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Independent amino acid residues in the S2 pocket of falcipain-3 determine its specificity for P2 residues in substrates

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ABSTRACT

Falcipain-3 (FP3) is an essential and drug target cysteine protease of the most lethal human malaria parasite *Plasmodium falciparum*. FP3 and its majority of homologs in malaria parasites prefer Leu at the P2 position in substrates and inhibitors, whereas its major host homolog cathepsin L prefers Phe. However, FP3 is much less active on peptide substrates and has negligible activity against a P2 Arg-containing substrate (Z-RR-AMC) compared to its paralog falcipain-2A (FP2A). To identify the specificity determinants, the S2/3 pocket residues of FP3 were substituted with the corresponding residues in FP2 or cathepsin L, and the wild type and mutant proteases were assessed for hydrolysis of peptide and protein substrates. Our results indicate that the S2 pocket residues I94 and P181 of FP3 are chiefly responsible for its P2 Leu preference and negligible activity for Z-RR-AMC, respectively. E243 in FP3 and the corresponding residue D234 in FP2 have a key role in Z-RR-AMC hydrolysing activity, possibly through stabilization of side chain interactions, as their substitution with Ala abolished the activity. Several FP3 mutants, which retained P2 Leu preference and showed similar or more activity than wild type FP3 on peptide substrates, degraded haemoglobin less efficiently than wild type FP3, suggesting that multiple residues contribute to haemoglobinase activity. Furthermore, P181 and E243 appear to contribute to the optimum activity of FP3 in the food vacuole milieu (≈pH 5.5). The identification of residues determining specificity of FP3 could aid in developing specific inhibitors of FP3 and its homologs in malaria parasites.

1. Introduction

Proteases have critical roles in development of all organisms from viruses to humans, and are considered attractive drug targets for a number of diseases, including malaria. Protozoan parasites of the *Plasmodium* genus cause malaria, which had an estimated 198 million cases and 0.58 million deaths in 2013 (WHO Malaria Report 2014). Five *Plasmodium* species cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*, *P. falciparum* and *P. vivax* are responsible for the majority of malaria cases, with the former causing most of the deaths. Infection in humans begins upon inoculation of the sporozoite stage by female *Anopheles* mosquitoes. Sporozoites invade and mature in hepatocytes, producing several thousand merozoites, which are released in the blood, and invade erythrocytes. Inside the erythrocyte, parasite develops through ring, trophozoite, and schizont stages. Each schizont matures into several merozoites, which are released in the blood upon rupture of the infected erythrocyte. These merozoites invade fresh erythrocytes, thereby restarting the erythrocytic stage development cycle, which is responsible for malaria. During this development, some parasites differentiate into gametocytes, which are taken up by the mosquito along with the blood meal, and undergo sexual development through multiple stages, finally producing hundreds of sporozoites.

Rapidly spreading resistance to most of the antimalarials is a major hurdle to control malaria. The emergence of resistance to artemisinins, the mainstay drug of current antimalarial chemotherapy, has further limited the choice of antimalarials. Hence, the need

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of today is to develop new drugs and identify new targets. Proteolytic repertoire of malaria parasites offers attractive targets for antimalarial development, and papain-like cysteine proteases of the parasite are both genetically and chemically validated drug targets. Initial studies with protease inhibitors suggested roles of cysteine and aspartic proteases in haemoglobin degradation, as their inhibitors blocked erythrocytic stage development of malaria parasites with accumulation of undegraded haemoglobin [1–3]. *P. falciparum* has 4 papain-like proteases that are known as falcipains: falcipain-1 (FP1), falcipain-2A (FP2A), falcipain-2B (FP2B), and falcipain-3 (FP3) [4]. FP1 shares 28–30% sequence identity with other three paralogs; FP2A and FP2B are 92% identical, and share 54% identity with FP3. All falcipains are expressed during erythrocytic stage development; FP2 and FP3 have been shown to be located in the food vacuole, a lysosome-like organelle wherein haemoglobin is degraded [5–10]. All four recombinant falcipains have been shown to be optimally active around the food vacuole pH (pH 5.5), degrade haemoglobin, and inhibited by the cysteine protease inhibitors that also block parasite development. Knockout of the FP1 or FP2B gene did not affect erythrocytic stage development, indicating that these two proteases are dispensable during erythrocytic stage development [11,12]. An independent study showed 70–90% lower oocyst production in FP1 knockout parasites than wild type parasites, suggesting a key role for FP1 during parasite development in the mosquito midgut [13]. Contrary to the FP1 gene knockout studies, a FP1 specific inhibitor apparently inhibited invasion of erythrocytes by *P. falciparum* merozoites, suggesting a crucial role of FP1 in invasion [14]. Disruption of the FP2A gene caused swollen food vacuoles due to accumulation of undegraded haemoglobin during trophozoite stage, which confirmed a major role of FP2A in haemoglobin degradation [12,15]. Independent FP2A knockout and knockdown studies further corroborate its involvement in haemoglobin degradation [16–18]. FP2A knockout parasites showed almost 2-fold and 1000-fold more sensitivity than wild type parasites to cysteine and aspartic protease inhibitors, respectively. On the other hand, FP1KO and FP2BKO parasites were as susceptible as wild type parasites. The FP3 gene could not be knocked out, but it was replaced by its functional myc-tagged allele, suggesting that it is essential for erythrocytic development of the parasite [12]. In addition to falcipains, a number of other proteases, including plasmepsins, metallopeptidases, dipeptidyl aminopeptidases, and aminopeptidases, have been implicated in different stages of haemoglobin catabolism [2,5–8,19–30]. Thus, haemoglobin is degraded by concerted action of multiple proteases, and multiple lines of evidence indicate that falcipains are the major haemoglobin-degrading proteases.

A number of falcipain homologs of other *Plasmodium* species have also been characterized in recent years. There are four falcipain homologs in other human and nonhuman primate malaria parasites, such as vivapains (VX1-4) in *P. vivax* and knowpains (KP1-4) in *P. knowlesi*. Rodent malaria parasites have only two homologs, which are known as bergheipains (BP1 and BP2) in the commonly used animal model malaria parasite *Plasmodium berghei* [31–35]. All FP1 homologs cluster together in a phylogenetic tree, forming the FP1 subfamily; the homologs of remaining three falcipains cluster together in a phylogenetic tree, forming the FP2/3 subfamily. Phylogenetic relatedness of FP2/3 subfamily proteases is also reflected in their properties, as the majority of these proteases are maximally active around pH 5.5, degrade haemoglobin, and located in the food vacuole. All FP2/3 subfamily proteases contain two unique inserts in the mature protease domain: one upstream of the catalytic cysteine residue in the N-terminal that has been shown to function as a refolding domain and the other one downstream in the C-terminal between the highly conserved catalytic histidine and aspargine residues, which has been shown to be required for binding to and degradation of haemoglobin by FP2A [36–39]. In addition to haemoglobin degradation, the FP2/3 subfamily proteases are also implicated in selective processing of several parasite proteins, including plasmepsins, erythrocyte skeleton proteins, and the gametocyte surface protein Pfs230, suggesting additional roles of these proteases [40–48].

Taken together, a major role of FP2A in haemoglobin degradation and indispensability of FP3 for erythrocytic stage parasite development validate drug target potential of FP2A and FP3. Considerable work has been done towards developing potent and selective inhibitors of FP2 and FP3, and a number of inhibitors have shown promising results in both in vitro culture systems and animal models [49–51]. Investigation of structure-activity relationship (SAR) of enzymes has greatly aided in designing/developing specific inhibitors of a number of clinically important enzymes, including the HIV protease and cathepsin K [52–54]. The active site of papain-family proteases contains distinct pockets for interacting with substrate residues on either side of the scissile peptide bond; pockets S1, S2, S3, and S4 bind to the substrate residues P1, P2, P3, and P4, with the numbering S1 for the immediate upstream residue P1; pockets S1′, S2′, and S3′ bind to the substrate residues P1′, P2′, and P3′, with the numbering S1′ for the immediate downstream residue P1′ [55]. Of these, the S2 pocket is the major specificity determinant and has mostly hydrophobic environment.

The majority of FP2/3 subfamily proteases, including FP2 and FP3, prefer Leu at the P2 position in substrates [31–34,56], whereas the most abundant homolog in the host, cathepsin L (CatL), prefers Phe [57]. Notably, the P2 Leu selectivity has also been demonstrated for inhibitors in the cases of FP2, FP3, VX2, and VX3 [32], which is consistent with over 20 times greater inhibition of parasite growth by compounds with P2 Leu compared to those with P2 Phe [58]. This difference in selectivity could be exploited for developing potent and specific pan-FP2/3 subfamily protease inhibitors. Additionally, the P2 Leu preference could be exploited for developing dual-target inhibitors of FP2/3 subfamily proteases and the proteasome [59]. Crystal structures of FP2 and FP3 complexed with inhibitors showed a typical papain-like fold in which the proteases contain L and R domains, with the active site residues (FP2/FP3: Gln36/45, Cys42/51, His174/183, Asn204/213) in between [39,60–62]. The structures identified amino acid residues forming the S2 pockets of FP2 and FP3, and proposed that the P2 Leu preference of FP2 and FP3 is due to their narrow S2 pockets compared to that of CatL. The crystal structures of protease-inhibitor complexes and docking studies also indicated substantial interactions of inhibitors with the S2 pocket residues, suggesting that these interactions could be exploited for achieving specificity [39,56,60,62,63]. Although these structures offered speculations about differences in biochemical properties of FP2 and FP3, and their homologs in other parasites and mammalian cells, a systematic investigation of the determinants of structure-activity relationship of FP2 and FP3 has not been done yet. Using a comparative structure-guided mutagenesis approach, we have identified the determinants of P2 Leu selectivity, activity towards substrates with P2 positively charged amino acid residue, and lower catalytic efficiency of FP3 compared to FP2.

2. Materials and methods

2.1. Materials

The *P. falciparum* 3D7 strain was obtained from the Malaria Research and Reference Reagent Resource Centre (MR4). All the biochemical reagents and solvents were from Sigma and Serva; plasmid isolation kits and Ni-NTA agarose resin were from Qiagen and MACHEREY-NAGEL; cell culture reagents were from Lonza and Invitrogen; restriction and DNA modifying enzymes were from New England Biolabs; peptide substrates and inhibitors were from