Dynamical footprint of falcipain-2 catalytic triad in hemoglobin-β-bound state

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Falcipain-2 (FP-2) is a member of papain family of cysteine proteases and the major hemoglobinase of the hemoglobin detoxification and hemozoin polymerization complex localized in the food vacuole of the plasmodium species. FP-2 is currently gaining clinical significance as the drug target of choice in combating malaria epidemic. Here, a theoretical FP-2/hemoglobin complex has been proposed and the dynamical footprint and energetics of binding have been investigated using molecular and quantum mechanics approaches. The mapped interaction interface comprises residues 34–51 of hemoglobin and cysteine-42/histidine-174/glutamine-36/asparagine-173/204 and subsites S1, S1′, and S3 of FP-2. In hemoglobin-bound FP-2, asparagine-173 preferentially partners histidine-174, while glutamine-36 is preferred in ligand-free state. Cysteine-42 exhibits dihedral switch from 110° to 30° in free and bound states, respectively, with exclusion of water from the binding core upon hemoglobin binding. Hemoglobin similarly exhibits high occupancy within .2 nm distance with charged amido acid-rich subsites S1 and S3 of FP-2 functioning in tandem to reduce conformational flexibility of hemoglobin and facilitate the formation of a stabilizing anti-parallel β-sheet between Leucine-172-valine-176 of FP-2 and phenylalanine-45-asparate-47 of hemoglobin and to overcome the +1.13e+5 eV activation energy required to optimize the FP-2/hemoglobin-β conformation that precedes hydrolysis.

**Keywords:** FP-2; hemoglobin-β; dihedral switch; activation energy; dynamical footprint

**Introduction**

Malaria is one of the most important infectious diseases in the tropical and subtropical countries of the world with a strong link to infant mortality and pregnancy-related deaths (Sadanand, 2010; Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009). The battle against malaria epidemic has been a multi-faceted one involving the control of mosquito breeding and targeting the major stages of plasmodium life cycle. Increasing number of research publications have supported that pyrethroids insecticides used for controlling mosquito population may have possible human toxicity (Omotuyi et al., 2006; Schleier & Peterson, 2012). Similarly, several available drugs inhibiting plasmodium development have either suffered from resistance or have become a source of clinical concern in terms of toxicity (Effern & Kaina, 2010; Omotuyi et al., 2008; Quashe, 2010). Therefore, new generation of anti-malarial drugs, either by redesigning the currently available types to ensure exclusive toxicity to plasmodium and enhanced anti-plasmodium activity or directed against new targets, is of immediate clinical importance.

Although, several macro-molecular candidates currently represent potential new targets for anti-malarial drug design; however, hemoglobin detoxification and hemozoin polymerization complex is arguably the most promising target (Chugh et al., 2013). This multi-protein complex is ~200-kDa and localized in the food vacuole of plasmodium. Potentially, each of the constituent protein (falcipain (FP)-2/2′, plasmepsin II, plasmepsin IV, aspartic protease, and heme detoxification protein) or in its entirety can be targeted for anti-malarial drug development via intra-erythrocytic hemoglobin breakdown inhibition.

The focus here is on the FP component. The four well-characterized FPs (FP-1, FP-2/FP-2′, FP-3) of *Plasmodium falciparum* are classical papain-family (family C1A) cysteine proteases (Pandey, Barkan, Sali, & Rosenthal, 2009; Pandey et al., 2005). Within the catalytic domain, FP1 shares 38% sequence identity with FP-2; FP-2 and FP-2′ are identical while sharing 68% identity with FP-3. Combined gene disruption and enzyme inhibition data have established the role of FP-2/FP-2′ in hemoglobin hydrolysis (Chugh et al., 2013; Hogg et al., 2006), restricted parasite development (Sijwali & Rosenthal, 2004) upon inhibition and in some reports an outright cure of mice subjected with murine malaria (Dominguez et al., 2005; Soni et al., 2005).

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To contribute to the developmental effort at designing drug candidates with inhibitory action on FP-2, a theoretical complex comprising FP-2 and beta-chain of hemoglobin was built based on current information available in literature, available crystal structures (FP-2 and other molecular complexes containing FP-2, crystal structure of hemoglobin) and protein-docking algorithms (Comeau, Gatchell, Vajda, & Camacho, 2004). Furthermore, a combined ~2.750-microsecond all-atom molecular dynamics simulation was conducted on various assemblies relevant to the complex in order to understand the dynamical workings of FP-2 during hemoglobin binding and possible hydrolysis.

**Materials and methods**

**Starting coordinates**

The starting coordinate of inactive FP-2 was derived from the crystal structure of E64-bound FP-2 (inactive) resolved at 2.90 Å and deposited at the protein data bank (PDB ID: 3BPF) (Kerr et al., 2009). The structure shows high conformational similarity with *Plasmodium berghei* inhibitors of cysteine proteases (PbICP-C)-bound FP-2 resolved at 2.90 Å deposited by Hansen et al. (2011). To generate a thermodynamically stable free FP-2, the E-64 coordinate was removed from the E64-bound FP-2 complex and the resulting FP-2 was subjected to 500 ns MD simulation (heavy-atom restrained MD simulation, Suppl. Table 1.0), this was followed by a plot of the 3D free energy landscape from the probability distribution \( P [\Delta G] \) of the protein along the root-mean square deviation (rmsd, nm) (of all the conformations generated at every 10 ps interval to the initial structure) and radius of gyration \( r_g \) using the equation \( \Delta G = -k_B T \ln P(\Delta G) \) Jain and Stock (in press). A single coordinate from the valley \( \Delta G = 0 \) kcal/mol, \( r_g \approx 1.83 \) nm, rmsd \( \approx .16 \) nm, Suppl. Figure 1A) was extracted as representative metastable structure (Suppl. Figure 1B) for free FP-2 used for the other simulations in this study. It is interesting to note that the selected structure here showed alpha carbon rmsd value \( \approx .34 \) Å from a single unit of free FP-2 structures (chain A, B, C and D) crystallized by Hogg et al. (2006) (PDB ID 2GHU). The crystal structure of oxyhemoglobin resolved at 2.1 Å (PDB ID 1HHO) was used as the starting coordinate for heme-bound hemoglobin-\( \beta \), while the removal of heme gave the coordinate for the free hemoglobin-\( \beta \) (Shaanan, 1983). At the time of this investigation and manuscript preparation, no crystal structure for FP-2/hemoglobin complex has been resolved. Consequently, no direct information is available on the stoichiometry of binding. However, the earlier works of Atha and Riggs (1976) revealed the hemoglobin tetramer to dimer dissociation during pH 8.5 to 6.0 transition. Furthermore, the structures of FP-2 co-crystallized with E-64 (Kerr et al., 2009), leupeptine, cystatin and PbICP-C (Hansen et al., 2011; Wang et al., 2006) did not only establish the likelihood of 1:1 FP-2-ligand stoichiometry, but also depicted the affinity of the FP-2 binding site for randomly coiled regions of the ligand. Therefore, using the aforementioned information, the tightly bound FP-2/hemoglobin-\( \beta \) conformation was generated using the cluspro-server (www.cluspro.bu.edu/) (Comeau et al., 2004); here, the flexible region valine-34 and proline-51 of (free) hemoglobin and all residues within 10 Å radius of cysteine-42 of FP-2 were designated as the binding partners for the rigid body docking on cluspro server. The docked complex was subjected to 500 ns simulation as described above to remove atomic clashes and to generate metastable complex at \( \Delta G = 0 \) kcal/mol to be used as the starting structure for tightly bound FP-2/hemoglobin-\( \beta \) conformation (Suppl. Figure 1C&D). The loosely bound conformation (center-of-mass distance \( \approx 20 \) Å) was derived from the tightly bound conformation where the hemoglobin coordinate was dragged from the complex to a distance of 20 Å using VMD (Humphrey, Dalke, & Schulten, 1996). The E-64-FP-2/hemoglobin-\( \beta \) was derived from the loosely bound complex by replacing the FP-2 subunit by the E64-bound FP-2 using the align module of PYMOL (PYMOL, Version 1.5.0.4).

**MD simulation and quantum mechanics calculation**

The pH of each biosystem (Suppl. Table 1.0) to be simulated was adjusted to 6.0 using the protein preparation module on Molecular Operating Environment (MOE, 2013.08) (MOE, Chemical Computing Group Inc.) to conform with the optimum pH of FP-2 as reported by Hogg et al. (2006). Each biosystem was inserted into the middle of the simulation box at 40 Å from the wall of the box in all directions. The boxes were solvated in single point charge water and neutralized in NACL. The molecular dynamics calculations were performed using GROMACS (ver 6.5) MD package (Van Der Spoel et al., 2005) and GROMOS force field. Prior to production runs, 100 ns equilibration simulations were performed on each biosystem at 300 K and 1 bar except in two biosystems ((E64-less)- FP-2 and docked hemoglobin-FP-2*(tight)), where the equilibration were extended to 500 ns. Semi-isotropic Parrinello–Rahman barostat and Nose–Hoover thermostat algorithms were used for pressure and temperature control during the equilibration, while the Lennard-Jones potential was adapted for estimating interaction between a pair of neural atoms at a cut-off distance of 9.0 Å. Particle mesh Ewald summation was used for estimating the long-range ionic interactions and each cut-off was maintained by Verlet scheme (Páll & Hess, 2013), while the equation of atomic motions was integrated using the leapfrog algorithm at a time step of 2.0 fs. For production runs, all positional...
restraints were excluded and all other simulation parameters were maintained as described for NPT equilibration. To drive the tightly bound FP-2/hemoglobin complex towards the loosely bound conformation, 1000 kJ mol/nm^2 force was applied to the hemoglobin-β subunit at the rate of .04 Å/ps. Fifty different coordinates separated by 4 Å were retrieved for potential energy estimation using quantum mechanics approach (Atkins & Friedman, 2008). The quantum mechanics calculation was carried out using MOPAC QM package; first, all residues within 12 Å radius of cysteine-42 (FP-2) and serine-44 (hemoglobin-β) of each complex were chosen using MOE, these coordinates were then used for generating MOPAC input files. Noting that MOPAC implemented on MOE has the capacity for a maximum of 60 heavy atoms, the input files generated from MOE were used as the input files for potential energy calculation on stand-alone MOPAC (MOPAC2012) using the PM3 semi-empirical SCF method without geometry optimization step.

**Data analysis**

The data generated during the simulation were analyzed using the GROMACS analysis tools (Van Der Spoel et al., 2005) such as g_mindist tool (for interatomic minimum distance analysis), g_dist tool (for estimating the center of mass distance between two groups), g_angle (for estimating the chi1 dihedral changes in group of atoms), trjorder tool (for the counting of water molecules at specified distance), and g_analyze (for estimating mean and standard deviation of data) or in-house scripts (3D free-energy landscape plot). The 3D plots were generated using Mathematica’09, the box-and-whisker plots were generated using QI-MACROS 2014, the line graphs were plotted using NUMBER’09 package, and the protein snapshots were taken using PYMOL.

**Results**

**The dynamical footprint of the FP-2 catalytic triad-hemoglobin-β interaction**

FP-2 like many members of cysteine proteases depend on a common catalytic triad contributed by asparagine, histidine, and cysteine (nucleophilic thiol) residues (Buller & Townsend, 2013). Based on crystallographic data, the nucleophile responsible for the attack of carbonyl group in FP-2 is cysteine-42, while histidine-174 functions in thiol deprotonation. Histidine-174’s polarization function is achieved by one of the three redundant glutamine (36) or asparagine (173, 204) located proximal to histidine-174 (Hogg et al., 2006; Kerr et al., 2009) (Figure 1(A) and (B)). The molecular dynamics data presented revealed that in hemoglobin-bound FP-2, asparagine-173 may play a key role in histidine polarization as its amide group existed at less than 3.5 Å from the imidazole side chain of histidine-174 (Figure 1(C)). This contrasts with free FP-2 and hemoglobin semi-bound states, where they are separated by at least 7.0 Å. In FP-2 free state, glutamine-36 represents the polarizing amide-group; the theoretical evidence for this was obtained in semi-bound state (Figure 1(D)), where glutamine-36’s amide group initially located at less than 3.0 Å distance from the imidazole ring of histidine-174 (hallmark of free FP-2 state) relocated to approximately .7 Å, a similar distance observed for hemoglobin-bound state of FP-2. Although, asparagine-204’s amide group does not show a clear distinction between free and hemoglobin-bound states of FP-2 (Figure 1(E)), its approximate distance (<2.5 Å) from the imidazole group of histidine-174 revealed that it is the preferred residue; this finding is consistent with the E-64, leupeptine, and chagasinc co-crystallized FP-2 (Hansen et al., 2011; Kerr et al., 2009) structures. Similarly, in agreement with the deprotonation mechanism prior to nucleophilic attack, the thiol side chain of cysteine-42 existed in approximately 1.5 Å away from histidine free FP-2 and in semi-bound states, these groups gyrate between 1.5 and 5.0 Å which contrasts the hemoglobin-bound state where they were persistently separated by 5.0 Å (Figure 1(F)). To establish that the features described above truly define putative hemoglobin-bound and semi-bound states, the minimum distance between FP-2 cysteine-42 sulfur atom and hemoglobin serine-44 carbonyl carbon atom was followed along the trajectories. Indeed, a gradual decrease in the interatomic distance to less than 5 Å at the last 100 ns of the FP-2/hemoglobin-bound complex provided a strong theoretical evidence for possible nucleophilic attack in cysteine-42 charged state compared with >1.50 Å distance between equivalent atoms in semi-bound state (Figure 1(G)). Furthermore, a possible dihedral footprint following hemoglobin attack was investigated. Indeed, cysteine-42 of FP-2 exhibited a distinct side chain chi1 dihedral of approximately 30° in hemoglobin-bound state and 120° in semi-bound and free states. Lastly, the interface between FP-2 and hemoglobin is scantily solvated in both hemoglobin-bound and semi-bound states. Within .3 nm radii in both cases, a maximum of two water molecules are detectable during the last 50 ns of the simulation (Figure 1(H)). This finding is not only consistent with an earlier statistical thermodynamic study detailing the displacement of explicit solvent from the ligand binding domains of FP-2 and FP-3 upon ligand binding (Shah et al., 2012), but also consistent with the strict exclusion of acid–base substance from interfering with the acid–base-nucleophile triad hydrolysis mechanism in operation in FP-2.
**FP-bound hemoglobin-β adopts distinct conformation stabilized by coil-sheet transition**

An important revelation in the crystal structure of FP-2 in free and E64 bound states is that a free FP-2 is structurally indistinguishable from the inhibitor-bound (Hansen et al., 2011; Kerr et al., 2009) and that raises a question here as to whether the ligand is responsible for the large structural alteration required to optimize binding. To answer this question, another molecular dynamics simulation was conducted on a free hemoglobin-β subunit and the results obtained were compared with the previous FP-2 bound and semi-bound states. Indeed, as indicated in Table 1.0, the conformations sampled by residues phenylalanine-41 to glycine-46 (bound regions) in FP-2-bound state were distinct as revealed by the large difference in the root-mean square distance values (average values after 250 ns showed .295 ± .049, .516 ± .013, and .0549 ± .028 nm for free, semi-bound, and bound states relative to the average conformation sampled by the bound state in the last 50 ns). Comparatively, the unbound hemoglobin did not sufficiently sample bound hemoglobin conformations and showed that free hemoglobin is not readily hydrolizable by FP-2. This finding raises the question and an observation; first, what structural ensembles were sampled by hemoglobin in bound state that distinguished it from the unbound state? (A) Snapshots of FP-2 active site pocket showing the catalytic triad residues. (B) Schematic arrangement of the triad with number indicating the sequence of function. (C, D, and E) the minimum distance (nm) between asparagine-173 (C), glutamine-36 (D), asparagine-204 (E), and histidine-174 side chain during the simulations. (F) The minimum distance (nm) between cysteine-42 and histidine-174 during 250 ns simulation. (G) The minimum distance (nm) between the thiol functional group of cysteine-42 (FP-2) and the carbonyl carbon atom of serine-44 of hemoglobin-β as a function of time. (H) Line plot of the dihedral angle of cysteine-42 as a function of time for 250 ns. (I) Concentric circle representing the number of water molecule at given radii from cysteine-42 core during the molecular dynamics simulation. (All plots represent a mean of two independent simulations, the color represents different biosystems; blue represents free FP-2, green represents FP-2 partially bound to hemoglobin-β separated at 20 Å, while yellow represents tightly bound FP-2/hemoglobin-β).
And the observation is that the induced fit mechanism is required for the interaction between hemoglobin and FP-2. To answer the question raised, the representative structures of the largest population of conformations adopted during the last 50 ns of the simulations were investigated (Figure 2(A)–(C)). Interestingly, in FP-2-bound state, a transition from coil to β sheet was observed within the bound region (Figure 2(A)), while in free and semi-bound states, this region of hemoglobin persistently sampled coil conformation (Figure 2(B) and (C)). It is interesting to note that similar secondary structure sampling by ligand had been reported in E64- and leupeptine-bound FP-2. It was observed that regions of the inhibitor extending beyond the FP-2 binding-pocket into the solvent appeared more disordered (butane moiety in E64 and the arginal moiety in leupeptin) indicating that the active site reduces the flexibility of the bound regions. Furthermore, the mechanism underlying such order within the pocket is a hydrogen-bond formation between the O and N atoms of the inhibitor backbone resulting in a typical β–sheet formation (Kerr et al., 2009); and the anti-parallel β–sheet formation between the bound portion of hemoglobin-β and FP-2 captured during the MD simulation (Figure 2(D)) also shows high consistency with the reported avid interaction between the dynamic β-hairpin of FP-2 and hemoglobin (Hogg et al., 2006).

**Free and E64-bound FP-2 is indistinct but different from hemoglobin-β-bound conformation**

Another valid question is whether the conformational space sampled by FP-2 tightly bound to hemoglobin is similar to those sampled by free FP-2, E-64-bound FP-2, and semi-bound to hemoglobin. To answer this question, the conformations generated during the last 50 ns of FP-2/ hemoglobin complex were clustered and two representative fragments (residues 170–175, and residues 40–45) covering the catalytic histidine and cysteine dyads were analyzed for similar conformation in free FP-2, E-64-bound FP-2, and semi-bound to hemoglobin during the last 150 ns of the trajectories. The rmsd values obtained were represented as box-and-whisker plots (Figure 3(A) and (B)) with the FP-2/hemoglobin complex serving as the control. The data here showed good agreement with observations deducible from the crystal data that free FP-2 is not structurally distinguishable from E-64 bound form; only differing by <.05 nm (alpha carbon rmsd, data not

![Figure 2. Representative snapshots of the most populated conformations sampled by free hemoglobin, semi-bound hemoglobin/FP-2 and tightly bound hemoglobin/FP-2 complexes. (A) Representative snapshot of tightly bound hemoglobin/FP-2 complex showing a secondary structure (cyan). (B,C) Representative snapshot of free (B) and semi-bound (C) hemoglobin/FP-2 complex showing coil structures in the same region. (D) A snapshot of ‘A’ above with the hemoglobin pair to underscore the captured anti-parallel formation between hemoglobin-β and FP-2.](image-url)
shown). Conversely, these structures differ slightly from the hemoglobin-bound conformation with alpha carbon mean rmsd values of .3 and .15 nm for free FP-2/E64-bound FP-2 and FP-2/hemoglobin, respectively. Similarly, while the presence or otherwise of E-64 has no significant structural effect on FP-2 in complex with a loosely bound hemoglobin-β, the presence of hemoglobin however drives the complex to sample distinct conformations with mean alpha carbon rmsd values >.6 nm for fragment 170–175. It is also interesting to note here that loosely bound hemoglobin and E-64 to FP-2 does not significantly alter the conformation sampled by the fragment 40–45 (the alpha carbon rmsd values for free FP-2/E-64/FP-2 is ≈ .2 nm vs. .27 nm for FP-2/hemoglobin (loose))/E-64/FP-2/hemoglobin (loose) (Figure 3(B)), thus, providing theoretical confirmation that fragments 170–175 may be required for stabilizing hemoglobin binding within the FP-2 active site cleft compared with fragment 40–45 which has the nucleophilic cysteine.

Accurate positioning of the hydrolizable fragment of hemoglobin-β and precise orientation of CYS-42 thiol towards the attackable carbonyl carbon of the ligand is the rate-limiting step

Next, starting from the tightly bound FP-2/hemoglobin-β conformation (starting conformation), we drove the complex towards the semi-bound conformation where FP-2 and hemoglobin were separated by center-of-mass distance of 20 Å using umbrella pulling simulations (see Materials and methods). Along the trajectory, 50 complexes separated by .4 Å were retrieved for potential energy calculations using quantum mechanics approach. Indeed, the starting conformation showed a center-of-mass distance <7.5 Å with the potential energy of $-2.28e + 5$ eV (Figure 4(A)). Surprisingly, the dissociation was unexpectedly simple with a single transition peak, where the two proteins were separated by center-of-mass distance of 1.0 Å, loss of the stabilizing β-sheet in the bound portion of hemoglobin-β and retraction of cysteine-42 side chain.

Figure 3. Active site conformational sampling in free, inhibitor-, and substrate-bound FP-2 and the occupancy of FP-2 subsites by hemoglobin-β. (A) A box-and-whisker plot showing the conformational relationship between structural ensembles sampled by residue 170–174 fragment in free and E-64-bound FP-2 compared with similar assembly in complex with semi- and tightly bound hemoglobin. (B) A box-and-whisker plot showing the conformational relationship between structural ensembles sampled by residue 40–45 fragment in free and E-64-bound FP-2 compared with similar assembly in complex with semi- and tightly bound hemoglobin. (C) Histogram representation of the percentage occupancy of hemoglobin-β residues within the S1, S1’, S2, and S3 subsites of FP-2; here, the residues for computation are derived from mature FP-2 and written in box bellow each histogram.
deeper into the protein core (Figure 4(B) and (D), transition conformation). This complex has the free energy value of $-1.15 \times 10^5$ eV; therefore, the estimated activation energy is $+1.13 \times 10^5$ eV; providing theoretical indications that hemoglobin binding by FP-2 may not be spontaneous. It is interesting how the estimated activation energy obtained here robustly define the findings of Hogg et al. (2006) who detected a low-affinity binding between human methemoglobin and FP-2 at pH 6.0. Noting also that the affinity of FP-2 for methemoglobin is significantly higher than hemoglobin. The two proteins dissociate into two modestly stable states (Figure 4(C) and (D), Unbound conformation).

**Discussion**

The dynamical footprint of FP-2 catalytic triad in hemoglobin-β-bound state has been investigated using rigid-body docking, molecular dynamics simulation, and quantum mechanics calculation. The data presented here have put forward a theoretical explanation for the interaction between FP-2 and hemoglobin, and answered a few key questions critical to the design of future anti-malaria drugs targeting FP-2 component of the heme detoxification protein complex (Chugh et al., 2013). First, at pH 6.0, residues 34–51 of hemoglobin-β subunit exist essentially in coil conformation separated by a short helix. Upon docking, the first coil residues 34–46 are...
inserted into the active site of FP-2, orienting the carbonyl group of serine-44 towards the thiol group of cysteine-44. Insertion of coiled regions of ligands into the active site of FP-2 is consistent with the crystallized cystatin- and PbICP-C-bound FP-2 (Hansen et al., 2011; Wang et al., 2006). Since there are four major coils separating five helices in the tertiary hemoglobin-β structure as revealed by X-ray crystal data (Shaanan, 1983), other experiments are therefore required to establish whether the residues 34–51 coil in the preferred region for FP-2 binding. However, the promiscuity of FP-2 at acidic pH provides preliminary evidence that complementary geometry rather than residue specificity may play important role in FP-2 (Hogg et al., 2006; Kerr et al., 2009; Ramjee et al., 2006). Therefore, leveraging on the promiscuity of FP-2 and choosing hemoglobin-β residue 34–46 coil as the interface of FP-2/hemoglobin-β, the molecular dynamics data revealed that in the bound state, glutamine-36, asparagine-173, and asparagine-204 present chemically equivalent amide group to the imidazole group of histidine-174 but with different ligand footprints. For instance, asparagine-204 partners with histidine-174 in both free and ligand-bound states of FP-2 (Hogg et al., 2006; Kerr et al., 2009; Rizzi et al., 2011), glutamine-36 preferentially partners with histidine-174 in hemoglobin-free state, while asparagine-173 is the preferred carrier of the amide group in hemoglobin-bound state. The importance of this ligand-dependent amide-footprint to FP-2/hemoglobin binding and hydrolysis activity requires further experimental investigation as it may hold key information for designing drugs capable of inhibiting different stages of hemoglobin binding and hydrolysis by FP-2. Another important ligand footprint within the FP-2 active site groove is the cysteine-42 side chain dihedral switching from 120° in ligand-free state to 30° in ligand-bound state. Although, in aspartic protease plasmepsins, such dihedral changes have been reported in apo- and inhibitor-bound states (Friedman & Caflisch, 2007), no information on such dihedral shift is available for FP-2. Speculatively, to allow such a large change in side chain dihedral angle, less bulky ligand side chain must be inserted into the active site of FP-2 in order to reduce the steric clash with the rotating cysteine-42. Here, hemoglobin-β achieved this structural feat by orienting all the side chains proximal to cyst-42 away from the binding interface and in some cases towards hemoglobin core. Evolutionarily, the presence of hydrophobic and bulky groups at multiple locations within the four well-defined subsites (S1, S1’, S2, and S3) bordering FP-2 active site groove may be key to restricting such bulky side chain amino acids of a ligand from inserting into the active site groove (Shah et al., 2011, 2012). Interestingly, deductions from homology model-}

ing of pro-FP-2 support that the inhibitory prodomain of FP-2 interacts via salt-bridge formation between charged groups conserved within the C-terminal of motif (ERF-NI/GNFD) and the FP-2 subsites and hydrophobic interaction established by the insertion of the conserved bulky phenylalanine side chain through the active site groove (Pandey et al., 2009; Sundararaj et al., 2012). The crystal structure of E64- and leupeptide-bound FP-2 revealed that moderately bulky and hydrophobic leucyl-phenyalanyl side chain contained in their P2-site may insert into FP-2 active site to produce inhibitory activities (Kerr et al., 2009).

In addition to the dynamical footprint in FP-2, the data here also showed that there exists optimum hemoglobin geometry for FP-2 binding. The data revealed that restriction might be placed on large structural flexibility within the hemoglobin residues proximal to serine-44 of FP-2. A key example of an arrested flexibility within hemoglobin-β is an anti-parallel formed between residues 170–175 of FP-2 and residues 45–48 of hemoglobin-β and a putative electrostatic interaction between the residues of hemoglobin-β and the occupant residues in S1, S1’, and S3 subsites of FP-2, these subsites have been tagged indispensible for hemoglobin binding (Cotrin et al., 2013). Similarly, the quantum mechanics-estimated activation energy (is + 1.13e + 5 eV) correlated that precisely complementary geometries are required for FP-2/hemoglobin-β interaction. This observation reiterates further that FP-2 subsites may become indispensible for hemoglobin-β binding as they ensure accurate and precise latching of hemoglobin-β to the surface of FP-2 prior to the insertion of hydrolyzable groups within the active site cleft (Cotrin et al., 2013; Farady & Craik, 2013; Hansen et al., 2011; Hogg et al., 2006). Similarly, the finding highlights the evolutionary importance of the multiprotein complex-heme detoxifying protein as a means of trapping and optimizing hemoglobin geometry for hydrolysis by FP-2 (Chugh et al., 2013).

In conclusion, for a design of peptidomimetic compounds with inhibitory action on FP-2, it is desirable that such compound must possess a non-hydrolyzable group as a replacement for carbonyl group in all known substrates of FP-2, this group must be connected to bulky and hydrophobic group with a less flexible bond as to insert into the active-site groove. Finally, a charged group capable of interacting with the amide group of asparagine-173 must be contained in the putative compound.

**Supplementary material**

The supplementary material for this paper is available online at [http://dx.doi.org/10.1080/07391102.2014.924878](http://dx.doi.org/10.1080/07391102.2014.924878).
Molecular dynamics and energetics of FP-2/hemoglobin-β binding

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