



Research paper

Molecular mechanisms of bio-catalysis of heme extraction from hemoglobin

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ABSTRACT

Red blood cell hemolysis in sickle cell disease (SCD) releases free hemoglobin. Extracellular hemoglobin and its degradation products, free heme and iron, are highly toxic due to oxidative stress induction and decrease in nitric oxide availability. We propose an approach that helps to eliminate extracellular hemoglobin toxicity in SCD by employing a bacterial protein system that evolved to extract heme from extracellular hemoglobin. NEAr heme Transporter (NEAT) domains from iron-regulated surface determinant proteins from *Staphylococcus aureus* specifically bind free heme as well as facilitate its extraction from hemoglobin. We demonstrate that a purified NEAT domain fused with human haptoglobin β -chain is able to remove heme from hemoglobin and reduce heme content and peroxidase activity of hemoglobin. We further use molecular dynamics (MD) simulations to resolve molecular pathway of heme transfer from hemoglobin to NEAT, and to elucidate molecular mechanism of such heme transferring process. Our study is the first of its kind, in which simulations are employed to characterize the process of heme leaving hemoglobin and subsequent rebinding with a NEAT domain. Our MD results highlight important amino acid residues that facilitate heme transfer and will guide further studies for the selection of best NEAT candidate to attenuate free hemoglobin toxicity.

1. Introduction

Sickle cell disease (SCD) is a fatal hemolytic disorder resulting in multiple organ failure, poor quality of life and shortened life expectancy. Rupture of Red Blood Cells (RBC) in SCD releases intracellular components including iron-heme containing hemoglobin (Hb), into the blood stream. Extracellular Hb exhibits a highly toxic nature by scavenging Nitric Oxide (NO) that reduces its bioavailability [1]. Hb and its degradation products – free heme and iron – perpetuate oxidative stress, and together with decreased NO availability promote many SCD complications. These include vaso-occlusion, thrombosis and hyper-coagulation, as well as tissue hypoxia, pulmonary hypertension and stroke [2]. Iron chelators [3] and heme scavengers [4], although shown to be partially protective in animal models, are not cell penetrable and, therefore, cannot completely extinguish toxic intracellular effects of iron and heme on vascular cells. One potentially more effective approach is to capture extracellular Hb by haptoglobin before it has degraded to free heme and iron, however a high dose (2–10 g per patient) required for such treatment significantly limits feasibility of this approach, in part, due to potentially high costs of the therapy [5]. Moreover, Haptoglobin consists of two subunits, alpha

and beta chains, and human haptoglobin has two genetic variants HP1 and HP2. In the plasma, HP1 and HP2 interact with hemoglobin to form a great numbers of different high molecular weight HP-Hb complexes via reduced cysteine residues available for cross-linking utilizing peroxidase activity of captured hemoglobin to produce those crosslinks. Macrophages and liver cells capture large HP-Hb complexes clearing the plasma from free hemoglobin. Thus, this system is very complex requiring many steps from formation of multimeric complexes to recognition, capturing and utilization those complexes in liver cells or macrophages.

In this work we propose and test *in vitro* a novel and unique system to sequester heme from extracellular hemoglobin using bacterial protein domain, NEAr heme Transporter (NEAT). To elucidate molecular mechanisms underlying a process of heme extraction by NEAT we perform extensive molecular dynamics (MD) simulations of the hemoglobin-NEAT domain complex in presence of a heme. We also carry out simulations of a heme transfer between two proteins. The modeling studies allow us to elucidate a heme-transfer process pathway, as well as to determine structural features of the proteins that make the heme extraction possible. Despite large theoretical and experimental literature available on the mechanisms of function of

Abbreviations: SCD, Sickle Cell Disease; NEAT, NEAr heme Transporter; MD, Molecular Dynamics; Hb, Hemoglobin; Isd, Iron-regulated surface determinant proteins; AMBER, Assisted Model Building with Energy Refinement; PDB, Protein Data Bank; COM, Center Of Mass; HP, Haptoglobin; HX, Hemopexin; RMSD, Root-Mean-Square Deviation; SD, Standard Deviation

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various heme-containing proteins, little is known about processes of heme binding and unbinding with the protein. Our study is the first of its kind, in which simulations are employed to characterize the process of heme leaving hemoglobin and subsequent rebinding with a NEAT domain. As a result of these simulations we will be able to design more efficient NEAT containing systems in the future work.

Iron-regulated surface determinant proteins (isd) are responsible for heme extraction and transfer process in *Staphylococcus aureus* [6–10]. Isd family consists of nine proteins facilitating heme transfer from outer region of the bacterial cell wall (isdH,B,A,C) to the cytoplasm through the inner membrane (isdD,E,F). A first protein in the cascade, heme capturing protein IsdH, contains three NEAT domains, of which two N-terminal domains (N1, N2) are shown to site-specifically bind hemoglobin or haptoglobin, whereas the C-terminal domain (isdH-N3) extracts heme directly from hemoglobin [11,12]. Recently, a partial structure of isdH bound to a hemoglobin tetramer was resolved [13]. In the structure of a Hb-isdH complex the isdH-N3 domain is coordinated with an alpha subunit of Hb in a complex conducive to heme transfer (see Fig. 3A).

The heme-binding NEAT domains, isdH-N3, isdB-N2, isdA, isdC, share a highly similar tertiary structure. NEAT domains are characterized by a hydrophobic heme binding pocket formed by a β -sheet that contains two conserved tyrosine residues implicated in heme iron coordination [14] (shown in Fig. 3B in complex with a heme molecule), as well as a histidine or a glycine in isdA, isdC proteins respectively. Propionate groups of the NEAT bound heme are oriented outside of the heme binding pocket and interact with the loop 1 (see Fig. 3B), which consists of mostly polar and charged residues [14]. Loop 1 sequence varies highly among NEAT domains, although it has a conserved serine (Fig. 3B). On the opposite side heme is coordinated by a short helix of variable sequence.

It was proposed that the mechanism of heme-transfer between NEAT containing proteins is based on increased heme affinity to the proteins in the isd heme transferring protein cascade. However, a specific mechanism and a pathway of heme extraction from Hb by NEAT or heme transfer between NEAT domains remains unknown. In this work we demonstrate a successful heme extraction by an isdH-N1 domain not shown before, and propose a mechanism of heme-extraction from Hb by an isdH-N3 domain based on Molecular Dynamics (MD) simulations of the process of heme transfer in the Hb - ISDH-N3 complex.

2. Methods

2.1. Molecular dynamics simulations

All MD simulations were performed using AMBER14 [15] software package, with AMBER99SB-ILDN force field [16]. Force field parameters for heme were obtained from Giammona et al., [17]. Equilibration procedure was as follows. The protein (PDB 4IJ213) was prepared for the simulations using AmberTools 14 Leap with the standard protocols. The protein was solvated with the TIP3P water, so that the distance between the protein and an edge of a simulation box was 11.0 Å. The charge of the simulated system was neutralized by adding the counter ions. Protonation state of the histidine residues in hemoglobin was obtained from Ohe and Kajita [18,19]. After a short minimization of the solvent and a subsequent minimization of the protein structure using steepest descents algorithm, the MD simulations were performed as described in the following. All simulations used 2 fs time step for integration with the covalent bonds to hydrogen atoms constrained via SHAKE [20] algorithm. The non-bonded interactions cutoff radius was 8.0 Å and a Particle Mesh Ewald (PME) method [21] for electrostatic interactions was used as implemented in AMBER14. The temperature was controlled using the Langevin thermostat and the pressure - via Berendsen barostat with isotropic scaling, also as implemented in AMBER14.

The system was first heated to 300 K at constant volume with harmonic restrain force applied to all protein Ca atoms ($k=10 \frac{\text{kcal}}{\text{molÅ}^2}$). This was followed by a simulation at constant pressure of 1 atm and constant temperature of 300 K, in which the protein atom restrains were decreased gradually from 1 to $0.25 \frac{\text{kcal}}{\text{molÅ}^2}$. Lastly, a 20 ns equilibration simulation without any restrains was performed. One of the criteria used to analyze the structure of the protein conformation was root-mean-square deviation (RMSD) of atomic positions, which is a measure of the average distance between the atoms of superimposed proteins. Post-processing of trajectories was done with VMD [22], Cpptraj v14.25 [23], and Pymol [24] software packages.

Production MD simulation trajectories, in part, were obtained using Steered Molecular Dynamics (SMD) [25]. SMD is a computational method specifically developed to increase the rates of reactions in MD simulations by applying a mild force along a reaction field [26]. To apply constrains, the centers of mass (COM) of the proteins were used during the SMD simulations. COM1, representing the COM of hemoglobin, is defined as a center of mass of Ca atoms of the residues M32, F43, H45, H58, K61, A65, L83, H87, F98, L101, L129. COM2, representing the COM of NEAT, is defined as a center of mass of Ca atoms of residues V564, F568, V569, Y593, W594, V633, V635, Y642, Y646. COM3 is another representation of the NEAT's COM defined using Ca atoms of residues R616, I619, V633, V635, Y642, Y646. Two COM representations were used for NEAT in order to control the direction of the heme movement towards the heme-binding pocket of NEAT as described in Results section.

2.2. NEAT protein purification

NEAT domain from ISDH-N1 fused at C-terminus with haptoglobin beta chain (human sequence) was purified using bacterial expression system for purification of the His-tagged protein. The BL-21 strain of *E. coli* was transformed with a polyHis-pET47b plasmid containing human NEAT ISDH-N1 fused with beta-chain of human haptoglobin at C-terminus. Isopropyl-beta-D-thiogalactopyranoside (IPTG, 1 mM) was added and the cells were incubated for 18–20 h at 25 °C. Bacteria were then harvested by centrifugation and the pellet was immediately lysed in 40 mM Tris-HCl, 5% glycerol, 1 mg/ml lysozyme, 100 mM NaCl, protease inhibitor cocktail, ribonuclease A (Sigma), and deoxyribonuclease I (Sigma). The pellet was gently rocked for 30 min, sonicated and subjected to ultracentrifugation. The supernatant was loaded onto a Hisprep FF 16/10 column using binding buffer (40 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 30 mM imidazole) at 0.1 ml/min flow. The column was washed with 40 mM Tris-HCl, 300 mM NaCl, 5% glycerol, 30 mM imidazole using a flow rate of 1.5 ml/min. Elution of the histidine-tagged protein was accomplished with elution buffer (40 mM Tris-HCl, 300 mM NaCl, 5% glycerol, 400 mM imidazole) at 1.0 ml/min flow. Collected fractions were loaded for size-exclusion gel filtration on a HiLoad 26/60 Superdex 75 column using gel filtration buffer (60 mM Tris-HCl, 100 mM NaCl, 5% glycerol) at 0.2 ml/min flow. Fractions were collected and analyzed by Coomassie blue staining and Western blot. All purification steps were performed at 4 °C, and purified protein was stored at –80 °C.

2.3. Heme extraction measurement

Purified NEAT domain (100 µg/ml) and porcine hemoglobin (Sigma-Aldrich) (10 µg/ml) in the molecular ratio 4:1 respectively were mixed for 18 h at 37 °C. As hemoglobin is rapidly oxidized by the air, in our preparation, hemoglobin predominantly was in the ferric/oxidized state (Fe^{3+}). After separation on the gradient gel (SDS sample buffer was added to the samples without heating/boiling), heme color in the hemoglobin band was obtained by scanning gel in transparent film mode (Epson Perfection 600). For total hemoglobin protein visualization, Imperial Blue staining was used. Assay was duplicated.

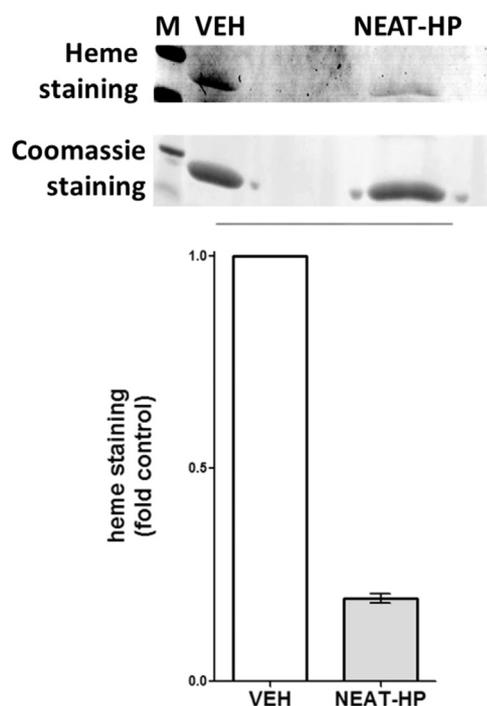


Fig. 1. NEAT-haptoglobin fusion protein scavenges heme from hemoglobin. Hemoglobin was incubated with NEAT-HP fusion protein and after SDS-electrophoresis was stained for heme and for total hemoglobin by the Coomassie stain (upper panel indicates heme staining, lower panel is a Coomassie stain of total Hb protein) NEAT HP treatment removed ~85% of the heme from hemoglobin (N=2, mean \pm SD).

2.4. Hemoglobin peroxidase activity

We measured peroxidase activity of human sickle hemoglobin (MyBioSource) at 2 μ g/ml incubated with vehicle, NEAT-HP (25 μ g/ml), haptoglobin (HP) (25 μ g/ml) and hemopexin (HX) (25 μ g/ml) (18 h, 37 °C) using Amplex Red protocol with known concentration of H₂O₂ (5 μ M). After 15 min of incubation with H₂O₂, Amplex red fluorescence was measured in 96 well plate in BioTek H1m multi-plate reader with excitation/emission at 571/585 nm. Each treatment was replicated 5 times.

3. Results

3.1. Experiments

In a proof-of-principle experiment, purified NEAT domain from ISDH (N1) was fused with the human haptoglobin β -chain to increase affinity of NEAT to the hemoglobin. A haptoglobin fused NEAT domain (NEAT-HP) protein was then mixed with hemoglobin for 18 h of incubation at 37°C (Fig. 1). Our data indicate that the in-gel heme staining (The upper panel of Fig. 2) was markedly reduced in Hb incubated with NEAT-HP. The lower panel of Fig. 2 indicates Coomassie stained Hb protein to serve as a Hb protein loading control. It is seen in Fig. 2 that after incubation with NEAT-HP, Hb has decreased the heme content by 85%, which confirms heme removal ability of NEAT-HP construct.

It is well known that in the presence of H₂O₂ hemoglobin exhibits a peroxidase-like activity, catalyzed by the heme moiety. At the same time, heme bound to NEAT does not catalyze the peroxidase reaction due to inability to interact with H₂O₂. To compare an “active” heme scavenging by NEAT-HP with a “passive” heme scavenging by hemopexin and haptoglobin, which are not capable to actively extract heme from Hb, we measured peroxidase activity of a human sickle hemoglobin (2 μ g/ml) incubated with PBS (VEH), NEAT-HP, haptoglobin (HP) and hemopexin (HX) (18 h, 37 °C). Our data demonstrate a significant

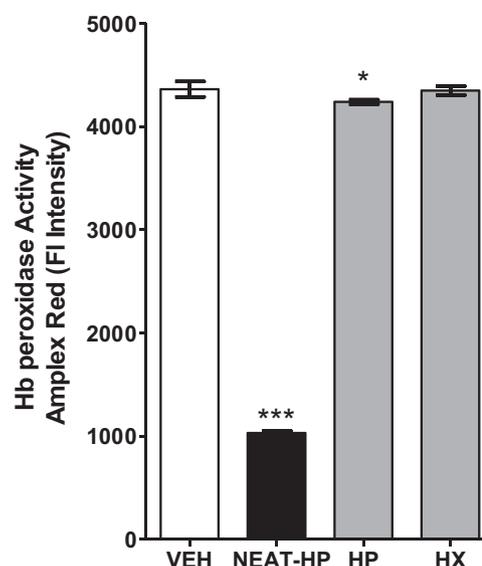


Fig. 2. NEAT-HP inhibits heme-dependent peroxidase activity of hemoglobin. Hemoglobin peroxidase activity measured by oxidation of Amplex Red was markedly attenuated by NEAT-HP treatment (***) $p < 0.001$, human haptoglobin (HP) mildly reduced peroxidase activity (*, $p < 0.05$) and human hemopexin (HX) did not affect hemoglobin peroxidase activity. (mean \pm SD, N=4–5*—different to veh control). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decrease in peroxidase activity of hemoglobin incubated with NEAT-HP, indicating heme transfer. However, no changes in peroxidase activity were observed after incubation with haptoglobin or hemopexin (Fig. 2). Therefore, we demonstrated that an active scavenging of heme by NEAT can reduce peroxidase activity of hemoglobin, whereas passive scavengers cannot.

3.2. Simulations

IsdH-N3 is the only NEAT domain crystallized in a complex with Hb in configuration conducive to heme transfer. Therefore, the molecular dynamics simulations of heme transfer from Hb to NEAT were performed with a protein complex of an alpha chain of Hb and an isdH-N3 NEAT domain (shown in Fig. 3A) extracted from a crystal structure (PDB 4IJ2 [13]). This structure consists of a Hb tetramer and four corresponding isdH-N2,N3 proteins bound to the Hb. Fig. 3A shows that initially the heme is buried deeply in the hydrophobic pocket of Hb in a T-deoxy state with proximal histidine coordinating the iron. The heme propionate groups extend outside of Hb pointing toward NEAT, particularly, Y642 and Y646, the key tyrosine residues shown in yellow sticks in Fig. 3B.

After equilibrating simulations of the protein-heme complex in water solution, performed as described in Methods section, an equilibrium MD simulation of the α Hb-isdH-N3 protein complex with heme bound to Hb was performed for about 200 ns. In this simulation we monitored an overall complex structure and dynamics. Both proteins remained stable in the simulations with maximum value of 2.5 of root-mean-square deviation (RMSD) of C α atoms in individual proteins (NEAT and Hb) from their crystallographic positions of 2.5 Å. However, the complex interface exhibited significant mobility, and relative positions of two proteins with respect to each other were fluctuating. The dynamics of the protein complex resembled the dynamics of two rigid bodies weakly bound at the interface. The distance between the centers of mass (COM) of the proteins fluctuated from 10 to 13 Å, and the angle of deviation of a vector pointing from Hb COM to NEAT COM was approximately 25° (data not shown). This result is consistent with the experimental observation that a stand-alone N3 domain is incapable of heme transfer, due to its low affinity to

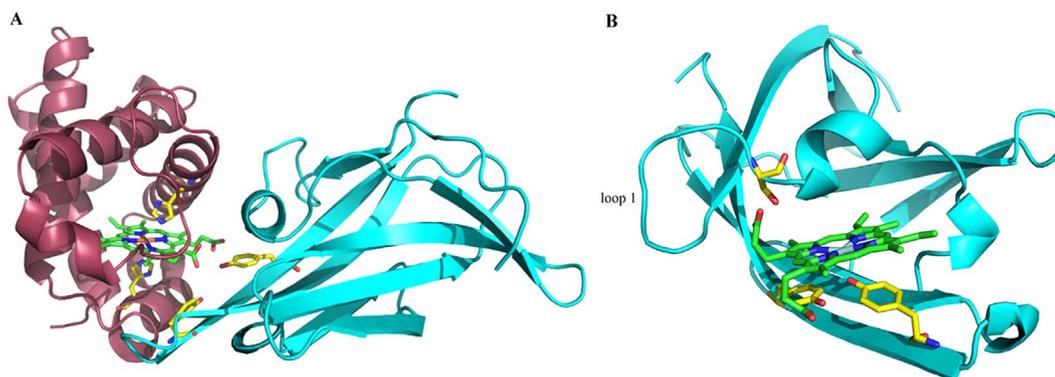


Fig. 3. A. A structure of the complex of the *isdH-N3* NEAT domain and the hemoglobin alpha subunit with heme bound to Hb, extracted from the crystal structure [28] and equilibrated in MD simulations (see text). Hb is shown in cartoon representation in raspberry red, NEAT is in cyan, heme is in green. Shown in yellow are two key tyrosine residues Y642, Y646 of NEAT, as well as proximal and distal histidine residues of the Hb heme binding site. **B.** A structure of the *isdH-N3* NEAT domain in complex with heme [13]. NEAT is shown in cyan, heme is shown in green, two Y642, Y646 residues coordinating iron and S563 (of loop 1) interacting with a propionate group of the heme are shown in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Hb.

It is expected that the process of heme transfer from the binding pocket of Hb to the binding pocket of NEAT is slow, with respect to the simulation times. This, in turn, means that it is practically impossible to capture the heme transfer event in a free unrestrained MD simulation. To model heme transfer process and pathway we used Steered Molecular Dynamics (SMD). The heme transfer process during the SMD simulations was split into two major steps: i) heme leaving the binding pocket of Hb, and ii) heme entering the binding pocket of NEAT. SMD simulations of the first step were set to restraint corresponding the reaction coordinate between the COM (see Methods for definition of COM) of the complex Hb - NEAT and Fe^{2+} atom of heme. This simulation is further referred to as the “Push simulation”. During the Push simulation heme was pushed out of the binding pocket in the Hb into the inter-protein region. The SMD simulations of the second step were set up to restraint corresponding the reaction coordinate between the COM of NEAT and Fe^{2+} atom of heme. This simulation is further referred to as the “Pull simulation”. During this simulation the heme was pulled into the binding pocket of NEAT. A combined representation of all simulated trajectories of the heme-transferring process is shown as a scatter plot in Fig. 4. Also, see

the Supplementary movie of heme transfer process in the Hb - *isdH-N3* complex.

Supplementary material related to this article can be found online at <http://dx.doi.org/10.1016/j.redox.2017.01.004>.

The Push simulation was performed using SMD for 400 ns with the following parameters: $k=100 \text{ kcal } \text{\AA}^{-1} \text{ mol}^{-1}$, $t=400 \text{ ns}$. The distance between the center of mass of the NEAT - Hb complex and the Fe^{2+} ion was gradually decreased from 7.5 \AA to 0 \AA . An extent of a heme motion in the Push simulation is shown in Fig. 5.

Following the Push simulation, in which relative position of the heme and the proteins was controlled by relatively mild restrains, a short 10 ns SMD simulation was performed to promote relaxation of the complex and the heme at the edge of the Hb outside of the Hb binding site. In this simulation the distance between Hb (COM1) and NEAT (COM2) (see Methods section for definition of COM1 and COM2) has been slightly increased, while the heme was not restrained. This separation has led to an adjustment of NEAT and heme relative to each other, with heme remaining bound to Hb. Heme positions resulting from this simulation are shown in Fig. 6.

Following the separation simulation, the Pull simulation of 500 ns and $k=100 \text{ kcal } \text{\AA}^{-1} \text{ mol}^{-1}$ SMD was performed. Fig. 7 represents struc-

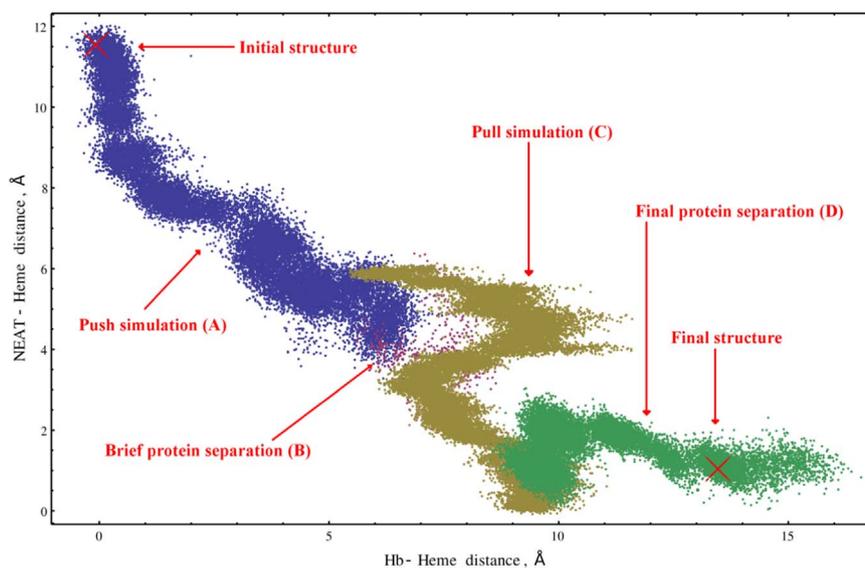


Fig. 4. Combined trajectory represented as the distances between heme iron and centers of mass of Hb and NEAT throughout the MD simulations. System transfers from large NEAT-heme, small Hb-heme distance (upper left corner), to small NEAT-heme, large Hb-heme distance (lower right corner). Blue dots represent the Push simulation (Fig. 5), red dots – first brief protein separation (Fig. 6), dark orange dots – the Pull simulation (Fig. 7), and the green dots represent the final protein separation (Fig. 8), see the text. Red crosses indicate the initial (Fig. 1) and the final (Fig. 9) structures as indicated in the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

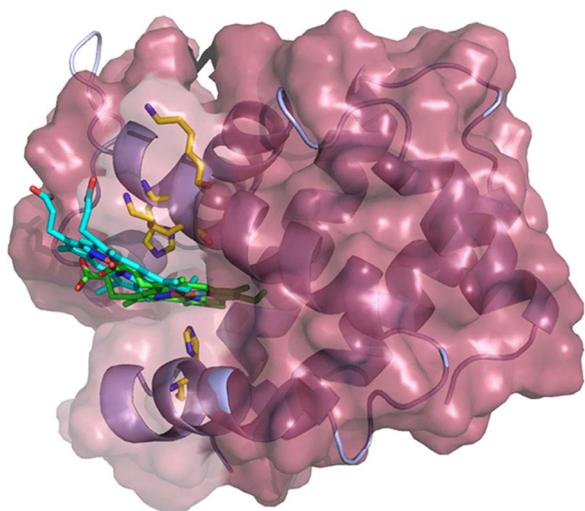


Fig. 5. Heme transition from hemoglobin pocket into inter protein space (NEAT protein is not shown). Raspberry red is the surface of initial structure of Hb, light blue is Hb after the Push simulation (see the main text), green and cyan colored – initial and final positions of heme relative to hemoglobin and the heme-binding pocket. Yellow histidine (proximal and distal) and lysine residues are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

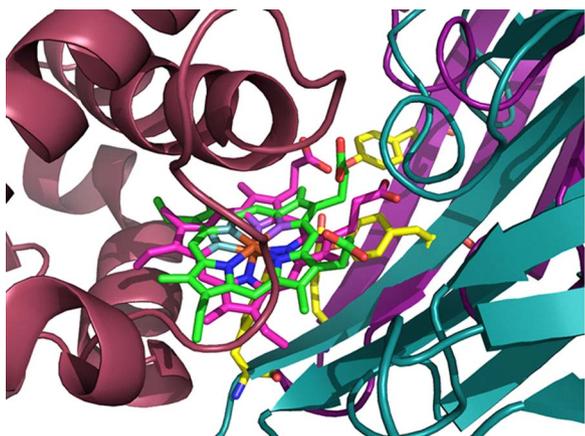


Fig. 6. Change in position of NEAT relative to hemoglobin after first brief (10 ns) Hb-NEAT separation simulation. Shown in teal and magenta are initial and final positions of NEAT respectively, and in green and magenta – initial and final positions of heme respectively. Shown in yellow are the iron-coordinating tyrosine residues of NEAT.

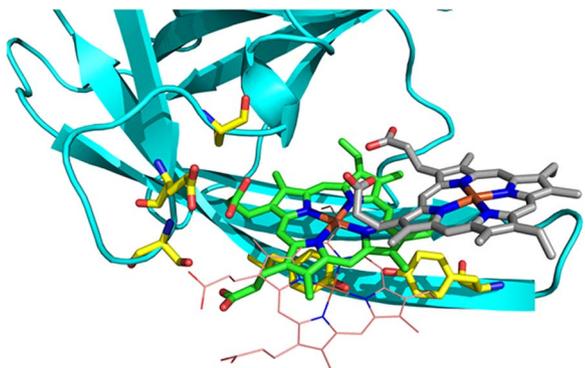


Fig. 7. Heme capturing by NEAT from inter protein space (Hb is not shown) during Pull simulation (Hb is not shown). Heme shown with sticks colored grey is at a position in the beginning of the Pull simulation. Heme shown with magenta lines is at a position achieved after 130 ns of the simulation. Heme colored green in stick representation is at the final position after 500 ns. Two yellow residues in the binding site are Y646, Y642, and three yellow residues on the loop 1 are E556, S557, and S563.

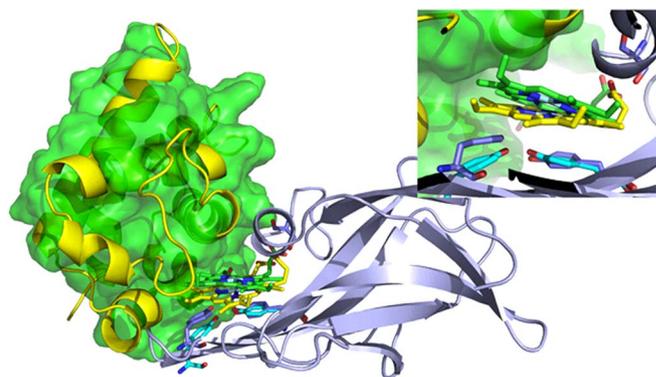


Fig. 8. Protein conformations after second brief (30 ns) Hb-NEAT separation. Initial position of Hb is shown in yellow cartoon, and Hb after separation is shown in green surface. Shown in yellow and green sticks are the initial and final position of heme relative to NEAT respectively, and shown in teal and cyan are the residues on the beta strand – Y642, Y646. Zooming into heme is in the insertion (top-right corner), where top-right blue residue is the serine S563 residue.

tures resulted from the Pull simulation. A different center of mass group of residues for NEAT, COM3, was used (see Methods section) to direct the heme into the binding pocket of the NEAT domain. In this simulation the distance between Fe^{2+} and the COM3 of NEAT was decreased to the value obtained from the PDB 2Z6F structure of a stand-alone NEAT domain bound with heme [27]. The resulting structure of the complex at the end of the Pull simulation is shown in Fig. 7. This simulation was designed to direct the heme towards the β -sheets of NEAT, which contain the tyrosine residues coordinating the iron of the heme when it is bound to NEAT. The initial distance between the heme iron and COM3 was 15.1 Å. This distance was decreased to 9.1 Å (see Fig. 4).

To test whether the heme will remain bound to the NEAT during the protein complex separation, a second short protein separation simulation was performed with the heme free from any restrains. Fig. 8 shows structures explored in this simulation. The corresponding representation of the simulated trajectory is shown in the scatter plot in the Fig. 4D (see the green dots in the lower right corner of the plot). According to this plot, although Hb is moving away from the heme, the heme remains bound to the corresponding binding pocket of the NEAT. This simulation shows that free from any restrains heme is firmly bound to NEAT.

Finally, a short equilibrium 30 ns MD simulation of the heme - NEAT complex was performed without any restrains. In Fig. 9 the final structures of the simulated complex are overlapped with the crystal

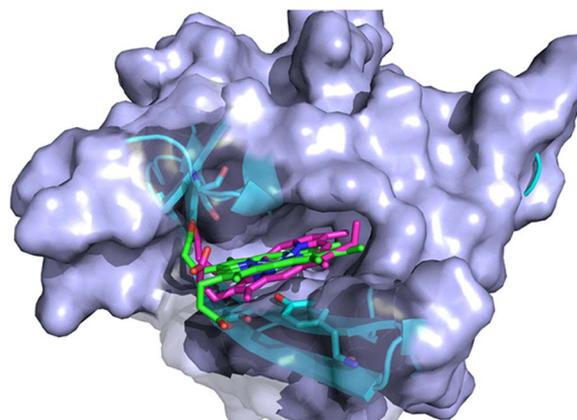


Fig. 9. Heme position after 30 ns equilibration of the NEAT structure with heme transferred to its binding pocket. Shown in green and magenta are heme in crystal and simulated structures respectively. Crystal structure and the simulated NEAT are shown in cyan cartoon and blue surface respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structures. RMSD of all protein Ca atoms and the nitrogen atoms of heme of the simulated NEAT-heme complex measured to the crystal structure (PDB 2Z6F [27]) was 2.1 Å. This result indicates that position of heme after the simulations is in an excellent agreement with the heme-bound crystal structure of NEAT, particularly, the iron is in the same position and one propionate group is interacting with S563.

4. Discussion

Nature perfected the ability of pathogenic bacteria to use hemolysis for acquiring nutrition and supporting bacterial growth. The iron-regulated surface determinant protein family (isd) is responsible for hemoglobin binding, heme extraction and heme transfer inside the bacteria. NEAT domain of isd protein family is responsible for heme extraction and transfer. In this paper we have presented the first successful attempt to design a novel heme scavenger that is capable of heme extraction from hemoglobin and arresting its peroxidase activity, using the property of NEAT domain to extract heme from free hemoglobin. To increase NEAT affinity to hemoglobin we developed a protein construct of a NEAT domain fused with haptoglobin. In the NEAT-HP construct, we utilized just a beta chain of haptoglobin to increase affinity of NEAT to hemoglobin. Beta chain alone cannot produce oligomeric complexes with hemoglobin, thus, cannot induce sequestration of free hemoglobin. Importantly, the structure of heme bound to a NEAT domain indicates that heme is tightly bound, and the binding site has no cavity that allows small molecules such as O₂, NO, CO, H₂O₂ to react with the heme. This result in heme shielding from ligand binding after the heme transfer from Hb to NEAT and heme moiety bound to NEAT does not participate in reaction with small molecules. This ability of NEAT can decrease the level of NO scavenging and oxidative stress in SCD. Moreover, scavenging heme from hemoglobin molecule will not only reduce Hb-mediated toxicity, but also prevent further Hb degradation with release of free heme and iron.

In our proof of concept experiment (Fig. 1), we found that NEAT-HP construct from isdH-N1, indeed, reduced heme signal from hemoglobin. Our experiments have successfully demonstrated the feasibility of using a NEAT domain to extract heme from the hemoglobin by employing a haptoglobin. Moreover, heme scavenging by NEAT-HP markedly decreased hemoglobin-mediated peroxidase activity (Fig. 2), whereas haptoglobin was capable of only a small attenuation of peroxidase activity and hemopexin did not affect it at all. Thus, the heme relocated from hemoglobin to NEAT-HP does not react with hydrogen peroxide, which suggests that heme bound with the NEAT domain is inaccessible to small molecule ligands. Importantly, our experiments demonstrate that isdH-N1 domain can successfully bind heme, contrary to an earlier claim [28].

To characterize the structural determinants of heme transfer, we have performed extensive molecular simulations of Hb-NEAT complex with heme transfer from the binding site of Hb to the binding site of NEAT. Due to the large size and absence of high resolution structure information on the whole complex of Haptoglobin bound with NEAT the simulated protein complex was truncated to include only domains that interact with HEME. Specifically designed constrains were placed to mimic haptoglobin presence. The simulations performed in this work allowed us, for the first time, to model a pathway of heme transfer between two proteins. Participating in this process amino acids were illuminated. Our results shed light on the process of heme extraction.

It has been previously shown that a wild-type isdH-N2,N3 construct is sufficient to transfer heme from Hb, while a separated isdH-N3, or an isdH-N2,N3 complex, with one of the heme-coordinating tyrosine residues mutated in isdH-N3, cannot extract heme from Hb. Also, it was shown that a stand-alone isdH-N2 binds to Hb, while replacing isdH-N2 can dramatically decrease the heme-transfer process. Therefore, it was concluded that a presence of an N2 NEAT domain stabilizes the interface of NEAT and Hb, while a stand-alone isdH-N3 has a low Hb binding affinity [14]. This low affinity of the stand-alone

Hb-isdH-N3 complex, indeed, was evidenced in the restraint-free simulation of the truncated complex. While we did not estimate the complex affinity *per se*, its mobility is indicative of a low binding strength. At the same time, it was implicated that isdH-N1 and isdH-N2 have a low affinity to heme, while isdH-N3 has low binding affinity to Hb, and high affinity to heme. This dramatically depend on the presence of iron coordinating tyrosine residues [14]. In this work we have demonstrated that despite a lower affinity to heme, stabilization of the Hb-haptoglobin complex allowed for an efficient heme transfer to an isdH-N1 domain. This indicates that formation of a stable complex with orientation of a NEAT domain conducive of heme transfer is one of the major factors for heme harvesting.

Simulating a heme transferring pathway with steered molecular dynamics reveals a number of specifically preferred protein – heme interactions. During the Push simulation heme moved from the binding pocket of Hb to the inter protein space, particularly, the iron ion has moved to the center of mass of the Hb-NEAT complex (Fig. 4A). The results of the Push simulation, where at the end of the simulations heme resides at the edge of Hb (see Fig. 5). This suggests that in order to exit hemoglobin, the heme has to initially fluctuate out of the Hb binding pocket to occupy an “intermediate” position, which may be the rate limiting step for the heme extraction. The interaction between the propionate groups of heme and the lysine residues K60 and K61 at the surface of Hb may play a role in transient stabilization of this heme position. It is worth mentioning that during the first quarter of the Push simulation, heme did not change its position in the binding pocket considerably, while NEAT domain approached Hb and the binding site, which is shown in the general scatter plot in Fig. 4 as blue dots (A). While the distance between heme and Hb did not change, and distance between NEAT and heme decreased. At the end of the Push simulation Y642 of NEAT (shown in yellow in Fig. 6) has already coordinated the heme iron. However, during the subsequent short protein separation simulation (Figs. 4 and 6), in which the heme is allowed to readjust its position freely, the distance between the heme iron and Y642 of NEAT increased. Importantly, the heme has rotated, such that its propionate groups followed the Y646 and Y642 tyrosine pair of NEAT. At this stage, the heme may be coordinated by either NEAT or Hb proteins and fluctuations can lead it to move back to the Hb binding site. Based on the duration of the simulation, we estimate that the step of Hb leaving the binding pocket is the main rate-limiting step of the heme-harvesting reaction.

Following the Push simulation, the protein separation simulation was included to account for the fact that isdH-N3 domain by itself has a relatively low binding affinity to Hb and is able to move when it is in a complex with Hb. In the full length isdH protein (not simulated) the Hb-isdH-N3 complex is strengthened by the presence of isdH-N1 and N2 domains, which have high affinity to Hb, but do not participate in heme extraction. Therefore, the relative positions of the Hb and N3 in the complex may be less affected by the heme relocation. Y646 coordinates heme iron after 130 ns of the Pull simulation. Based on the results of our simulations, it is possible to suggest that the weak binding between NEAT domain and Hb is important for the successful heme extraction. Changing a relative position of Hb and NEAT during the heme transfer may be conducive for a complex process of heme rotation and transfer. Thus, NEAT cannot strongly bind to hemoglobin at the site of extraction and requires an additional binding site through the different NEAT domain, or as in our case – through haptoglobin.

In all simulations performed in this work with similar strength of constrains, two processes of heme transfer took relatively longer time: first, a heme unbinding from the Hb binding pocket, and secondly, a heme final coordination by the Tyr of NEAT.

It is known that the system of two Tyr residues in NEAT is required to bind heme. In the case of both Tyr residues protonated, MD simulation shows that these residues are directed towards the exterior of the binding site in NEAT. Although, when one of the Tyr residues is deprotonated, the relative position of the residues in a simulation

remains the same as in the crystal structure, where the side chains face each other. The hydroxyl hydrogen of Tyr residues could easily transfer from one residue to another stabilizing the local conformation; that facilitates the coordination of an iron in heme by the deprotonated Tyr residue.

By the end of the Pull simulation the heme moved deeper into the binding pocket of NEAT (see Fig. 6 for structure of the complex). Importantly, the heme propionate groups interact with the S557 and S563 residues of the loop 1, although E556 repels one of the propionate groups. During the heme transfer two serine residues of loop 1 capture heme while it is moving in the inter-protein space. Therefore, altering residues of the loop 1 region can dramatically affect heme capturing mechanism, and perhaps, the rates of the heme extraction from hemoglobin. However, the main driving force for the heme binding is hydrophobicity of the NEAT heme-binding pocket, as well as the presence of Y646. In the second protein separation simulation with free heme (Fig. 4, trajectory D), as Hb moved away from NEAT, the heme further rotated within the NEAT binding pocket, thus, the hydrophobic part of the heme moved deeper into the hydrophobic pocket of the protein, and the propionate groups of heme remained exposed to water or coordinated by the serine residues of loop 1. In the final separation simulation and the simulation free of any constraints heme remained firmly bound to NEAT coordinated by the residues of loop 1 and the tyrosine of the binding pocket (as shown in Fig. 8). The final simulated structure is in an excellent agreement with the crystal structure of the NEAT in the complex with heme, indicating plausibility of the suggested pathway of the heme transfer. Binding affinities of heme to the NEAT domains increase in the following order: *isdH-N3* < *isdA* < *isdC*, and it was proposed that the increase in heme binding affinity to be the main factor for heme transfer through the *isd* protein cascade. In spite of importance of NEAT heme-binding pocket hydrophobicity, it has been shown that the loop 1 plays important role in facilitating heme transfer. For example, *isdH-N3* wild type and *isdC* with loop 1 replaced from *isdH* have similar binding affinities to heme, while the wild-type *isdC* binds heme stronger than *isdH* does. Indeed, engineered *isdH-N3* with the loop 1 from *isdC* has increased affinity to heme [14]. Based on these mutations it was suggested that loop 1 plays an important role in heme binding. Our results corroborate the importance of loop 1. In the simulations the propionate groups of the heme interact with serine residue of loop 1 to anchor heme in the final state in the NEAT binding pocket.

It is important to notice that Steered molecular dynamics used in this study is a method designed to accelerate conformational transitions in protein systems that otherwise have rates inaccessible by the simulations. Using SMD does not guarantee finding the lowest energy path for the conversion, therefore it is possible that heme may proceed between its endpoint positions along multiple pathways that all have similar energy barrier. To ensure that a pathway that we have discovered in the simulations presented in this paper belongs to the lowest energy and therefore most probably pathway family we used very weak biasing forces, no larger than 40 kcal/mol/Å². Considering that conformational rearrangement of the system such as HEME transfer happens in a dense environment of two well-formed binding pockets of the Hb and NEAT proteins any deviation from a low energy pathway would result in a need for a much stronger biasing force. Thus, without rigorously calculating energetics of the proposed pathway the confidence in its plausibility remains high.

5. Conclusions

Hemolysis occurring in SCD patients releases extracellular hemoglobin that plays a key role in many SCD complications. Free hemoglobin and its degradation products – heme and iron – induce oxidative stress, inflammatory response and vascular damage that can lead to complications in multiple organs. The iron chelators, hemopexin and haptoglobin were recently explored as a possible therapeutics

for SCD. However, current chelating approaches have different limitations. Hemoglobin bound to haptoglobin can still inactivate NO and has even an increased peroxidase activity after haptoglobin binding [29]. Hemopexin can only attenuate heme-mediated toxic effects, but it cannot affect hemoglobin and iron mediated toxicity. In this work, we have developed a novel approach for inhibition of hemoglobin toxicity using bacterial protein domain. We have demonstrated that NEAT domain fusion with haptoglobin beta chain can successfully transfer heme from hemoglobin and shield heme from interaction with hydrogen peroxide, thus, attenuate peroxidase activity of free hemoglobin.

We have also used the computational biology approaches to resolve a molecular pathway of heme transfer from Hb to *isdH-N3* NEAT domain. Our data indicate that heme binding to Y642 and Y646 is important to stabilize heme in NEAT pocket. The inter protein space residues K60 and K61 of Hb, and S557 and S563 of NEAT are important for heme transfer via interaction with propionate groups, that facilitate heme rotation during the transfer. This analysis is important for understanding molecular determinants contributing to heme transfer from Hb to NEAT and for selection of best NEAT therapeutic candidate to help eliminate the extracellular Hb toxicity.

The success of this initial study of the HEME transfer between binding pockets of hemoglobin and Neat domain proteins opens up a multitude of possibilities future directions in development and analysis of these HEME scavenging systems. A number of questions that immediately come to mind are what is the best choice of a neat domain for heme scavenging, what is the energetics of the heme transfer and what is the rate of the process of such transfer; how do specific groups of the external NEAT loop facilitate the process of transfer, do they influence energetics of the final state or process rates; what is the minimum energy pathway for the process of heme transfer; how presence of haptoglobin affects energetics and rates of heme transfer? These other questions will be the subject of our future research.

Author contributions

S.S. performed, analyzed and interpreted molecular dynamics data and wrote the article; O.R. performed, analyzed and interpreted biochemical characterization, M.K. and R.R. designed research, interpreted data, and wrote the article.

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