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Atomistic simulations of heme dissociation pathways in human methemoglobins reveal hidden intermediates

Premila P. Samuel¹, David A. Case^{*,1,2}

¹Institute for Quantitative Biomedicine, Rutgers University, Piscataway, NJ 08854;

²Department of Chemistry & Chemical Biology, Rutgers University, Piscataway, NJ 08854.

Abstract

Heme dissociations disrupt function and structural integrity of human hemoglobin and trigger various cardiovascular complications. These events become significant in methemoglobins that have undergone autoxidation of ferrous into ferric heme. We have structurally characterized the heme disassociation pathways for adult tetrameric methemoglobins using all-atom molecular dynamics simulations. These reveal that bis-histidine hemichromes, characterized here by the coordination of heme iron to both the F8 (proximal) and E7 (distal) histidines, are seen as intermediates following dissociation of the water molecule distally bound to each heme iron. Later, the breaking of coordination between heme iron and proximal histidine disrupts the F helix and pushes it away from heme cavity, enabling both bulk solvent penetration and disruption of tetramer interface interactions. The interactions inhibiting heme dissociation were then seen to be (i) either a direct or a water molecule mediated interaction between distal histidine and heme iron; and (ii) stacking between heme and the α CE1/ β CD1 phenylalanine residue. These interactions are less important in the β than in α subunits due to a more flexible β subunit CE loop region. The absence of distal histidine interaction in the H(E7)L mutant, and increased heme cavity volume in the V(E11)A mutant both promoted heme escape from the protein interior. Adult and fetal hemoglobins were seen to share a general heme disassociation pathway and intermediates due to conservation of key heme pocket residues. The intermediates seen here are analyzed in light of experimental studies of heme dissociation and pathways of certain hemoglobinopathies.

Graphical Abstract

Corresponding Author: David A. Case - Department of Chemistry & Chemical Biology, Rutgers University, Piscataway, NJ 08854, United States; Phone: +1-609-751-8668; david.case@rutgers.edu.

Supporting Information

Heme parameters (Table S1-S2), water ligand parameters (Table S3-S4), summary of simulation conditions (Table S5), and average incidence of electrostatic interactions involving heme propionates (Table S6-S9). HbA heme disassociation trajectory at 310 K (Fig. S2), C- α RMSD of E helices and FG loops in HbA (Fig. S3), electrostatic interactions involving HbA heme propionates (Fig. S4), water penetration pathways into the proximal side of HbA heme cavities (Fig. S5), secondary structure changes of HbA E helices (Fig. S6), and changes in dihedral χ 1 angles of CE1 and distal His residues for HbF (Fig. S7).

Accession Codes

UniProt IDs: HbA, P69905 and P68871; HbF, P69905 and P69892.

INTRODUCTION

Metal cofactors often play critical structural and/or functional roles in the host proteins^{1–3}. The tetrameric human hemoglobin (Hb) protein contains a metal cofactor heme (Fe(II)-protoporphyrin IX) group within a cavity in each of the Hb subunit. The adult human hemoglobin A (HbA) in red blood cells functions as the oxygen transport protein of our cardiovascular system by regulating oxygen binding to each of the ferrous heme iron center^{4–6}. In many instances, cardiovascular and cerebrovascular complications (including heart disease, stroke, and pulmonary hypertension) during hemoglobinopathies can be traced to heme disassociation events in human hemoglobins^{7–9}. Structural characterizations of the heme disassociation pathways may aid in identifying mechanisms and potential therapeutic targets for the related hemoglobinopathies. Resolution of the pathways is also critical for engineering acellular hemoglobin-based oxygen carrier (HBOC) products for use in transfusion medicine^{10,11}. One of the key steps in resolving the pathways is identifying the pathway intermediates and determining their functional roles¹².

Heme disassociation events can lead to cardiovascular and cerebrovascular complications by triggering Hb disassembly, causing accumulation and precipitation of the unstable apo (heme-free) Hb disassembly intermediates in red cells, and introducing toxic free heme in

the blood plasma^{13–18}. Accelerated heme disassociation may be triggered by genetic mutations occurring in the heme pocket that are either (i) causing unfavorable heme binding environment^{19,20} or (ii) facilitating heme autoxidation into ferric heme (hemin) and so resulting in the autoxidized methemoglobin (metHb) accumulation^{20,21}. MetHb accumulation, which leads to increased heme disassociation, can also occur due to disease or aging-related disruption of the normal reductive mechanisms within red blood cells or when Hb is introduced into non-reductive acellular environment^{22–25}.

Our recent equilibrium experimental studies on both HbA and fetal Hb (HbF) highlight the critical roles played by heme in mediating reversible Hb assembly in red blood cells and in enabling Hb to adopt its stable folded quaternary structure¹⁴. In the study, we were able to draw connectivity between reversible heme binding parameters and apoHb equilibrium folding constants. HbA, composed of α and β subunits, forms the dimeric $\alpha_1\beta_1$ and then the tetrameric $\alpha_1\beta_2$ interfaces during assembly^{14,26}. Each heme molecule in a Hb is held within a specific subunit cavity formed by the F helix at the proximal side and the E helix at the distal side (Fig. 1A). The mouth of this heme cavity, enclosed by the FG corner and the CE loop regions, is located at the Hb tetramer interfaces^{4,6}. The deprotonated Ne2 atom of the proximal F8 His on the F helix coordinates heme iron, while the distal E7 His is critical for stabilizing heme iron bound ligands such as oxygen and water (Fig. 1A)²⁷. In HbF, the γ subunits replace the β subunits with prevalent amino acid differences between the heme cavities existing in the E and D helices.

HbF is responsible for transporting oxygen via the placenta²⁸. Generally, at about 6 months post-natal, HbF completely disappears from the blood circulation. However, in certain patients of either sickle cell anemia ($\beta^E(A3)V$) or β -thalassemia, elevated levels of HbF in the blood circulation as a form of the body's response for modulating these blood disorders has sometimes been observed. Therefore, there has been much interest in clinically inducing synthesis of HbF in adults^{29–31}.

MetHb is much more susceptible to heme loss relative to ferrous Hb, due to the weaker His(F8)-Fe³⁺ coordination bond^{17,32,33}. In physiological conditions, a water molecule is coordinated distally via the O atom to each heme Fe³⁺ atom of a metHb, which therefore is then also referred to as aquometHb (Fig. 1A)³⁴. Previous spectroscopy and crystallography studies have extensively explored and established the critical role of hydrogen bonding between the distal histidine and this bound water in slowing down heme exit in both met-myoglobin and Hb^{20,32,35,36}.

Several recent simulation studies have explored the mechanism of the iron-regulated surface determinant (Isd) receptors of *Staphylococcus aureus* in extracting heme from HbA^{37–40}. The presence of the receptors, however, likely introduces changes to the heme disassociation pathways. Correspondingly, these studies did not report the various unique characteristics such as the bis-histidine hemichromes that have been observed during the present heme disassociation pathway simulations. In a 1999 study, Ramadas and Rifkind⁴¹ reported that they observed bis-histidine hemichrome occurrence in human methemoglobin at 235 K during a short 100 ps molecular dynamics (MD) simulation. However, the actual starting structure they used for the simulation is horse metHb⁴². Given both the extremely short

time-scale of the simulation as well as the significant protein sequence differences existing between horse and human hemoglobins, we could not draw any meaningful conclusions from their study in terms of MD studies of human metHbs.

In earlier kinetics experiments, heme disassociation from each metHbA subunit was always observed to be a simple two-state process^{33,43,44}. However, in our recent thermodynamics experiments of met-HbA and HbF disassembly, spectroscopic evidence clearly indicates that hemichromes act as intermediates¹⁴ and that a 2-state description is incomplete for assessing heme disassociation. We have also already shown experimentally that when heme is extracted from native protein, both HbA and HbF unfold with ~ 30 % loss of helical content and undergo complete disruption of the tetramer interface to form the extremely unstable apoHb^{14,26}. Our simulations now further demonstrate and characterize the multi-state nature of the heme disassociation pathways in Hb.

As discussed above, the heme group in metHb is chemically connected to the protein via fairly weak bonds to a proximal histidine and a distal water molecule. These interactions are expected to spontaneously break and perhaps re-form on a microsecond or longer time scale^{32,33,45}. In order to simulate these events in HbA within the MD time scales available to us, we artificially first removed the bond between distal water and the heme iron generally at about 150–250 ns into each of 7 replicate simulations done; the bond to the proximal His was removed next generally at about 300–450 ns. The breaking of bond between the ligand water and heme iron was explored first because such interactions are known experimentally to be weaker relative to the coordination between the proximal His and heme iron^{14,33}. Some of the intermediates (hemichromes and pentacoordinated Hbs) observed during our MD simulations have been previously detected through either spectroscopic or X-ray crystallographic measurements^{14,46}. Our MD simulations also provide a description of likely accompanying secondary and quaternary structural changes. Transient hemichrome intermediates, identified previously by characteristic electronic spectral signatures, are seen to arise in 007e30 % of the simulations, allowing for a more detailed description of their likely structures. Interestingly, when parallel simulations on HbF were conducted, we observed intermediates corresponding to that seen in HbA to occur despite the differences of 39 amino acids between β and γ subunits.

Parallel simulations on two HbA mutants (H(E7)L and V(E11)A Hb variants) that accelerate heme dissociation were also analyzed. For the H(E7)L mutation, the disassociation is facilitated by the disruption of the interactions between the heme iron and distal His. Clinical studies have shown that the presence of this same mutation in the Hb Kirklareli (Hb α H(E7)L) variant leads to increased heme loss and thereby resulting in mild anemia²⁰. The V(E11)A mutation, meanwhile, introduces heme packing deficiency and Hb Sydney (Hb β V(E11)A) is known clinically to be an unstable variant manifesting through hemolytic anemia¹⁹.

METHODS

1. Protein structure preparation

All simulations used the molecular mechanics AMBER ff14SB protein force field⁴⁷ and TIP3P explicit solvent model⁴⁸. Hb structures were solvated in octahedral boxes at a minimum distance of 20 Å from the box boundaries. Starting X-ray crystallography structures used for simulating the metHbs were: (i) PDB code 3P5Q (metHbA); and (ii) PDB code 4MQJ (ferrous HbF). The metHbF structure was prepared from 4MQJ by substituting both the carbon monoxide molecule bound to the heme iron of α subunits and oxygen molecule bound to the heme iron of γ subunits with water molecules.

E7, F8, and α F9 histidines of the Hbs were simulated in the neutral HID protonation state, for which the N δ atom is protonated. All other histidines were simulated in the neutral HIE protonation state, for which the N ϵ atom is protonated. The F9 histidines in the α subunits were simulated in the HID state to explore the alternative hemichrome formation involving the hexacoordination of heme iron with both F9 and E7 histidines as was observed to occur during heme extraction by *Staphylococcus aureus*³⁷. The E11A mutations were prepared using AMBER18⁴⁹ *tleap* module by deleting the original side chain atoms in metHbA and allowing the module to rebuild the mutated side chains. The rotamer for the E7L side chain was initially modeled based on the PDB code 3QJB crystal structure²⁰ of the HbA mutant α H(E7)L in the CO bound form.

The metHb heme parameters were developed through quantum mechanics (QM) methods using the B3LYP/6–31G* model by applying the *MCPB.py* program^{50,51} and Gaussian software (Gaussian, Inc., Wallingford, CT). The general heme model used for the QM parameterizations consists of the heme, a heme bound water molecule, and both the distal and proximal histidines. Prior to the parameterizations, all the models used were geometry optimized. In order to derive partial charges for the heme and the water ligand, complete histidine molecules were modeled with each N terminus capped by an acetyl group and each C terminus capped by a methylamine group. Reduced representations of His were used during the parameterization of bonds, angles, and non-bonded interactions of heme, with the imidazole side chains of the His groups being capped by methyl groups. For the heme iron bound water ligand, the GAFF2⁵² force field was utilized to define the bond, angle, and non-bonded parameters. All the heme (Tables S1 and S2) and water ligand (Tables S3 and S4) parameters generated and used in this study are included in the supporting information.

Electrostatic potentials around the heme model were initially calculated in Gaussian (Gaussian, Inc., Wallingford, CT). Then, starting with these potentials, restrained electrostatic potential fitting method (RESP)⁵³ was further applied in 3 iterations using AMBER18⁴⁹ in order to determine partial charges on the heme atoms and water ligand. During the RESP iterative fittings, charges on the histidines and their caps are restrained to the values in the ff14SB⁴⁷ force field that reflect partial charges on the His when it is not bonded to the heme iron. During the 2nd and 3rd iterations, charges for degenerate hydrogens on the heme were fitted in a constrained manner to have same values, unique for each set of degenerate group of hydrogens.

2. Equilibration of Hb systems during MD simulations

MD simulations of the solvated Hb systems were conducted through Particle Mesh Ewald Molecular Dynamics (PMEMD) modules, using graphical processing unit (GPU) accelerations⁵⁴, and under periodic boundary conditions with a 9 Å non-bonded cutoff. Simulations performed at 298 K and 310 K were done at 2 fs time steps and 1 fs time steps, respectively. SHAKE algorithms were used to constrain the hydrogen bonds. Pressure and temperature were regulated respectively by a Berendsen barostat with a pressure relaxation time of 10 ps and a Langevin thermostat with a collision frequency of 5 ps⁻¹. The MD simulations were primarily carried out as isothermal-isobaric (NPT) ensembles at 1 bar with pressure relaxation time of 10 ps, except during the minimization and initial equilibration stages.

Initially, energy minimization of each system was conducted for 2000 steps. Next, the system was heated to and held at the equilibrium temperature for 20 ps. These minimization and initial equilibration stages were conducted in the canonical (NVT) ensemble with a 12 kcal(mol⁻¹)(Å⁻²) force constant restraining the coordinates of the solute heavy atoms to the initial X-ray crystallography reference structure. Then, the system was equilibrated in NPT ensemble sequentially in three stages, with a gradual decrease in the force constant restraining the solute heavy atoms to the reference structure. The general time scale for each of these stages was 60 ns. The general sequence of force constants used was 8, 1, and 0.1 kcal(mol⁻¹)(Å⁻²). Unrestrained equilibrations of the systems with the aforementioned force constants set to 0 kcal(mol⁻¹)(Å⁻²) were next conducted for periods from 100 to 200 ns. These equilibrated systems were then either used as starting states for heme disassociation simulations or underwent further equilibration for at least 60 ns in order to generate different starting states for replicate sampling of the heme disassociation events.

3. Varying the Fe-ligand bond parameters

The bond energies for heme iron bonds with (i) Nε2 atom on proximal His; and (ii) O atom on the distal water ligand (the initial water ligand present in the metHb crystal structures) were parameterized using the AMBER18⁴⁹ restraint facility with *ialtd=1*⁵⁵ in order to artificially simulate heme disassociation events by breaking the distance restraints or “bonds” between the atoms. Based on the X-ray structure of metHb, the equilibrium distance (R_{eq}) restraints between Fe and Nε2 and between Fe and O were respectively set to 2.3 Å and 2.1 Å³⁴. We assigned the breaking of the “bonds” to occur when the distance (R) between iron and either Nε2 or O atoms got larger beyond $R_{Max} = R_{eq} + 0.5$ Å. The restraint energy (E_R) (as shown in Fig. S1), characterized in terms of R values and the equilibrium force constant (k_{eq}), is a smooth parabolic function when $R < R_{Max}$ but switches to a hyperbolic function when $R \geq R_{Max}$ ^{49,55}. For the distance restraint between iron and water, an initial k_{eq} of 40 kcal(mol⁻¹)(Å⁻²) was assigned based on our QM parameters. For the distance restraint between Fe and Nε2, k_{eq} values in α and β subunits were respectively set to 74 kcal(mol⁻¹)(Å⁻²) and 68 kcal(mol⁻¹)(Å⁻²). These k_{eq} values were assigned based on approximations of heme affinities of the subunits^{14,33}. During the minimization and the subsequent different restrained and unrestrained equilibration stages, all these initial k_{eq} values assigned were maintained.

Additionally, we had set both these specific heme iron interactions, using AMBER18⁴⁹ ParMed program and independent of the distance restraint definitions, to be dummy bonded interactions⁴⁷ throughout the MD simulations conducted. This was done by redefining the default harmonic force constant for each of these bonds in the protein topology file to be 0 kcal(mol⁻¹)(Å⁻²).

4. Heme disassociation simulation setup and analysis

Heme disassociation trajectories were initiated by first decreasing the k_{eq} for the equilibrium distance restraint between the heme iron and water from 40 kcal(mol⁻¹)(Å⁻²) to 0 kcal(mol⁻¹)(Å⁻²) in a step-wise manner. The decrease of the force constant to 0 kcal(mol⁻¹)(Å⁻²) completely disrupted the restraint bond between heme iron and water, allowing for the water to disassociate. Bulk solvent molecules were later able to interact with heme iron via electrostatic interactions.

Next, the Hb system was equilibrated in NPT ensemble for at least 50 ns to 100 ns in order to allow for the system to relax. Then, the k_{eq} for the distance restraint between the heme iron and proximal His in both types of the subunits were brought down to 0 kcal(mol⁻¹)(Å⁻²) in a step-wise manner. The Hb system was further equilibrated for a few hundred nanoseconds to allow for the system to relax. In this study, we defined a hemichrome to form when the coordination distance between each axial His Nε2 and heme iron is less than 2.6 Å. This distance was chosen based on the equilibrium distance range observed in our simulations between the proximal His Nε2 and heme iron atoms for aquometHbA (Fig. 1). The interaction that was observed to form between distal His Nε2 and heme Fe was a non-bonded interaction. We did not parameterize this interaction for this present work as we were only pursuing an exploratory study of possible intermediates that can occur during heme disassociation. Electrostatic interactions involving heme propionate groups meanwhile were calculated at distances ≥ 4 Å.

Overall, the MD simulation trajectories including the heme disassociation events were analyzed using AMBER18⁴⁹ CPPTRAJ program, VMD⁵⁶, Chimera⁵⁷, and MATLAB R2019b (The MathWorks). The root-mean-square-deviation (RMSD) calculations were done by superimposing all Ca residues of the simulated structures against the reference structure. Hydrogen bond estimation algorithm (DSSP) was used to assign secondary structure⁵⁸. The occurrence of heme disassociation was characterized both visually and through the calculation of solvent accessible area of heme using the linear combinations of pairwise overlaps (LCPO) algorithm⁵⁹.

RESULTS AND DISCUSSION

Simulations of initial heme disassociation events were sampled at both 298 K and 310 K for HbA, HbF, Hb α/βH(E7)L, and Hb α/βV(E11)A (Table S5). For each of the different native and mutant Hbs examined, five to seven replicate simulations were conducted. The H(E7)L and V(E11)A mutations were introduced into HbA. We discuss first results for HbA, and then discuss results for HbF and the two mutants at the end. The heme disassociation trajectories were generally consistent across both the temperatures, so we have provided a combined analysis of the simulations conducted at both temperatures.

In our simulations, we observed heme to span through 6-coordinate, 5-coordinate, and 4-coordinate states via changes at the heme iron axial sites. The heme group also formed non-covalent interactions with their surroundings through pi stacking and electrostatic interactions (average length was generally $\sim 2.9\text{--}3.1$ Å). The regions spanning from C helix to FG loop predominantly formed both the heme pockets and the tetramer interfaces. The breaking of coordination between proximal His and heme iron initiated losses of both secondary structure along the proximal side of the heme pockets and tetrameric interface interactions as well as increases in protein backbone positional RMSD around the proximal side of the heme pockets relative to the reference starting crystal structure. As heme disassociation progresses, there will be an increase in the solvent accessible surface area of the heme due to increased exposure to external bulk solvent. Over the timeframe of our simulations ($\sim \mu\text{s}$), we did not however observe complete heme disassociations to occur in the native Hbs (both HbA and HbF).

1. Hemichrome formation in methHbA

The original distance between heme iron and distal His Ne atom in native aquometHbA was at a non-coordinating distance of ~ 4.1 Å (Fig 1A). When we initially sampled the disassociation of the distal water ligand from the hexa-coordinated heme iron at 298 K (occurring approximately at 260 ns in Figs. 2 - see the black circles in the figures), the hydrogen bonding (Fig. 1A) between the water ligand and the distal His Ne atom became disrupted first (Fig. 1B) before the water molecule exited the cavity. In the intermediate penta-coordinated heme iron states that formed as a result, the distal His sometimes moved closer to the iron ($\sim 3\text{--}3.5$ Å), albeit still at a non-coordinating distance (occurring between (i) 200–300 ns in Figs. 2A and 2B; and (ii) 200–400 ns in Fig. 2C - see the dark blue circles in these figures). At this point, the bulk solvent is likely restricted spatially from approaching the heme iron.

However, as the simulation proceeded further, the penta-coordinated heme iron was observed to favor reverting back to a hexa-coordinated state by forming either an aquomet state again or by generating a new bis-histidine hemichrome state, occurring when the Ne atom of the distal His (His(E7)) formed a new coordination with the heme iron at an average distance of $\sim 2.2\text{--}2.5$ Å. Therefore, a hemichrome structure, characterized by a hexa-coordinated heme iron axially coordinating with the Ne atoms on both proximal and distal histidines, was observed to form as an intermediate during heme disassociation in α and β subunits (Fig. 1B; and see dark blue and cyan circles between 300–420 ns in Figs. 2A and 2B). The aquomet state on the other hand was regenerated when a bulk solvent molecule re-entered the heme pocket distal side and bound the heme iron (see solid green circles between 260–420 ns in Fig. 2D). It should be noted that both the aquomet and penta-coordinated structures are of high spin states, while the hemichrome is of a low spin state^{14,27}. However, since the present simulations were conducted using molecular mechanics force field, the spin state of the structures were irrelevant for this study.

In order to compare the relative energetics for the formation of the hexa-coordinated heme structures from the penta-coordinated structures, we pursued energy calculation in AMBER18 following structure minimization in the gas phase. For simplicity sake, each

structure evaluated had been reduced to just consist of a heme, the proximal His, and either a bulk solvent or distal His. All the His residues were also capped by acetyl and methylamine groups, respectively, at the N- and C- terminals. The hemichrome formation energy involved -28.5 kcal/mol while binding of the bulk solvent at the distal side of the penta-coordinated heme iron involved -26.4 kcal/mol energy. While the computed numbers might not completely represent the physical system, these values obtained do show that the relative binding energies of the distal His and bulk solvent to the distal side of the heme iron are comparable to each other.

Both aquomet- and hemichrome conformers were sampled in some of the α and β subunits at both 298 K (Fig. 2) and 310 K (Fig. S2). The total percentage of hemichrome occurrence across all our HbA simulations was $\sim 29\%$ (18% in α subunits and 11% in β subunits). The percentage of aquomet conformers was much higher at $\sim 61\%$ (21% in α and 39% in β).

No large structural changes in the heme cavities were observed to occur with the hemichrome formation (Figs. 3 and S3). This was surprising, as we had expected a contradictory observation given that hemichrome occurrence has not been observed as being energetically favorable in native Hb states at room temperature^{14,60}. Exit of the water molecule, however, enabled subtle and local structural dynamics around the heme cavity to be just enough to bring the E7 His closer to heme iron for hemichrome formation (Figs. S3a and S3b). The movement of E7 residue into the heme cavity also removed the cavity packing deficiency introduced by the water exit.

2. Interaction changes at the proximal side of the heme cavity

Next, disruption of the bonded interaction between proximal His Ne2 atom and heme iron was introduced (Fig. 1D; see cyan circles at 420 ns in Figs. 2 and S2). The disruption of this interaction between His(F8) Ne2 and heme iron was seen to be often accompanied by distinct displacement of the residues around the C-termini of the F helix including the F8 residue, as depicted by the relative increase observed for the average C- α RMSD of F helices (occurring after 420 ns in Figs. 3e, 3g, and 3j). We did observe that the coordinating distance (< 2.6 Å) between the proximal His Ne2 atom and heme iron can be recovered via electrostatic interactions later on if we re-defined the interaction between the atoms to be non-bonded by removing the “dummy” bonded interaction in terms of the ff14SB force field (data not shown). Therefore, the Hb was not undergoing non-reversible structural changes in the heme pocket at this point.

Past studies have discussed the idea that bulk solvent penetration at the proximal side of the cavity is a promoting factor for coordination breaking between proximal His and heme iron^{10,61}. However, in our current simulations, no bulk solvent penetration into the proximal side of the heme cavity was observed prior to the F8-Fe coordination-breaking event (Figs. 2 and S2). The coordination breaking event actually was seen to facilitate the bulk solvent penetration followed by solvent binding to the heme iron (Fig. 1D; and see red solid circles in Figs. 2 and S2). The iron bound bulk solvent was not seen to interact with the Ne2 atom on proximal His due to either large movements of proximal His away from heme iron (panels e, g, and j in Fig. 3), or due to rotation of the deprotonated Ne2 to a configuration of facing away from the heme (seen in Fig. S4). The distinct structural changes along the F

helix resulting from the coordination breaking event are likely then causing the observed solvent penetration.

One dominant pathway observed for solvent penetration is through the disruption of the hydrophobic cage encapsulating the heme iron at the F helix side of the heme cavity, thereby creating the space for solvent molecule penetration directly from the surrounding bulk solvent (Fig S5). The specific residues making up the hydrophobic cage in both α and β subunits are: Leu(F4), Leu(F7), Leu(FG3), Val(FG5), Phe(G5), and Leu(α H19/ β H20)^{39,62}. Water entry was observed to occur via the parting of one the following pair of the hydrophobic cage residues: (i) F4 and F7; (ii) F7 and FG3; and (iii) FG3 and FG5. Another minor mode of solvent penetration observed was via the Xe5 cavity, which is both a xenon atom binding site and a part of heme ligand migration pathways, in the α subunit^{63,64}.

3. Heme stabilization by axial coordination with the distal His

Following breaking of the coordination between proximal His and heme iron, a critical limiting step for heme disassociation from the cavity was observed to be the disruption of the interaction between heme iron and distal His. The interactions involving distal His were observed to occur either as direct coordination between heme iron and N ϵ 2 atom (Figs. 1D; see after 420 ns and 350 ns in Figs. 2A–2C and S2C–S2D, respectively), or as hydrogen bonding between the heme iron bound bulk solvent and N ϵ 2 atom (see after 420 ns and 350 ns in Figs. 2D and S2A–S2B, respectively). No structural disorder was observed along the E helices (Fig. S6) during the course of the simulation, and therefore, these helices were able to provide more consistent interactions for interacting with heme.

4. The role of CE loop flexibility in heme stabilization

The CE loop region was observed to dampen heme exit in our simulations through the following 3 interactions: (i) restriction of distal His from flipping away from the heme cavity - provided by the Phe residues on α CE1/ β CD1 and α CE4/ β CD4 (Fig. 1); (ii) stacking between heme porphyrin group and Phe(α CE1) or Phe(β CD1) (Fig. 1); and (iii) a strong electrostatic interaction between heme propionate and N ϵ -H on His(α CE3) (average length is 2.9–3 Å) (Figs. 1A–1C; and Tables S6 and S7).

However, we observed significant differences between the α and β subunits in the degree of stabilization provided by the CE loop for heme due to the increased flexibility of CE loop in the β subunits. Due to the additional presence of a D helix between the CD loop and E helix in the β subunits, the CE loop region of the subunit is composed of both CD loop and D helix. Therefore, β CE loop region is longer by 6 residues relative to the α subunits. This increase in length contributes towards the higher flexibility of this region, as demonstrated by a high C- α RMSD of the CD loop (Figs. 3c and 3h) and D helix (Figs. 3d and 3i) across the trajectories overall. The D helix in the β subunits of HbA further demonstrated higher flexibility by means of the extensive structural disorder across the whole segment occurring throughout the time frame of the simulations, independent of the heme disassociation events. The α -helical content was rapidly exchanging with 3–10 helical, bend, and turn elements (Fig. 4E).

The increased flexibility of β CE loop region for movement away from the distal heme pocket reduced the restraints imposed on distal His by both CD1 and CD4 phenylalanines. In the α subunits, the distal histidines were restrained from flipping due to a relatively more constrained CE loop region (Figs. 3a and 3f). Flipping events of distal His imidazole ring away from the heme cavity, leading to disruption of the His interactions with heme iron, were observed exclusively in the β subunits during the simulations (Figs. 5A and 5B). In Figure 5B, His flipping events are quantified by the occurrence of the 90–100° changes in dihedral χ_1 angle formed by N, C α , C β , and C λ atoms in the histidines. The distal His acts as a gate for ligand entry, and therefore, the flipping events also facilitate water penetration into the distal side of the heme cavity⁶⁵.

The higher flexibility of the β CE loop region also caused the stabilization stacking between CD1 and heme to be often disrupted, relative to α subunits. With disruption of the stabilization between the Phe benzene ring and heme porphyrin ring, the benzene ring will be more susceptible to flipping events which can be quantified by the ~90° changes in the Phe dihedral χ_1 angle (Figs. 5C).

The His(α CE3) residue meanwhile demonstrated a high incidence for forming an electrostatic interaction with heme propionate (average fractional incidence was ~0.5–0.7 at 298 K and ~0.7–0.8 at 310 K, see Tables S6 and S7). The occurrence of hemichrome was observed to eventually promote the breaking of this interaction between heme propionate and His(α CE3) (Fig. 1D), with an increase in the C- α RMSD of the His(α CE3) residue observed (dotted cyan lines in Fig. 3a). In the β subunit, this electrostatic interaction is not present as the corresponding CD3 residue is a Cys.

5. Intermittent electrostatic interactions involving heme propionates

Intermittent electrostatic interactions between heme propionate groups and H atom of either Nd atom on proximal and distal histidines or lysyl N δ atoms on specific lysines were observed (Fig. S4; and Tables S6 and S7). The lysine residues involved were β E3, FG2, and E10. Lys(β E3), Lys(FG2), and distal His all exhibited low average fractional incidence (~0.1) for forming electrostatic interactions. In comparison, the Lys(E10) residue exhibited a much higher average fractional incidence (~0.3). Electrostatic interaction involving the proximal His was generally more pronounced in the β subunits, with the highest average fractional incidence was seen in the β 1 subunits at 0.3 ± 0.2 at 298 K.

6. Structural changes resulting from heme disassociation events

By the end of the trajectories, the average C- α RMSD of the heme pocket regions had overall increased in β subunits from an initial 1.6 Å at equilibrium to a final value of 2.2 Å and had also shifted in α subunits from being initially at 0.9 Å to 1.6 Å. These regions generally underwent some displacement from equilibrium positions during the simulations (Figs. 3 and S3) due to changes occurring in the interactions between heme and Hb. For example, the axial coordination disruption between proximal His and heme iron sometimes led to an increase in C- α RMSD along both the N-termini region of F helix (Figs. 3e, 3g, and 3j) and the adjacent FG loop (Figs S3g and S3h). In comparison to the heme cavity, the average C- α RMSD for the ABGH core across the trajectories were relatively unaffected

and remained at approximate values of 0.9–1.0 Å for α subunits and 1.1–1.2 Å for β subunits.

The coordination breaking event between the proximal His and heme iron was seen to initiate loss of α -helical content along the F helix in both α and β subunits (Figs 4A and 4C). A complete loss of secondary structure, however, was not observed; instead the α -helical content underwent transformation primarily into 3–10 helical, turn, and bend structural elements while streaks of π (3–14) helical segments also sometimes appeared. We propose that these secondary structure elements are forming as intermediates along the pathway of F helix unfolding and apoHb formation. Soman, Karimi, and Case had previously conducted MD simulations of a single myoglobin H helix in water and observed the occurrence of turns and 3–10 helical elements as intermediates prior to the structure being completely disordered⁶⁶. Different studies and theories have also proposed the occurrence of 3–10 and π helices as intermediates during α -helix unfolding or transition into coils^{67,68}.

The key coordination breaking event between His(F8) and heme iron was observed to initiate disruption of different interactions at the $\alpha 1\beta 2$ interfaces that likely occurred via protein backbone displacement and dynamics observed along the C helix and FG loop in α subunits, and the FG loop and beginning of G helix in β subunits. In Fig. 6, we provide specific examples observed of the breaking of HbA tetramer interface interactions as follows: (a) hydrogen bonding between the carboxyl group of Asp($\alpha 1G1$) and amine group of Asn($\beta 2G4$) (Fig. 6A); (b) side chain stacking between Tyr($\alpha 1C7$) and Arg($\beta 2C6$) (Fig. 6B); (c) side chain stacking between Arg($\alpha 1FG4$) and Arg($\beta 2C6$) (Fig. 6C); and (d) hydrogen bonding between the hydroxyl group on Thr($\alpha 1C6$) and the guanidino group on Arg($\beta 2C6$) (Fig. 6D).

7. Changes to the heme dissociation trajectories due to His(E7)Leu and Val(E11)Ala mutations

Heme dissociation simulations were done on Hb $\alpha H(E7)L$, Hb $\beta H(E7)L$, Hb $\alpha V(E11)A$, and Hb $\beta V(E11)A$ mutants at both 298 K and 310 K. Heme disassociation was observed to be facilitated by either the H(E7)L (Figs. 7C and 8A–8E) or the V(E11)A (Figs. 7D and 8F) mutation when the mutation is present in the β subunits. The reduced flexibility of the α subunit CE loop region relative to of the β subunit likely caused the α mutation effects to be dampened and therefore not be demonstrated along the time scale of the simulations.

The initial Hb H(E7)L mutant structures had been prepared without any water molecule bound to the heme iron distally. This was done based on observations seen in previous spectroscopy and crystallography studies^{20,69} that noted when the distal His is mutated to apolar residues such as Leu, only a pentacoordinated heme iron occurs due to the disruption of hydrogen bonding between the distal His and water. During the simulations however, water molecules sometimes did end up binding to the heme iron in the H(E7)L (Fig. 8B) heme pockets at the distal side.

We observed complete disassociation of heme iron from the $\beta 1$ subunit into bulk solvent during one of our replicate simulations for Hb $\beta H(E7)L$ done at 310 K, following the

breaking of coordination between proximal His and heme iron (Figs. 7C, and 8A–E). However, accompanying heme disassociation events were not observed in the neighboring subunits. The disassociated heme was later seen adsorbing on the external surface of the $\beta 1$ subunit around the CE loop region.

The V(E11)A mutation, on the other hand, introduces protein packing deficiency within the cavity and so facilitates the heme to be dislodged from its equilibrium position. We did not observe complete heme disassociation for the Hb V(E11)A variants. However, we did observe heme being dislodged in the $\beta 2$ subunit during one of our replicate simulations for Hb $\beta H(E11)A$, done at 298 K, following the breaking of coordination between proximal His and heme iron (Figs. 7D and 8F). Interestingly, a distal His flipping event also occurred around the time of the heme displacement event, disrupting the hydrogen bonding between distal His and heme iron bound water and thereby giving heme more flexibility for movement.

8. Heme disassociation trajectories in HbF

HbF demonstrated heme disassociation trajectories (Fig. 9) and associated structural changes (panels B and D in Fig. 4) generally consistent with that of HbA, despite having a number of residue differences between the adult β and fetal γ heme pockets especially along the D and E helices. The F8-Fe-E7 bis-histidine hemichromes were still observed to occur in both α and γ subunits (see the dark blue circles in Figs. 9B and 9C between 260–420 ns). The total percentage of hemichrome occurrence was ~29 % across all the simulations, comparable to the occurrence observed in HbA, with the distribution being ~21% across α subunits and 8.3% across γ subunits. Since there are no sequence differences between the $\alpha_1\beta_2$ and $\alpha_1\gamma_2$ interfaces, we did not observe differences in the interactions of the interfaces during the trajectories. The electrostatic interactions involving heme propionate groups (Tables S8 and S9) as well as the heme stacking Phe residues on the CE loop region are conserved in the γ subunits. Increased CE loop flexibility (Fig. S7B) relative to the α subunits and distal His flipping (Fig. S7A) are also observed in the γ subunits. However, rapid secondary structures exchanges were not observed in the D helix of γ subunits (Fig. 4F), in contrast to the β subunits.

While complete heme disassociation was not observed during the trajectories, heme exit was observed to be facilitated more in the γ subunits, relative to the β subunits. This was demonstrated by the propensity of heme in the γ subunits for increased solvent exposed surface area following the breaking of coordination between proximal histidine and iron (Fig. 7B). While our recent experimental studies on the other hand concluded that heme binding affinities are quite similar between native HbA and HbF¹⁴, there might be certain subtle differences in the heme disassociation trajectories between the proteins beyond the resolution of experimental errors that are being amplified in our simulations.

One difference between the β chain and the γ chain that could be directly affecting the interaction with heme is the $\beta(E15)Phe \rightarrow T(E15)Leu$ variation. In β chains, the benzyl side chain of (E15)Phe is directly parallel and is positioned to form almost stacking like interactions with the heme porphyrin at equilibrium, while this interaction is absent with T(E15)Leu.

CONCLUSIONS

Our MD simulations reveal structural pathways that trigger the initial Hb disassembly events as a result of destabilization of heme within the cavities. These events have been investigated in the past either through conventional equilibrium and kinetic spectral measurements^{33,60,70} or by X-ray crystallographic methods^{46,71}. MD simulations complement experimental measurements, suggesting details of both the evolving bond breaking or formation events and structurally dynamic regions of pathway intermediates^{72,73}. It should be noted that the interactions which formed between heme iron and either the bulk solvent or distal His during the simulations are non-bonded but in a physical system these interactions are actually bonded. We had pursued molecular mechanics simulations in order to sample a range of different possible interacting atomic pairs and structural changes that can occur during heme disassociation in Hb. In order to further model the chemistry of bond formation and breaking accurately including changes in the spin state of the heme iron, the hybrid quantum mechanics/molecular mechanics simulation method needs to be pursued in future. The implementation of this method however is computationally more expensive and beyond the scope of our present study.

We initiated heme destabilization within methHb subunits by first disrupting the axial interaction between heme iron and water ligand at the distal side of the heme cavity, and then disrupting the axial interaction between heme iron and proximal His. Artificial disruption of these interactions led to either disruption or formation of other interactions within the heme cavity, including the disruption of the hydrogen bonding between water ligand and distal histidine, and the formation of hemichrome structure. The significance of the interaction between distal His and iron in stabilizing heme within the cavity is further demonstrated during one of our simulations by the distal His(E7) to Leu mutation in HbA facilitating heme exit in a β subunit (Figs. 7C and 8A–8E).

In addition to interactions with axial ligands, heme is stabilized through compact packing within the cavity, stacking between heme porphyrin and Phe residues, and electrostatic interactions between heme propionates and either histidine or lysine residues lining the heme cavity. Even though some of the electrostatic interactions are intermittent in nature, they do help dampen heme exit at the later stages of trajectories (Figs. 8C and 8D), especially after heme iron interactions with both axial partners have been completely disrupted. The critical contribution of residues lining the cavity towards providing a compact heme packing environment, and therefore dampening heme loss, is demonstrated by the V(E11)A mutation that introduces increased heme cavity volume. During one of our simulations with this mutation in the β subunits, the heme in one of the subunits showed increased flexibility in moving around the heme cavity away from viable coordinating distances with the axial histidines (Fig. 8F).

The structural and dynamics differences of the CE loop region observed between α and β subunits resulting in the disruption of some of the heme stabilizing interactions in the β subunits also now provide mechanistic explanations for the experimentally observed higher β subunit heme loss rates^{33,43,44,74}. No atomic correlations of motions were observed between the heme pocket regions of the α and β subunits during our simulations (data not

shown). These observations correspond with our previous experimental studies in which we have already determined that heme binding and disassociation processes in hemoglobin are non-cooperative¹⁴.

In our past experimental studies, we had concluded that the hemichrome accumulation during different adult hemoglobinopathies likely results from hemichromes being the major intermediate populations at different stages of Hb disassembly in red cells¹⁴. In the present MD studies, we determined a critical intermediate for the heme disassociation, following the disassociation of the distally bound water ligand from heme iron, in both adult and fetal human metHbs to be a bis-His hemichrome state (Figs. 1B, 2A, 2B, 9B, 9C, S2C, and S2D). This hemichrome has iron coordination with the Ne atoms on both distal and proximal histidines. Interestingly, some experimental studies have observed hemichrome formation with hemoglobin dehydration^{75,76}. Various other experimental and clinical studies in the past have linked hemichrome occurrence with denaturation or significant unfolding events of Hb^{13,14,18,77}. However, this present study demonstrates that (i) large protein structural changes are not necessary to facilitate hemichrome formation; (ii) hemichrome formation can actually precede the unfolding events as inconsequential loss of helical content is observed at this point; and (iii) hemichromes may actually contribute towards delaying of the heme exit.

Our MD simulations also provide structural insight that both the disruption of inter-subunit interactions at the tetramer interface (Fig. 6) and the loss of helical content along the F helix (Figs. 4A, 4B, 4C, and 4D) during heme disassociation are triggered by the coordination disruption between iron and proximal histidine. During the time scale of the simulations, however, neither the tetramer dissociation into dimers nor the near-complete loss of secondary structure in the F helices were observed. Solvent penetration into the proximal side of the cavity as a result of the coordination breaking event could also likely be part of the pathway leading to initial unfolding of Hb^{14,78,79}.

While the coordination breaking event between proximal His and heme is assumed to be a key event for heme exit, we surprisingly observed that following this event, either through direct or water mediated indirect interactions with heme iron, the distal His later played a role in delaying heme exit from the cavity (Figs. 1C, 2, 9, and S2). For both of the aforementioned distal His-iron interactions, the heme iron was seen to be either penta-coordinated (Figs. 2C, 9A, 9C, S2A, and S2C) or hexa-coordinated (Figs. 1D, 2, 9, and S2) when a water molecule becomes bound to the heme at proximal side. In a recent crystal structure of a complex formed by Hb dimer bound to both a IsdH receptor and a haptoglobin⁴⁶, a new intermediate state of the heme extraction process was captured. The heme iron of the β subunit was observed to exist in two alternative penta-coordinated states where the heme iron was either bound to the proximal or, more surprisingly, the distal His. This structure lends support to our conclusions on the occurrence of direct interaction between distal His and heme iron during the later stages of heme disassociation. Additionally, we have observed in our present work both the intermediate states from the previous study (distal His penta-coordinated state is seen in Figs. 2C, 9A, 9C, and S2C; and proximal His penta-coordinated state is seen in Figs. 2A, 2B, 2C, 9A, 9C, 9D, S2A, and S2C).

This present work is also a pioneering MD simulation study of HbF. Our work reveals that fetal and adult Hbs follow a parallel heme disassociation trajectory, further lending structural evidence to help explain our recent spectroscopic studies concluding that both Hb variants follow a parallel Hb disassembly pathway¹⁴. In addition, our study also reveals new structural facets pertaining to the HbF dynamics and stability. For example, in contrast to the Phe(β E15) in HbA, Leu(E15) is present instead in both γ and α subunits. The combined effects of the (i) absence of stacking interactions between Phe(β E15) benzyl group and heme porphyrin and (ii) presence of highly dynamic CE loop could help explain our observations of the increased heme propensity for displacement in the γ subunits (Fig. 7B). We note that Hb Christchurch, a HbA variant with the β Phe(E15) $\rightarrow$ β Ser(E15) mutation, demonstrates unstable Hb phenotype clinically, which has been attributed to increased heme loss^{13,80}. The Phe(E15) $\rightarrow$ Leu(E15) change also increases cavity volume, thereby further promoting heme flexibility. Another interesting difference observed between γ and β subunits was that D helix in the β subunits demonstrated rapidly exchanging secondary structure elements, whereas the D helix in the γ subunits maintained its α -helicity content. There are 5 residue differences in the D helix from β to γ as follows: Thr(D1)Ser, Pro(D2)Ala, Asp(D3)Ser, and Val(D5)Ile. The Pro to Ala change might likely be contributing towards the stabilization of α -helical characteristics in the D helix (Fig. 4F).

Our work lays the foundation for deriving structural mechanisms of various hemoglobinopathies associated with increased heme disassociation and relatedly leading to unstable or non-functional Hb phenotypes. In our previous Hb disassembly pathway paper, we had concluded some intermediates such as hemichromes can be potential precursors for hemoglobinopathy pathophysiology due to introduction of toxicities following Hb trapping as intermediates. The inherent instabilities or dynamic natures of evolving intermediates on a pathway are often major barriers towards identifying the intermediates and further characterizing their roles and biophysical properties. MD simulations has provided us the means to overcome such barriers for the Hb heme disassociation pathways. Our results also provide new insights for examining various clinically unstable CE loop variants such as Hb Hammersmith (β Phe(CD1)Ser) resulting in severe hemolytic anemia²² and Hb Torino(α Phe(CE1)Val) resulting in Heinz bodies anemia⁸¹.

Finally, our results can contribute towards new hemoglobin protein engineering strategies for designing therapeutic agents in the fields of transfusion medicine and hematology. For example, HbF has been identified clinically as a therapeutic agent to address β -thalassemias^{82–85}, and our study can therefore provide further insights for enhancing efficacy of recombinant HbF in terms of heme pocket dynamics and heme loss modulation through genetic engineering. Meanwhile, high heme disassociation rates are one of the major challenges currently for designing a robust and clinically safe acellular HBOC¹⁰. Therefore, recombinant Hb engineering strategies can be pursued for modulating heme exit by restricting the flexibility of the CE loop or by introducing residues around the heme cavities that can form strong electrostatic interactions with the heme propionate groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Hb	hemoglobin
HbA	human hemoglobin A
HBOC	hemoglobin-based oxygen carrier
metHb	methemoglobin
MD	molecular dynamics
HbF	fetal hemoglobin
QM	quantum mechanics
RESP	restrained electrostatic potential fitting method
PMEMD	Particle Mesh Ewald Molecular Dynamics
GPU	graphical processing unit
R eq	equilibrium distance
R	distance
E_R	restraint energy
RMSD	root-mean-square-deviation
DSSP	hydrogen bond estimation algorithm
LCPO	linear combinations of pairwise overlaps

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Figure 1.

Snapshots along a molecular dynamics simulation trajectory of heme disassociation in the $\alpha 1$ heme pocket of methHbA. **A.** Native aquometHbA at equilibrium. At the distal side of heme pocket, a water molecule is both binding to the heme iron and hydrogen bonding to the Ne2 atom of His(E7). Heme iron is coordinating to the Ne2 atom of His(F8) at the proximal side of heme cavity. **B.** A penta-coordinated heme iron has formed following the initial disassociation of the distally bound water molecule. **C.** A bis-histidine hemichrome conformer has formed, following the penta-coordinated heme structure, with axial coordination of the heme iron to Ne2 atoms of both F8 and E7 histidines. **D.** The coordination between His(F8) and heme iron has been broken finally and the F helix has moved away from the cavity. A bulk solvent water molecule is now bound to the iron at the proximal side of heme cavity. The black arrows in panels **A** and **D** represent water exit and entry respectively. Panels A, B, C and D represent different time points in sequence along a simulation trajectory.

Figure 2.

MD simulation trajectory of heme disassociation in metHbA at 298 K illustrate changes in coordination between heme iron and bound ligands in the **A.** $\alpha 1$, **B.** $\beta 1$, **C.** $\alpha 2$, and **D.** $\beta 2$ subunits. The coordination between heme iron and the originally bound water molecule (black circles) breaks at around 260 ns. Following this, either a bulk solvent molecule (green circles) (panel **D**) or the distal His (panels **A**, **B**, and **C**) binds to the heme iron distally. The coordination between heme iron and proximal His (cyan circles) breaks at around 420 ns. Subsequently, a bulk solvent binds to the heme iron proximally (red circles).

Figure 3.

C- α RMSD of the CE loop regions and F helices in HbA during heme disassociation simulation at 298 K. The dotted lines in the color key bar on the right maps each line plot to a specific residue number of a protein segment (CE/CD loop, D helix, and F helix). For example, the dashed cyan line in panel (a) represents residue CE3. Each panel represents a specific protein segment in either α or β subunit as follows: CE1-CE9 in (a) α 1 and (f) α 2; F1-F9 in (b) α 1, (e) β 1, (g) α 2, and (j) β 2; CD1-CD8 in (c) β 1 and (h) β 2; and D1-D7 in (d) β 1 and (i) β 2. The coordination between heme iron and the originally bound water molecule is broken at 260 ns. The coordination between heme iron and proximal His is broken at 420 ns. Hemichromes were seen in α 1 and β 1 subunits between 300–420 ns

Figure 4. Secondary structure changes of D and F helices in HbA and HbF during heme disassociation simulations at 298 K. DSSP⁵⁸ algorithm was used to calculate the measurements. The panels represent $\alpha 1$ F helix in A. HbA and B. HbF; $\beta 2/\gamma 2$ F helix in C. HbA and D. HbF; and $\beta 2/\gamma 2$ D helix in E. HbA and F. HbF. The coordination between heme iron and proximal His is broken at 360 ns for HbA and at 580 ns for HbF.

Figure 5. Changes in the dihedral $\chi 1$ angle of **A.** distal histidines and **B.** CE1/CD1 residues in HbA during heme disassociation simulation at 298 K. **A.** Flipping of distal His observed in $\beta 2$ subunit of HbA at 298 K. Dihedral $\chi 1$ angle distribution of residues **B.** His(E7) and **C.** Phe(α CE1)/Phe(β CD1). The $\chi 1$ angle is defined as the dihedral angle formed between the N, C α , C β , and C γ atoms.

Figure 6.

Changes in HbA tetramer interface interactions during heme disassociation simulation. The interactions probed are **A.** hydrogen bonding between the carboxyl group of Asp(α 1G1) and amine group of Asn(β 2G4); **B.** side chain stacking between Tyr(α 1C7) and Arg(β 2C6); **C.** side chain stacking between Arg(α 1FG4) and Arg(β 2C6); and **D** hydrogen bonding between the hydroxyl group on Thr(α 1C6) and the guanidino group on Arg(β 2C6). In all panels, the coordination between heme iron and proximal His is broken at 420 ns. Simulation was conducted at 298 K.

Figure 7.

Solvent accessible surface area of heme calculated for heme disassociation trajectories of **A.** HbA, **B.** HbF, **C.** Hb β H(E7)L, and **D.** Hb β V(E11)A using the LCPO⁵⁹ algorithm. All the trajectories shown here were measured at 310 K except of Hb β V(E11)A, which was measured at 298 K. The coordination between proximal His and heme iron was broken for (a) HbA at 1.9 μ s, (b) HbF at 250 ns, (c) Hb β H(E7)L at 250 ns, and (d) Hb β V(E11)A at 333 ns.

Figure 8.

A-E. H(E7)L and **F.** V(E11)A mutations facilitate heme destabilization within the heme pockets of the HbA β subunits. Heme (green porphyrin) disassociation simulations were conducted at 310 K and 298 K for Hb β H(E7)L and Hb β V(E11)A, respectively. The β 1 subunit is depicted in panels A-E, and the β 2 subunit is depicted in panel F. For the H(E7)L mutant, in **A**, proximal His had moved backward from the heme pocket and the stacking between Phe(CD1) and heme is seen to be disrupted. Next, in **B**, the F helix has moved completely away from the heme pocket, heme has been dislodged from equilibrium, and a water molecule is now bound at each axial site of the heme iron. This is followed by in **C**, where the heme is held weakly in the heme pocket by both the heme porphyrin stacking with Phe(CD4) and heme propionate interaction with Lys(E10). In **D**, heme is almost completely out of the heme pocket with the heme propionate group still forming a weak electrostatic interaction with Lys(E10). In **E**, heme is completely out in solution. For the V(E11)A mutant in **F**, heme has dislodged to almost an orthogonal position from its equilibrium state while the porphyrin is surprisingly forming a stacking interaction with Phe(C7). The imidazole group of distal E7 His has flipped away from heme iron.

Figure 9.

MD simulations trajectory of heme disassociation in methHbF at 298 K illustrate changes in coordination between heme iron and bound ligands in the **A.** $\alpha 1$, **B.** $\gamma 1$, **C.** $\alpha 2$, and **D.** $\gamma 2$ subunits. The coordination between heme iron and the originally bound water molecule (black circles) is broken at around 420 ns. The coordination between heme iron and proximal His (cyan circles) is broken at around 580 ns.