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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 theisdH-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 Cα 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 Cα 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 Cα atoms of residues V564, F568, V569, Y593, W594, V633, V635, Y642, Y646. COM3 is another representation of the NEAT's COM defined using Cα 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.