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Crucial residues in falcipains that mediate hemoglobin hydrolysis

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ABSTRACT

Falcipain-2 (FP2) and falcipain-3 (FP3) constitute the major hemoglobinases of *Plasmodium falciparum*. Previous biochemical and structural studies have explained the mechanism of inhibition of these enzymes by small molecules. However, a residue-level protein-protein interaction (PPI) with its natural macromolecular substrate, hemoglobin is not fully characterized. Earlier studies have identified a short motif in the C-terminal of FP2, an exosite protruding away from the active site, essential for hemoglobin degradation. Our structural and mutagenesis studies suggest that hemoglobin interacts with FP2 via specific interactions mediated by Glu^{185} and Val^{187} within the C-terminal motif, which are essential for hemoglobin binding. Since FP3 is also a major hemoglobinase and essential for parasite survival, we further demonstrate its interactions with hemoglobin. Our results suggest that Asp^{194} of FP3 is required for hemoglobin hydrolysis and residue-swap experiments confirmed that this position is functionally conserved between the two hemoglobinases. Residues involved in protein–protein interactions constitute important targets for drug-mediated inhibition. Targeting protein–protein interactions at exosites may likely be less susceptible to emergence of drug resistance and thus is a new field to explore in malaria.

1. Introduction

Malaria poses a serious risk to human health with an approximate 445,000 deaths caused in 2016 alone (WHO, 2017). While Artemisinin Combination Therapies (ACTs) continue to be the front line treatment for uncomplicated malaria, emerging resistance in parts of SE Asia necessitate the development of novel drug targets and new mechanisms of drug administration (Dondorp et al., 2009). It is well established that malarial proteases play crucial roles in parasite development and survival. Malarial cysteine proteases, falcipain-2 (FP2) and falcipain-3 (FP3) have roles ranging from facilitating heme conversion to hemozoin to erythrocyte breakdown and albeit indirectly in artemisinin resistance (Chugh et al., 2013; Conrad et al., 2014; Marques et al., 2015). However, the major role of falcipains lies in degradation of host hemoglobin into constituent amino acids, which are used by the parasite (Francis et al., 1997). Substrate assays using peptides with partial or complete sequences of hemoglobin demonstrated that FP2 and FP3 rapidly cleaved hemoglobin at multiple sites (Subramanian et al., 2009). Thus, inhibition of these enzymes is important to check parasite growth. While hemoglobin hydrolysis is a co-operative process involving proteases of multiple classes, including cysteine, aspartic and metalloproteases, the abnormal food vacuole phenotype is primarily caused by inactivation of cysteine proteases (Rosenthal, 1995).

Falcipains consist of a prodomain and mature domain which dissociate under acidic conditions. The prodomain contains a cytoplasmic and luminal trafficking motif that play roles in endocytosis and vesicular transport to the food vacuole respectively, followed by a transmembrane domain that is involved in localization of FP2 and FP3 into the ER (Dahl and Rosenthal, 2005). At the C-terminus of the prodomain lies an inhibitory domain containing two highly conserved motifs ERFNIN and GNFD that bind at the active site and thus control the activity of the mature falcipain enzymes (Kumar et al., 2004; Pandey et al., 2009). The mature domain consists of a refolding domain, active site cleft and a hemoglobin binding domain (Pandey et al., 2004).

Along with the active site, the surface of a protease contains several sites for regulatory interactions with other proteins. Proteases have been shown to contain such regulatory motifs, called exosites that either act as regulators of enzyme activity or improve substrate specificity

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(Mikhailova et al., 2012). For instance, matrix metalloproteases share a structural domain present at the C-terminal called hemopexin (HPX) domain, essential for mediating catalysis of their natural substrate, collagen (Arnold et al., 2011; Robichaud et al., 2011). The well characterized human aspartic protease BACE1 (β-APP Cleaving Enzyme 1), has been shown to be inhibited by an exosite binding inhibitor, that impairs the ability of BACE to hydrolyze its natural protein substrate, APP (Amyloid precursor protein) (Kornacker et al., 2005; Wang et al., 2013). Further, cathepsin K, a papain family protease forms pentameric complex with chondroitin sulfate at an exosite to facilitate hydrolysis of collagen (Li et al., 2002). Similarly, the inhibition of serine protease thrombin by its macromolecular inhibitor, hirudin, via specific motif distant from the active site of the protease was observed (Grütter et al., 1990). Thus, exosites serve to facilitate biologically relevant protein-substrate and protein-inhibitor interactions.

Falcipains contain such short exosite interaction motif at C-terminus of the mature domain that are unusual for papain family proteases (Pandey et al., 2005). This motif is observed in all studied malarial proteases including FP2, FP3 and vivapains, but not in falcipain-1 (FP1). Solved structure of FP2 indicated that this structure (10 amino acids: Glu^{185} to Gly^{195}), formed a distinct β-hairpin that protrudes away from the mature enzyme. While removal of this domain in FP2 had no effect in activity against small and large substrates, the mutated enzyme however lost its activity against hemoglobin indicating involvement of C-terminus motif in mediating hemoglobin hydrolysis (Pandey et al., 2005).

Structural and biochemical studies have been valuable in explaining the mechanism of inhibition of FP2 and FP3 by small molecule inhibitors and different fluorogenic substrates (Kerr et al., 2009; Rosenthal et al., 1996, 1988). However, a detailed view of the protein-protein interactions (PPIs) between larger macromolecular substrate, hemoglobin and falcipains remains to be established. In this study, we investigated the role important residues in falcipains that mediate hemoglobin hydrolysis using bioinformatics and biochemical techniques. We also investigated the functional conservation of such residues within the hemoglobin degrading falcipains.

2. Materials and methods

2.1. Mutagenesis and cloning of FP2, FP3 mutants

Primers were generated using NEBaseChanger tool (http://nebasechanger.neb.com) (Table 1). Mutants were generated using Q5 Site-Directed Mutagenesis Kit (NEB) using wild FP2 and FP3 templates. The mutated FP2 and FP3 mature domain constructs were PCR amplified, digested with restriction enzymes BamHI and HindIII (NEB), ligated into the 6XHis-tagged expression vector pQE30 (Qiagen) and transformed into M15 (pREP4) E. coli cells (Qiagen).

Table 1
List of primers used to generate FP2 and FP3 mutants from wild mature templates. Mutants were generated as per the Q5 Site-Directed Mutagenesis Kit (NEB) protocol.

Primer	Sequence
^{E185A,V187A}FP2 for:	5′-TGCGAATCCATTAACCAAGAAAGG-3′
^{E185A,V187A}FP2 rev:	5′-ATCGCTTTCATACCAAAACCTACAAG-3′
^{E185A}FP2 for:	5′-TGGTATGAAAGCGATTGTTAATCCATTAAC-3′
^{E185A}FP2 rev:	5′-AAACCTACAAGCATAACG-3′
^{D194A}FP3 for:	5′-TGGTATGAAAGCGATTTATAATGAAGATACTG-3′
^{D194A}FP3 rev:	5′-TAACCTACAAGTATAACCG-3′
^{E185D}FP2 for:	5′-TGGTATGAAAGATATTGTTAATCCATTAAC-3′
^{E185D}FP2 rev:	5′-AAACCTACAAGCATAACG-3′
^{D194E}FP3 for:	5′-TGGTATGAAAGAAATTTATAATGAAGATAC-3′
^{D194E}FP3 rev:	5′-TAACCTACAAGTATAACCG-3′

2.2. Expression, purification and activation of wild/mutant FP2 and FP3 enzymes

Large scale expression, purification, refolding and activation of the wild and mutant enzymes of FP2, FP3 were followed as described earlier (Sijwali et al., 2001). Briefly, *E. coli* M15 cells containing the wild and mutant plasmids were induced with 1 mM IPTG and purified from inclusion bodies by Ni-NTA affinity chromatography (Ni-NTA beads, Qiagen) followed by ion exchange chromatography. The recombinant proteins were refolded as described earlier, activated by addition of 100 mM Sodium Acetate (NaAc, pH 5.5) and stored at −20 °C.

2.3. Hemoglobin hydrolysis assay

To analyze the activity of the mutant enzymes, their hemoglobin degradation capabilities were assessed. Briefly, 6 μg of human hemoglobin (Sigma) was added to 4 μg of active enzyme and incubated in 100 mM NaAc (pH 5.5), 5 mM DTT for 3 hrs at 37 °C. Hemoglobin hydrolysis capabilities of the mutants and wild enzyme were determined by analysis on 15% SDS-PAGE.

2.4. Fluorogenic substrate assay

To measure the hydrolysis of fluorogenic peptide, FP2, FP3 and different described mutants with similar concentration (20 nM) were incubated with 25 μM Z-Leu-Arg-AMC substrate in 100 mM NaAc, 8 mM DTT (pH 5.5). Hydrolysis of Z-Leu-Arg-AMC was continuously measured in fluorescence units (Excitation - 355 nm; Emission - 460 nm) for 15 min at room temperature as specified previously (Na et al., 2004; Pandey et al., 2005).

2.5. Enzyme kinetics experiments

The enzymatic activities of wild FP2, FP3 and mutant enzymes were studied by incubating fixed (1 nM) concentration of enzyme with increasing Z-Leu-Arg-AMC substrate concentrations (0–1 mM) in the presence of 100 mM NaAc, 8 mM DTT (pH 5.5). Hydrolysis of substrate was continuously for 15 min at room temperature (Ex.-355 nm; Em.-360 nm) as described earlier. Errors were calculated from separate quadruplicate reactions.

3. Results

3.1. Prediction of functionally important residues for binding to macromolecular substrate, hemoglobin

Our previous study suggests that FP2 captures its natural substrate, hemoglobin, using a unique C-terminal motif (Pandey et al., 2005). Structure of FP2 showed that this motif forms a β-hairpin that protrudes away from the enzyme active site (Fig. 1A and B). Protein-protein docking based on solved structure of enzyme and hemoglobin to find important interactions between FP2-hemoglobin and FP3-hemoglobin revealed that FP2 interacts with α as well as β subunits of hemoglobin via two important residues, Val^{187} and Glu^{185} (Fig. 1C and D). Similarly, the docking study also suggested that FP3 interacts with α as well as β subunits of hemoglobin via a functionally conserved residue, Asp^{194} (Fig. 1E and F).

Protein-protein docking of FP2, FP3 and hemoglobin monomers (α, β) were performed with Hex 6.12, a tool that analyzes binding affinities and molecular interactions and develops lowest energy clusters for possible conformers at every interaction site (Macindoe et al., 2010). The coordinates of crystal structures of mature domains of FP2, FP3 and hemoglobin (PDB ID: 3PNR, 3BPM and 1HHO, respectively) were obtained from the protein database (Hansen et al., 2011; Kerr et al., 2009; Shaanan, 1983). Docking results generated 100 lowest energy cluster

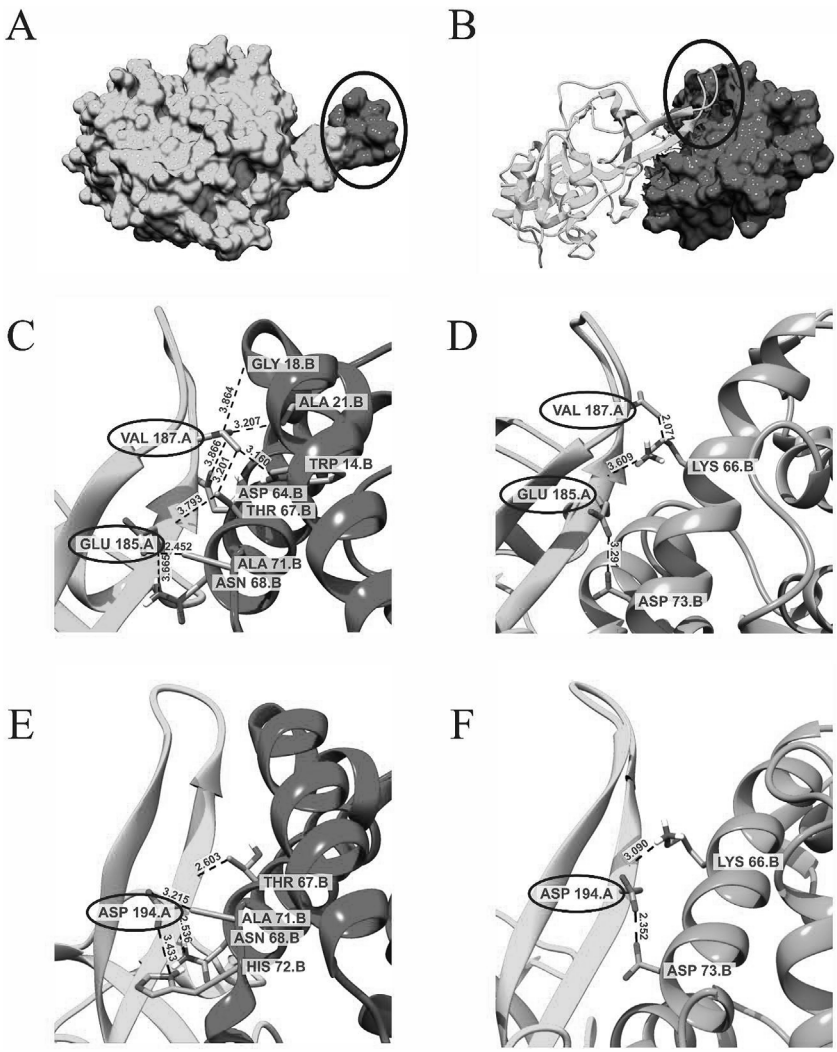

Fig. 1. Structural view of hemoglobin binding domain (C-terminal motif) and its interactions with Hb. (A) Structure of FP2 indicating hemoglobin binding motif (red). (B) Hemoglobin binding motif with an unusual 14 amino acid interacts with hemoglobin (monomer; red). Close view showing interactions of a motif of FP2 (green) with α chain (C; red) and β chain (D; orange) of hemoglobin. Similarly, close view of interactions between C-terminus motif of FP3 (blue) with α chain (E; red) and β chain (F; orange) of hemoglobin has been shown. Bond lengths of interactions have been indicated in angstrom units (Å). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

confirmation models for every docking complex. The best docked complex, selected based on lowest docked energy value, was further analyzed based on solvent accessible surface area (SASA) of residues by InterProSurf. InterProSurf uses a propensity scale for interface residues and a clustering algorithm to predict interacting residues in a protein and identify surface regions with residues of high interface propensities (Negi et al., 2007). Two residues, Val^{187} and Glu^{185} of FP2, were found to have a large difference in SASA after complex formation suggesting involvement of these residues in non-bonded interactions (Table SI, SII). Protein-protein interactions were also analyzed by LIGPLOT (Wallace et al., 1995), that generates a graphical representation of interacting residues with their strength of interaction, predicted these residues to interact with α as well as β subunits of hemoglobin. Non-bonded interactions include electrostatic interactions, hydrogen bonding, partial charges, dispersion, repulsion, Lennard-Jones potential and Van der Waals radii. Non-bonded atomic contacts are much closer than the sum of the Van der Waals radii of the two atoms. On the basis of above analysis, Val^{187} and Glu^{185} of FP2, and Asp^{194} of FP3 were selected for mutagenesis study (Fig. 2).

3.2. Prediction of binding affinity of FP2 motif-Hb complex and the effect of mutations on binding affinity

To predict the binding affinity between FP2 Hb binding motif and Hb, computational methods that calculated the effect on binding affinity upon mutation of selected residues were used. Previous studies suggest that FP2 motif is responsible for the initial recognition of Hb substrate which is then transferred to the active site for hydrolysis (Pandey et al., 2005). The recognition of hemoglobin by FP2 motif is an intermediate step and occurs at a very short time scale, thus determination of the experimental binding affinity of FP2 motif-Hb complex remained unfeasible. We have thus applied computational methods for predicting the binding affinity of the FP2 motif-Hb complex and observe changes in binding affinity upon mutagenesis. First, the binding affinity between FP2 motif-Hb complex was calculated by using the webserver PRODIGY (Protein Binding energy prediction) (Vangone and Bonvin, 2017, 2015). PRODIGY calculates the binding affinity based on the properties of residues at both the interacting and non-interacting surface. The predicted binding affinity (ΔG) and dissociation constant (K_d at 25 °C) is −12.4 kcal/mol and 8.3×10^{-10} M, respectively. Using bioinformatics analysis, two residues were selected which could play important roles in hemoglobin recognition. Further, the BeAtMuSic (prediction of Binding Affinity Changes upon Mutations) server was used to predict the effect on binding affinity upon mutation of these selected residues to Alanine (Dehouck et al., 2013). This webserver calculates the change in binding free energy ($\Delta\Delta G_{Bind}$) resulting from each mutation in a complex, and solvent accessibility of mutated residues, in complex and in the individual interacting partners. Our results have shown a decrease in binding affinity upon mutagenesis and support the claim that selected residues have a significant role in hemoglobin capture (Table SIII).

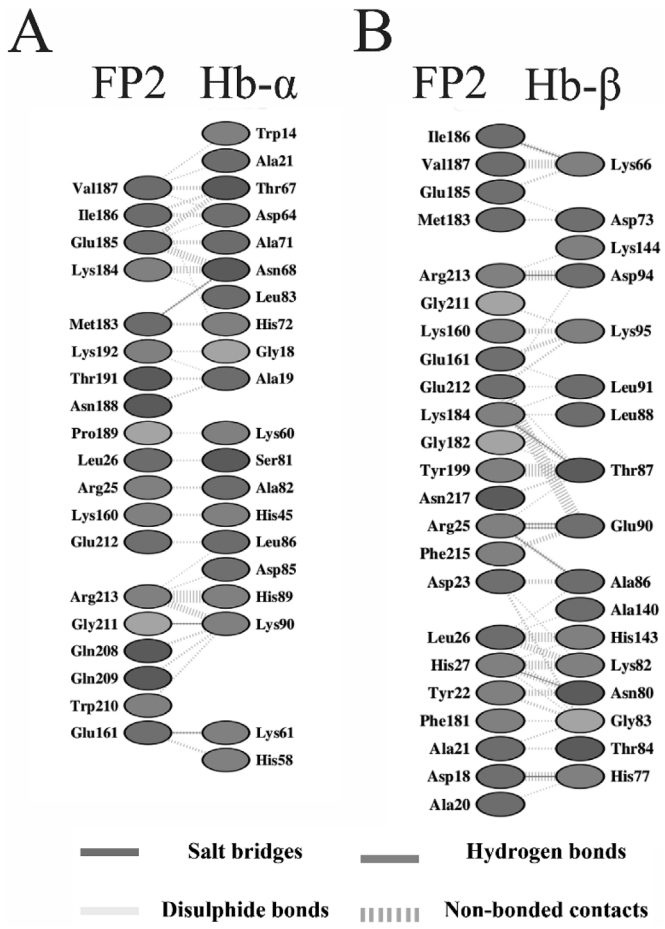

Fig. 2. Interactions between FP2 and Hb α, β chains. LIGPLOT analysis of interface residues of FP2 with interacting chains (α, β) of Hb joined by colored lines, each representing a different type of interaction. The number of lines for hydrogen bonding indicates number of hydrogen bonds between any two residues. For non-bonded contacts, the width of the striped line is proportional to the number of atomic contacts. Interactions of FP2 with HB-α (A) and FP2 with HB-β (B) show that residues Glu^{185} and Val^{187} of Hb motif of FP2 interact with α and β chains.

3.3. Expression and purification of different mutants of C-terminus motif of FP2 and FP3

Based on the above predictions, we constructed two mutants of FP2, and one mutant of FP3. A double mutant was generated by substituting Glu^{185} and Val^{187} residues of FP2 with alanine: ^{E185A,V187A}FP2 (Fig. 3A). Further, two single mutants were constructed by replacing Glu^{185} of FP2, and Asp^{194} of FP3 to alanine: ^{E185A}FP2 and ^{D194A}FP3, respectively (Fig. 3A). The wild and mutant proteases were expressed in *E. coli*, purified by affinity chromatography, further purified by ion-exchange chromatography and refolded in optimized refolding conditions (Fig. 3B–D). We further expressed, purified and refolded the C-terminus deletion motif of FP2 ($^{\Delta}$FP2) described earlier as a control (Pandey et al., 2005).

3.4. Enzyme activity of wild and different mutants of C-terminus motif of FP2 and FP3

To test the enzymatic activities, FP2, FP3 and different mutants (^{E185A,V187A}FP2, ^{E185A}FP2 and ^{D194A}FP3) were incubated with or without fluorogenic substrate under acidic conditions as described previously (Na et al., 2004; Pandey et al., 2005). Fluorescence unit was determined for each enzyme resulting from hydrolysis of a given substrate. The hydrolysis profile suggested that all mutants were active against fluorogenic substrate and had similar activity to that of wild enzyme, indicating that the mutants have refolded similarly as compared to wild enzyme and the active site was not considerably altered after mutagenesis (Fig. 3E). These data further corroborate with earlier finding that the deletion of 10aa motif is not required for activity against small peptides (Pandey et al., 2005).

The wild and mutant enzymes also had similar catalytic efficiencies, confirmed by calculating the specificity constant K_{cat}/K_m (Table 2). The enzymes also had similar activity to wild FP2 ($106{,}000 \pm 2180\ M^{-1}sec^{-1}$) and whole deletion mutant $^{\Delta}$FP2 ($103{,}000 \pm 3530\ M^{-1}sec^{-1}$) calculated from our earlier study (Pandey et al., 2005).

3.5. Crucial residues in C-terminus motif are required for hemoglobin hydrolysis

We compared the hydrolysis of hemoglobin, a natural substrate of falcipains, by wild and mutant enzymes. The wild type enzyme efficiently hydrolyzed hemoglobin, however, hydrolysis profile differed in comparison with C-terminus deletion mutant ($^{\Delta}$FP2) as observed earlier (Fig. 4A). The hydrolysis profiles of ^{E185A,V187A}FP2, ^{E185A}FP2 and ^{D194A}FP3 clearly showed that these mutants were unable to degrade hemoglobin, indicating that these residues are crucial for hemoglobin hydrolysis (Fig. 4B–D). Computational docking of FP2 and FP3 with hemoglobin and enzymatic activities of three mutants suggested that Glu^{185} and Val^{187} in FP2 and Asp^{194} in FP3 formed non-bonded interactions with α as well as β subunits of hemoglobin.

3.6. Functional conservation of Glu^{185} of FP2 and Asp^{194} of FP3

The amino acids Glutamic acid (Glu) and Aspartic acid (Asp) are both polar and acidic in nature. Thus their presence at the same position within the C-terminal motif of FP2 and FP3 indicated identical functional roles. To test this hypothesis, we interchanged the residues and created two mutants: ^{E185D}FP2 and ^{D194E}FP3. Hydrolysis and fluorogenic substrate analysis demonstrated that both ^{E185D}FP2 and ^{D194E}FP3 mutants were active and managed to degrade hemoglobin with similar efficiencies as wild enzymes (Table 2), suggesting functional conservation (Fig. 5).

4. Discussion

It is well known that *Plasmodium* cysteine proteases hydrolyze hemoglobin. As hemoglobin is hydrolyzed, heme component is changed into a non-toxic pigment, hemozoin. The proteinous part, globin is hydrolyzed to its constituent amino acids. Hemoglobin hydrolysis provides amino acids for protein synthesis of parasites (Francis et al., 1997; McKerrow et al., 1993), maintains the osmotic stability of malaria parasites and also provides space for growing intra-erythrocytic parasites (Lew et al., 2003).

How hemoglobin binding domains of FP2 and FP3 interact with hemoglobin has not yet been explored. In this study, we first mapped the hemoglobin binding domain by modeling a complex of FP2 with hemoglobin, based on solved structure of enzyme and substrate. The importance of interactions with Glu^{185} and Val^{187} in the hemoglobin binding domain of FP2 has been investigated by site directed mutagenesis and further validated by hemoglobin hydrolysis as well as fluorogenic substrate assays. Our study confirmed that C-terminus motif interacts via Glu^{185} and Val^{187} with hemoglobin by means of non-bonded interactions. Our result suggests that the enzyme initially captures hemoglobin via non-bonded interactions including hydrogen bonds and/or Van der Waals interactions before cleaving the substrate at the active site. Structural analysis studies at the surface of protein-protein interactions reveal that binding sites at the interface of PPIs are enriched with polar residues (Ma et al., 2003). Structurally, Glu^{185} of

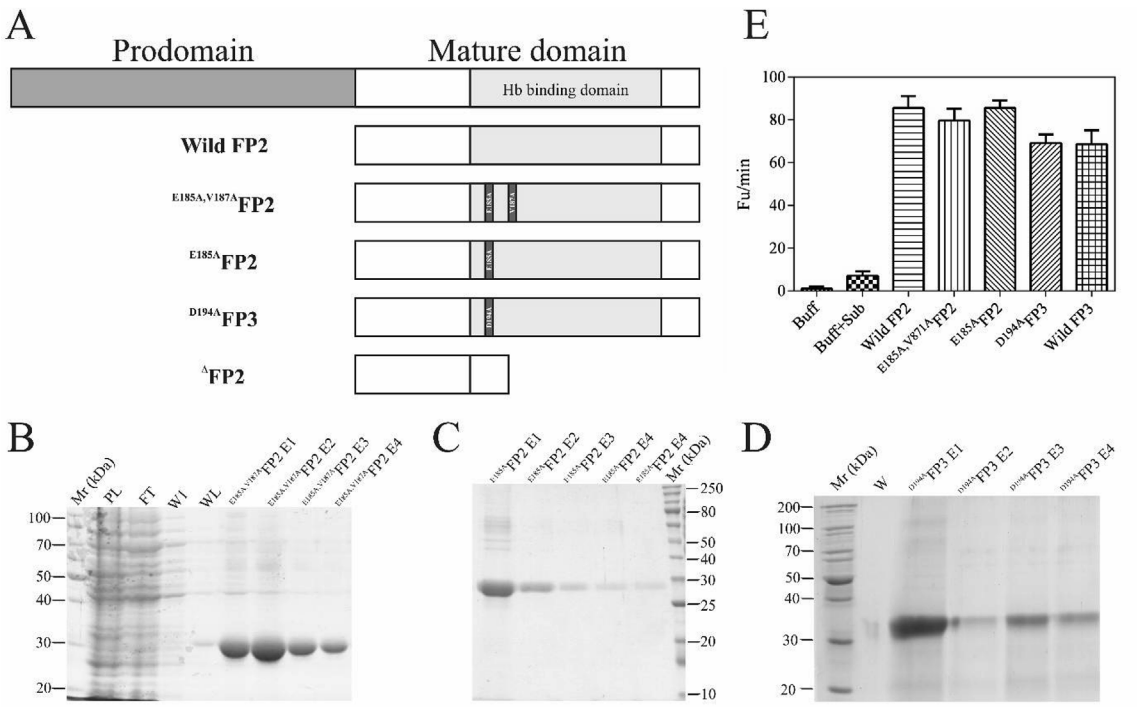

Fig. 3. Expression and functional assay of mutants. (A) Schematic showing design of two mutants (^{E185A}FP2 and ^{E185A,V187A}FP2) of FP2 and one mutant (^{D194A}FP3) of FP3. Mutants were expressed in *E. coli* and purified by Ni-NTA chromatography using imidazole gradient (B–D). PL (Preload), FT (Flow-through), W1 (wash 1), (WL) wash last, (E1-E4) elution of mutants and the positions of molecular wt. markers (kDa) are indicated. (E) The enzyme activity of different mutants (^{E185A}FP2, ^{E185A,V187A}FP2 and ^{D194A}FP3) and wild enzymes (FP2 and FP3) were tested by incubation with fluorogenic substrate, Z-Leu-Arg-AMC. Hydrolysis of substrate was measured as fluorescent units (Fu). Error bars represent the standard error of two independent measurements, each performed in duplicate.

Table 2
Catalytic efficiencies of different wild and mutant enzymes.

Enzyme	K_{cat}/K_m ($M^{-1}sec^{-1}$)
FP2 wild	130,300 ± 8069
FP3 wild	102,200 ± 7637
^{E185A,V187A}FP2	81,100 ± 6689
^{E185A}FP2	89,300 ± 6724
^{D194A}FP3	98,010 ± 7272
^{E185D}FP2	72,810 ± 6477
^{D194E}FP3	127,070 ± 8097

FP2 and Asp^{194} of FP3 are polar residues positioned at the interface of Hb domain-Hb interactions and thus are important in mediating specific interactions with residues in both hemoglobin monomer units. Val^{187} could have a role in stabilization of FP2-Hb interactions via Van der Waals interactions.

Computational and mutagenesis study suggested that both α and β subunits of hemoglobin interact with enzyme via non-bonded interactions. This finding is further supported by the stoichiometric analysis of FP2-hemoglobin by gel filtration chromatography, which showed a 4:1 ratio of enzyme to hemoglobin in the complex (Pandey et al., 2005). Hemoglobin being a tetramer, each subunit seems to interact with one molecule of enzyme via non-bonded interactions provided by specific residues, Glu^{185} and Val^{187} present in hemoglobin binding domain of FP2. Since the secondary structure of hemoglobin binding domain is well conserved among FP2 and FP3, we also tried to find out the crucial residues in FP3. Our mutagenesis and hemoglobin hydrolysis study showed that Asp^{194} is crucial for hemoglobin binding and also verified that Glu^{185} of FP2 and Asp^{194} of FP3 are functionally conserved.

Similar to FP2, matrix metalloprotease (MMP-2) binds to its natural substrate, collagen, through HPX domain, away from active site (Mikhailova et al., 2012). It seems FP2 and MMP-2 use similar means of biological control by binding natural substrate to exosite. There are evidences that exosites, specific substrate binding sites located outside the catalytic clefts, are essential for positioning of substrate molecule for binding and required for efficient hydrolysis (Lauer-Fields et al., 2008; Robichaud et al., 2011; Xu et al., 2007). It may be possible that evolution of such exosite interaction motifs in proteases is to specifically aid in scavenging and capturing of their natural macromolecular substrates. While molecular dynamic simulation studies have indicated the FP2 β-hairpin loop to be highly dynamic (Musyoka et al., 2016), this feature could be essential, as only a flexible structure can efficiently wriggle and assist in capturing and shuffling of Hb substrates to the active site, while a rigid structure may not.

Majority of drug development efforts in proteases were targeted to active site, however techniques such as high-throughput screening (HTS)/virtual screening of inhibitor libraries, generation of monoclonal antibodies against and incidental discovery of potent inhibitory compounds can also be used to identify exosite inhibitors and block exosite mediated interactions. The crucial enzymatic subunit Lethal factor (LF), a zinc-dependent metalloproteinase of *Bacillus anthracis* that causes anthrax, is responsible for cleavage and inactivation of host mitogen-activated kinase kinases (MKKs). A small molecule inhibitor, stictic acid (StA), a depsidone based natural product was identified through HTS of > 2500 compounds using a full length protease substrate which potently inhibited LF (Bannwarth et al., 2012). Human cathepsin K (CatK) a crucial target for treatment of osteoporosis was discovered to have two exosite regions that modulate enzyme activity. Blocking of active site led to inhibition of cleavage of other essential substrates as well, indicating detrimental side-effects of active site inhibition. A specific inhibitor DHT (*p*-dihydrotanshinone) was identified through a small molecule inhibitor screen that blocked exosite 1 which specifically inhibited elastase and collagenase activity but left activity against other substrates intact (Panwar et al., 2016; Sharma et al., 2015). The human metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) enzymatically cleaves both insulin-like growth factor (IGF)-binding proteins IGFBP-4 and -5. While many inhibitors target the

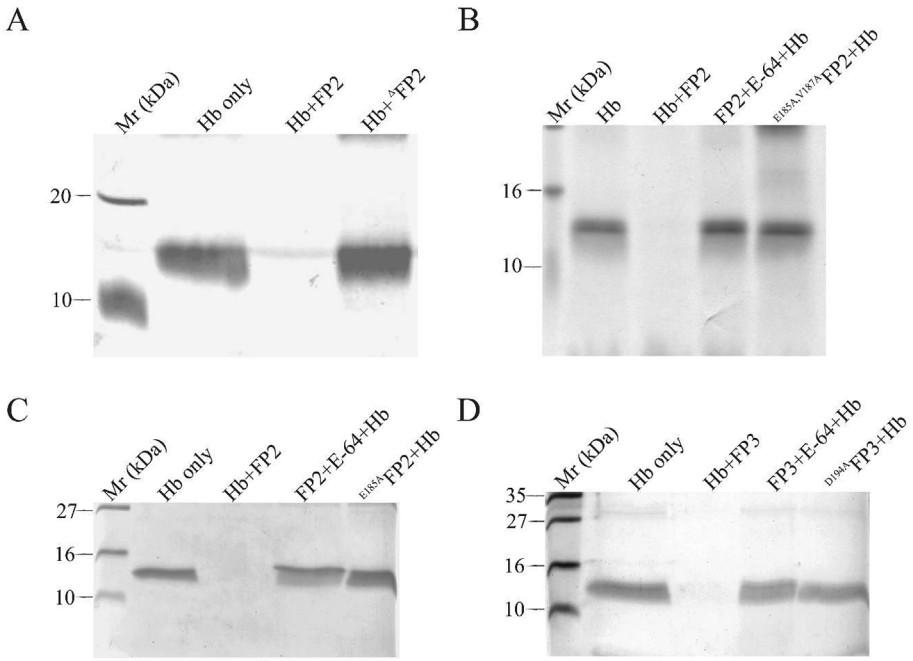

Fig. 4. Hydrolysis profile of mutant and wild enzymes. (A) Hemoglobin hydrolysis assay of C-terminus deletion mutant ($^{\Delta}$FP2) shown in 15% SDS-PAGE. The wild type enzyme (FP2) was used as a control. (B, C) Hemoglobin hydrolysis patterns of two mutants (^{E185A,V187A}FP2 and ^{E185A}FP2) of FP2 were analyzed under optimal conditions. (D) Hemoglobin hydrolysis of FP3 mutant (^{D194A}FP3) was also assessed with wild FP3 used as control. The positions of molecular wt. markers (kDa) are indicated.

downstream IGF signaling for treatment of several diseases, a set of monoclonal single chain fragment variable (scFv) antibodies were generated that specifically bound to a C-terminal exosite region in PAPP-A and selectively inhibited proteolytic activity against IGFBP-4 but not IGFBP-5 (Mikkelsen et al., 2008). Overall, such strategies suggest exosite targeting of FP2 to be highly feasible, through techniques such as inhibitor screening or developing novel compounds complementary to the structure of Hb binding domain or through monoclonal antibody generation specific to the exosite region.

Hemoglobin degradation is the major source of nutrition for malarial parasites and thus this pathway needs to be targeted for arresting asexual stage development. Currently, falcipains have been able to be inhibited by an array of peptidomimetic compounds that mimic and irreversibly bind to the active site. However, considerable success in developing potent drugs is yet to be achieved. Targeting exosites provide an alternative mechanism of inhibition of substrate binding to proteolytic enzymes. As the exosites reside further away from the active site, targeting exosite mediated PPIs could avoid drug resistance, as a drug resistance point mutation may not be complemented by the right compensatory mutations that can successfully stabilize a large protein-protein interface.

Targeting protein–protein interactions is a new field in malaria. Therefore, new compounds that may block exosite mediated substrate interactions have gained interest as a novel class of inhibitors with enhanced selectivity which could be less prone to drug resistance. However, targeting PPIs give rise to unique challenges as the target's structure (an interaction site between two proteins or hot-spot) is often not easily determined and/or occupies a larger area with different physicochemical properties than a typical 'catalytic site' (Zinzalla and Thurston, 2009). However, our recent study targeted such hot-spot interactions in falcipains and demonstrated allosteric inhibition of falcipain processing (Pant et al., 2018). Current literature suggest that PPIs have great potential as therapeutic targets but remain as challenging areas in drug discovery. In the near future, designing inhibitors based on PPIs will be a new approach in the field of malaria.

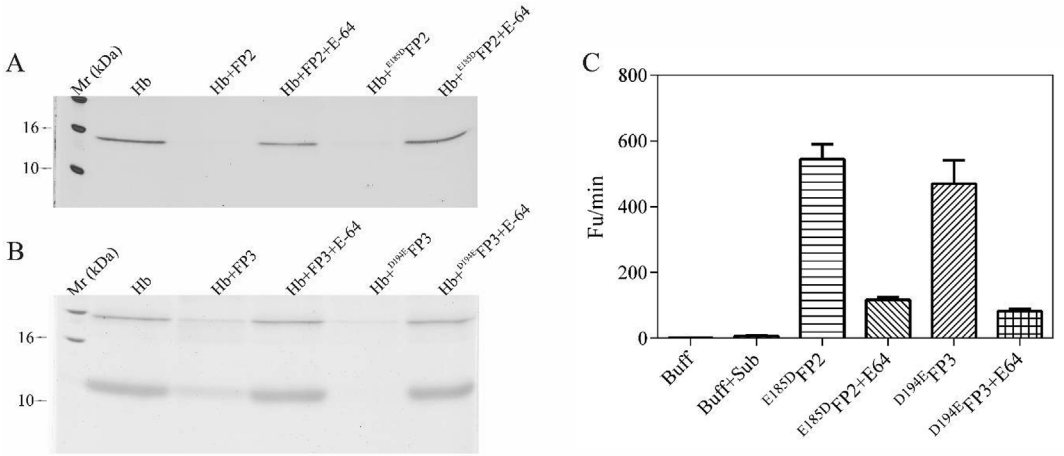

Fig. 5. Enzymatic activity of residue-swap mutants. Two mutants ^{E185D}FP2 and ^{D194E}FP3 were designed and their enzymatic activities assayed. Hemoglobin hydrolysis patterns of the two mutants ^{E185D}FP2 (A) and ^{D194E}FP3 (B) were analyzed under optimal conditions with wild FP2 and FP3 as controls. The positions of molecular wt. markers (kDa) are indicated. The enzyme activity of mutants ^{E185D}FP2 and ^{D194E}FP3 and wild enzymes (FP2 and FP3) were tested by incubation with fluorogenic substrate, Z-Leu-Arg-AMC (C). Error bars represent the standard error of two independent measurements, each performed in duplicate.

Author Contributions

RP, SV, AP, RS, KCP designed and performed the experiments. RD, SS assisted in the writing; AS and RD provided the valuable reagents; KCP, RP, SV wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2019.01.005.

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