MUT F 5′-AATTGTGGATCT(**GCC**)TGGGCCTTTAGTAG-3′	C42A	TGC → GCC C → A
C MUT R 5′-CTACTAAAGGCCCA(**GGC**)AGATACACAATT-3′		
H MUT F 5′-GGTGATCAATTAAAT(**GCT**)GCCGTTATGCTT-3′	H174A	CAT → GCT H → A
H MUT R 5′-AAGCATAACGGC(**AGC**)ATTTAATTGATCACC-3′		
N MUT F 5′-TATTATTATATAATTAAG(**GCC**)TCATGGGGACAACAATGG-3′	N204A	AAC → GCC N → A
N MUT R 5′-CCATTGTTGTCCCCATGA(**GGC**)CTTAATTATATAATAATA-3′		
W MUT F 5′-ATAATTAAGAACTCA(**GCG**)GGACAACAATGGGG-3′	W206A	TGG → GCG W → A
W MUT R 5′-CCCCATTGTTGTCC(**CGC**)TGAGTTCTTAATTAT-3′		
F MUT F 5′-GAATTAAACAGA(**GGT**)GCCGATTTAACTTATC-3′	F−30G	TTT → GGT F → G
F MUT R 5′-GATAAGTTAAATCGGC(**ACC**)TCTGTTTAATTC-3′		
D MUT F 5′-CAGATTTGCC(**GAG**)TTAACTTATCATG-3′	D−28E	GAT → GAG D → E
D MUT R 5′-CATGATAAGTTAA(**CTC**)GGCAAATCTG-3′		
D MUT F 5′-GTAGCCGTATCAGAT(**GCT**)TTTGCTTTTACAAA-3′	D155A	GAT → GCT D → A
D MUT R 5′-TTTGTAAAAGCAAA(**AGC**)ATCTGATACGGCTAC-3′		

Numbers denote the position of amino acid from mature protein start site.
(-) Represents position before mature protein start site. Nucleotides shown in bold represent the triplet for substituted amino acid.

under non-reducing conditions with gel co-polymerized with 0.1% (w/v) gelatin. Fluorimetric assays for falcipain-2 (wild type and mutants) were carried out in 3 ml assay buffer (0.1 M sodium acetate, 10 mM DTT) containing 200 nM enzyme. Fluorogenic substrate Z-F-R-AMC was added at 50 μM concentration and activity was monitored as the increase of fluorescence (excitation 355 nm; emission 460 nm) for 30 min at RT with a LS50B Perkin–Elmer fluoremeter. For all assays, substrate concentration was saturating within the time course studies, such that curves for fluorescence over time remained linear.

Enzyme kinetics. Rates of hydrolysis at varied concentrations of the peptide Z-F-R-AMC substrate were determined at constant enzyme concentration (200 nM) in 3 ml assay buffer at RT. Enzyme activity was monitored as described above. K_m, V_{max}, and K_{cat} were determined using the GCG program (Wisconsian package).

Hydrolysis of hemoglobin. Recombinant falcipain-2 proteins (wild type and mutants) were evaluated for their ability to hydrolyze native hemoglobin. Equal amounts of protein were taken in 35 μl reaction mixtures containing 100 mM sodium acetate, pH 5.5, 10 mM DTT, and 5.0 μg hemoglobin. Reaction mixtures were incubated at 37 °C for 2 h and stopped with 5.0 μl of reducing SDS–PAGE sample buffer. Reaction products were resolved on a 15% SDS–PAGE under reducing conditions as described by Laemmli [28].

Production of antibodies and immunoblotting. Six female BALB/C mice (6 weeks old) were immunized intra-peritoneally with purified recombinant falcipain-2 in complete Freund's adjuvant on day 0 followed by boosting with incomplete Freund's adjuvant on days 21 and 42. Pre-bleed sera were collected a day before immunization from each mice. Mice were sacrificed on day 49 and sera were pooled. Sera from each mice were analyzed by ELISA assay for the presence of antibodies against falcipain-2. For immunoblotting, refolded recombinant falcipain-2 was separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane. Nitrocellulose membrane was blocked at RT for 90 min with 2% nonfat milk in PBS, washed with PBS containing .05% Tween 20, and incubated with a 1:10,000 dilution of immune serum against recombinant falcipain-2 for 90 min at RT. Blot was washed once with PBS and incubated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) at 1:5000 dilution for 90 min at RT. Blot was washed again thrice with PBST and finally, washed once with PBS. Blot was developed with developing buffer (PBS having 10 mg diamino benzoic acid (DAB) and 10 μl of 20% H_2O_2 per 10 ml buffer).

Homology modeling of falcipain-2 to map active site residues. Molecular modeling was performed using 3D-PSSM software on the server [29]. Protein sequence of *falcipain-2* having full mature region was submitted to 3D-PSSM server. Modeling of *falcipain-2* was based on the sequence and crystal structure of procathepsin K (PDB code 7PCK) [30]. Results sent by the server were further analyzed using RASMOL.

Results

Site-directed mutagenesis of putative active site residues, Q36, C42, H174, N204, and W206 and pro-domain residues, F-30 and D-28

Sets of primers were designed to replace all the five probable active site and a non-active site residues with alanine and to replace conserved pro-domain residues; phenylalanine to glycine and aspartic acid to glutamine (Table 1). Wild type and mutant forms of recombinant falcipain-2 proteins were expressed, purified, and refolded up to homogeneity. SDS–PAGE analysis of these proteins revealed that all the mutant forms of falcipain-2 were expressed at the same level as the wild type protein

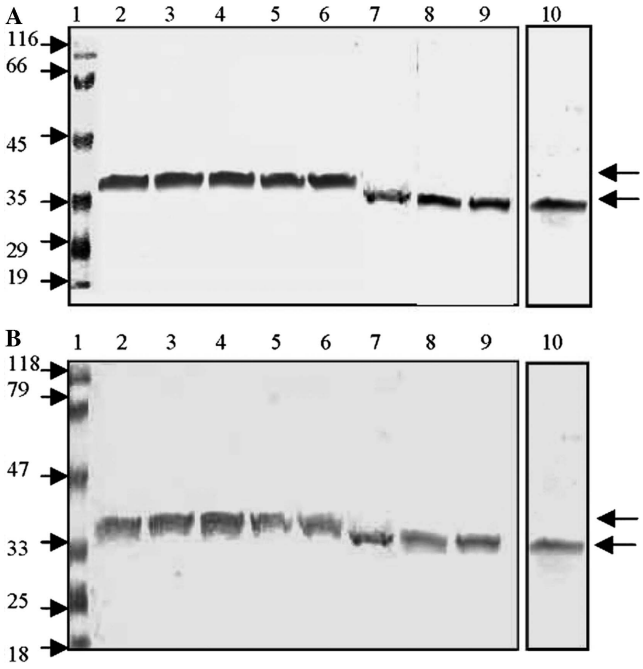

Fig. 2. Refolding of recombinant *Falcipain-2* proteins. SDS–PAGE showing purification and auto-processing of recombinant *Falcipain-2* from wild type and mutant constructs by (A) Coomassie blue staining and (B) Western blot using *falcipain-2* antibodies. Loading patterns of both the gels are as follows: (1) Marker (in kDa), (2) Q36A, (3) C42A, (4) N204A, (5) H174A, (6) W206A, (7) FP-2, (8) F-30G, (9) D-28E, and (10) D155A.

and the final yields of these proteins were comparable after purification and refolding. Of the eight mutants that were constructed in the present study, only D155A mutant in the mature region and both the pro-region mutant proteins showed shift in mobilities on SDS–PAGE similar to that seen with wild type falcipain-2 (Figs. 2A and B). No such shift in mobility was observed in proteins with active site mutations. This shift in mobility has been earlier shown due to auto-processing of the protein. These results indicated that mutations in the probable active site amino acid residues affect the processing of falcipain-2 protein.

Enzyme activity of wild type and the mutant forms of recombinant falcipain-2

Cleavage of specific substrate

To determine the effect of mutagenesis on the activity of protein, we carried out activity analysis of different forms of falcipain-2 proteins. Both, native and recombinant falcipain-2, have been previously characterized for their substrate specificies [16]. We also characterized wild type recombinant falcipain-2 for its proteolytic activity with three different substrates, Z-F-R-AMC, Z-R-R-AMC, and Z-R-AMC. As reported earlier, the recombinant falcipain-2 cleaved Z-F-R-AMC effectively while it did not cleave the other two

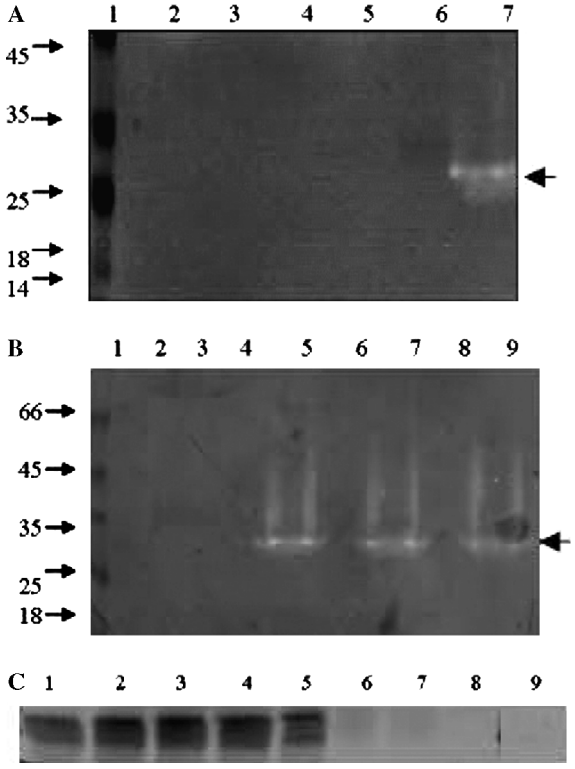

Fig. 3. Activity analysis of refolded *falcipain-2* proteins (wild type and mutants). (A) Activity analysis of *falcipain-2* constructs on SDS–PAGE containing gelatin as a substrate. Loading is as: (1) Marker (in kDa), (2) Q36A, (3) C42A, (4) N204A, (5) H174A, (6) W206A, and (7) FP-2. (B) *Falcipain-2* proform mutants on gelatin substrate SDS–PAGE to assess activity. Loading is as: (1) Marker (in kDa), (2) BLANK, (3) H174A, (4) BLANK, (5) F-30G, (6) BLANK, (7) D-28E, (8) BLANK, and (9) D155A. (C) Activity analysis to show hydrolysis of hemoglobin by the various *falcipain-2* constructs on a 15% SDS–PAGE loading is as: (1) Q36A, (2) C42A, (3) N204A, (4) H174A, (5) W206A, (6) D-28E, (7) F-30G, (8) FP-2, and (9) D155A.

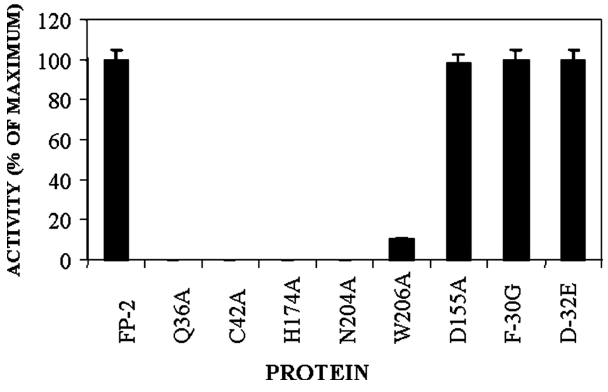

Fig. 4. Proteolytic activity of recombinant wild type and mutant *falcipain-2* protein. Equal quantities of recombinant falcipain-2 (FP-2) and mutant falcipain-2 proteins were analyzed for the hydrolysis of Z-F-R-AMC and represented as the release of fluorescence in arbitrary fluorescence units (activity percentage of maximum). Reaction was set up in activity buffer and hydrolysis of substrate was analyzed as release of fluorogenic product. Error bars represent the standard deviations of the results from three refolding reactions.

Table 2
Kinetic parameters for wild type and mutants of *Falcipain-2*

Enzyme	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1} M^{-1}$)
FP-2	18.9	0.25	13,200
W206A	19.0	0.003	157

Kinetic parameters were determined for FP-2 and W206A with fluorogenic substrate Z-F-R-AMC only. Q36A, C42A, N204A, and H174A did not degrade Z-F-R-AMC therefore none of the constants were determined. F-30G, D-28E, and D155A showed similar activity as the wild type falcipain-2, therefore similar constants were observed.

substrates significantly. Based on this cleavage specificity, we tested all the mutants for their ability to cleave Z-F-R-AMC. As shown in Fig. 4, the wild type, D155A, and pro-domain mutants showed comparable proteolytic activities. Four of the five putative active site mutants (Q36A, C42A, N204A, and H174A) showed complete loss of activity, while the fifth mutant (W206A) showed only marginal activity when compared with the wild type recombinant protein. Michaelis constant for the wild type falcipain-2 and W206A observed was almost same but there was significant change in the K_{cat} values (Table 2).

Gelatinase and hemoglobin degradation assay

The proteolytic activity of the wild type falcipain-2 and its mutant forms was also assessed by gelatin substrate SDS–PAGE. As shown in Figs. 3A and B, the wild type falcipain-2 and its pro-domain mutants showed similar gelatinase activity. However, as seen with fluorimetric assay, the putative five active site mutants did not show any gelatinase activity.

The principal natural substrate for aspartic and cysteine proteases in *P. falciparum* parasite is hemoglobin. To find out how mutant forms of falcipain-2 affect its hemoglobin degrading ability, we compared the activities of mutant forms with those of the wild type enzyme. The five mature site mutations, i.e., Q36A, C42A, N204A, H174A, and W206A completely abolished falcipain-2 hemoglobin degrading activity. On the other hand, D155A and two pro-domain mutations did not affect hemoglobin-hydrolyzing activity of falcipain-2, when compared to wild type recombinant falcipain-2 (Fig. 3C).

Homology modeling

Falcipain-2 has nine cysteine residues and eight of them are conserved in cruzain and form disulfide bonds. It has been shown that C42 positioned in the active site does not form a disulfide bond [31]. To understand the structural organization of *falcipain-2* and the positions of amino acids mutated in the present study, a homology model of *falcipain-2* was derived, based on sequence homology of the *falcipain-2* with procathepsin K. Procathepsin K also belongs to papain family of cysteine

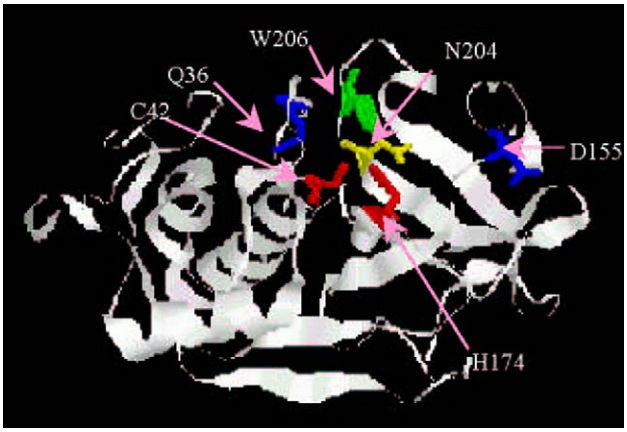

Fig. 5. Homology-based model for falcipain-2. Web-based homology modeling was done to display the position of putative active site amino acids. All the five active site amino amino acids are shown in color with their side chains. (Q36-blue, C42-Red, H174-Red, N204-yellow, and W206-green). Also, position of D155 is shown in blue color, which lies away from the active site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

proteases and shows high homology with *falcipain-2*. As shown in Fig. 5, homology model of *falcipain-2* showed that all the five probable active amino acids substituted in this study form the active site pocket in *falcipain-2*. Among these five amino acids, C42 and H174 are very much buried inside the active site loop and lie very close to each other. Q36 and N204 also lie close and are a part of the active site loop. W206 among these amino acids lies at the periphery of the active site loop and its side chain also points towards the outer side, while D155 resides outside the active domain pocket.

These modeling data supported our mutagenesis results where mutation in C42, Q36, N204, and H174 abolished the activity but in case of W206, 10% of the activity was retained. Mutation involving amino acids D155, away from the active site loop, did not show any effect on the activity of enzyme. These data are also in the line with alignment and modeling studies done for other papain family of cysteine proteases.

Discussion

Falcipains play a critical role in hemoglobin hydrolysis to provide amino acids for the development of parasite in erythrocytes. Of the three falcipains, *falcipain-2* has been identified as a principal trophozoite cysteine protease and a potential drug target. Structure based drug design of inhibitors of *falcipain-2* represents an effective route for finding and optimizing therapeutic agents for the treatment of malaria [31]. In the absence of a crystal structure for this enzyme, mutagenesis followed by functional screening of mutants and homology-modeling studies provide the best alternative for insights into binding specificities of *falcipain-2* and can be useful for structure-based inhibitor design.

In the present study, we attempted to analyze the role(s) of putative active site and pro-domain residues by site-directed mutagenesis. Six amino acids from the mature protease region were substituted individually with alanine using site-directed mutagenesis. These residues were selected based on site-directed mutagenesis studies and crystal structures of members of papain family [32]. The catalytic residues of papain are C25 and H159, and these are conserved in all members of the family that are peptidases. Other residues important for catalysis include Q19, which helps form the "oxyanion hole," and N175, which orientates the imidazolium ring of H159 [33]. There is also strong conservation of sequence in the vicinity of essential C19 and H159 and N175 residues. The two conserved residues in the pro-region (GNFD); phenylalanine and aspartic acid residues were mutated as they have been shown to be important for processing of the papain precursor [34]. We did not carry out any site-directed mutagenesis of another pro-region conserved motif; ERFNIN motif, as it has been already shown that this motif does not play any role in the activity of *falcipain-2* [16].

Among the mutants from the mature region, W206A showed marginal activity while D155A and the wild type falcipain-2 showed similar activities. However, all other mutant forms in the mature region were inactive. Refolded and inactive mutant proteins were stable and did not undergo auto processing as seen for wild type falcipain-2, D155A, and the pro-region mutants. This processing is because of the auto-catalytic activity, as it was not observed when refolding was done in presence of inhibitor [16]. These results were in line with the previous studies carried out on other members of papain super family. Mutations in the active site cysteine and histidine residues have been shown to cause loss of function among papain family of proteases [32,35]. Complete loss of activity for the Q36A and N204 mutants in the present study suggested their direct involvement in the catalytic activity of falcipain-2. *Falcipain-2* differs from papain in this regard as similar substitutions have not shown complete loss of activity in papain [33]. These results suggest that though active site residues are conserved among *falcipain-2* and other papain family of enzymes, they differ in their role for each enzyme.

The W206A showed marginal activity as observed by degradation of Z-F-R-AMC observed as fluorescence. It has been shown for enzyme barnase, a ribonuclease, that interaction of a protonated histidine side chain with a tryptophan residue results in an increase of pK_a of the former [36]. This suggests that the adjacent aromatic group can preferentially stabilize the protonated form of

histidine. Thus, this His–Trp pairing in cysteine peptidases is probably important for the ion pair stability and/or the catalytic activity of the enzyme through its combined effect of increasing the pK_a of histidine and shielding His and Asn hydrogen bond from the external solvent [36]. However, W206A did not show any significant hemoglobin and gelatin degradation, which may be due to the sensitivity of the technique. No significant change in K_m was observed but the turnover, i.e., K_{cat} changed significantly in comparison to the wild type falcipain-2. Results of these site-directed mutagenesis studies were further corroborated by homology modeling study done on *falcipain-2*. This study indicated that the five probable active site amino acids, which were mutated in the present study and the resultant proteins were inactive, form the active pocket of *falcipain-2*.

In addition to the conserved active site residues, pro-region of papain family enzymes has also been shown to play a crucial role in protein folding and localization [34]. In some members of papain family it has been shown to act as an inhibitor of the cognate protease [6]. Site-directed mutagenesis studies have shown that GNFD motif is crucial for the folding of papain family of enzymes [34]. Recently, Sijwali et al. [37] showed that N-terminal region of mature *falcipain-2* and not the pro-region help in folding of the enzyme. However, in the present study, we show the role of specific pro-region (GNFD motif) amino acids by carrying out point mutations of the two important residues, phenylalanine and aspartic acid, with residues glycine and glutamine, respectively. Even though these substitutions have been shown to produce non-functional protein in papain family members [26], in case of *falcipain-2*, these substitutions did not affect the activity/processing of the enzyme. These studies suggested that pro-region of *falcipain-2* does not play any role in folding/activity or processing of the enzyme. It remains to be elicited that whether this region plays any role in the localization of the enzyme in the parasite.

In conclusion our site-directed mutagenesis studies show that functionally the pro-domain of *falcipain-2* does not behave as that of other members of papain family while active site conserved amino acids have functional similarity with that of other papain family members.

Falcipain-2 and other *P. falciparum* hemoglobinases are the promising chemotherapeutic targets. Indeed, inhibitors of both *falcipain-2* [11,38–40] and the plasmepsins [13,41] are potent anti-malarials, and combination of inhibitors of both classes of proteases yields synergistic anti-malarial activity in vitro [42,43] and in vivo [43]. Designing specific inhibitors is greatly facilitated by structural studies of a protein. As these mutated *falcipain-2* proteins are stable, they can be used for crystallization purpose. Since these proteins have not shown autocatalytic activity, it will be interesting to study the pro-domain interaction with the mature form. Structural studies of *falcipain-2* will further help in designing novel inhibitors, which can be used as anti-malarials.

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