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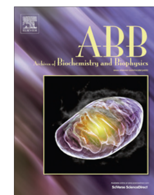

Kinetics of heme transfer by the Shr NEAT domains of Group A Streptococcus

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ABSTRACT

The hemolytic Group A Streptococcus (GAS) is a notorious human pathogen. Shr protein of GAS participates in iron acquisition by obtaining heme from host hemoglobin and delivering it to the adjacent receptor on the surface, Shp. Heme is then conveyed to the SiaABC proteins for transport across the membrane. Using rapid kinetic studies, we investigated the role of the two heme binding NEAT modules of Shr. Stopped-flow analysis showed that holoNEAT1 quickly delivered heme to apoShp. HoloNEAT2 did not exhibit such activity; only little and slow transfer of heme from NEAT2 to apoShp was seen, suggesting that Shr NEAT domains have distinctive roles in heme transport. HoloNEAT1 also provided heme to apoNEAT2, by a fast and reversible process. To the best of our knowledge this is the first transfer observed between isolated NEAT domains of the same receptor. Sequence alignment revealed that Shr NEAT domains belong to two families of NEAT domains that are conserved in Shr orthologs from several species. Based on the heme transfer kinetics, we propose that Shr proteins modulate heme uptake according to heme availability by a mechanism where NEAT1 facilitates fast heme delivery to Shp, whereas NEAT2 serves as a temporary storage for heme on the bacterial surface.

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Introduction

The bulk of the iron in the mammalian body is found in the form of heme, most of which is bound to hemoglobin and other proteins. To take advantage of the main iron reservoir in the host, pathogens compete for the heme and import it into the cytoplasm, where they incorporate the heme into the bacterial machinery or degrade it to release the iron [1–3]. An emerging theme for heme acquisition in Gram-positive organisms is the use of a protein relay apparatus to shuttle heme from the surface across the thick cell wall to a dedicated membrane ABC transporter [4,5]. The *isd* (Iron-regulated surface determinant)[3] locus of *Staphylococcus aureus* [6] encodes the best-described heme relay system in Gram-positive bacteria. Analogous mechanisms for heme uptake are used by several other pathogens including species of Bacillus, Listeria and Streptococcus. In *S. aureus*, the surface accessible receptors IsdH (encoded outside of the *isd* locus) and IsdB capture heme from the host [7–9] and transfer it directly or via IsdA to the membrane proximal receptor, IsdC [10–13]. In the last step of the cell wall passage, IsdC delivers the heme to the substrate-binding protein, IsdE, for transport into the cell. Heme shuttle from IsdH and IsdB through cell wall proteins is rapid and is a unidirectional process that involves protein–protein interactions [10,13,14]. IsdC plays a central cogwheel role in this pathway, receiving heme from several proteins and delivering it only to IsdE. *In vitro*, IsdC was shown to cycle between the apo and holo forms as it delivers the heme from IsdA to IsdE [15]. The Isd receptors in Staphylococcus and Listeria are covalently bound to the peptidoglycan. However *Bacillus anthracis* employs two protein hemophores, IsdX1 and IsdX2, which extract heme from hemoglobin and carry it to the cell wall IsdC [16–18]. The Bslk surface receptor, which is associated with the bacterial S-layer, can also deliver heme to IsdC in *B. anthracis*[19].

The Isd receptors, along with BslK, capture and transport heme using a protein domain named NEAT (for NEAr-iron Transporter) [4,5,20]. Multiple NEAT domains may be found in a single receptor, in which case they are numbered from the N-terminus of the protein. The ~120 amino acid NEAT domain adopts an eight-strand β-sandwich structure [21]. The heme is bound in a hydrophobic groove where the iron is typically pentacoordinated to tyrosine.

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[3] *Abbreviations used:* isd, iron-regulated surface determinant; ECM, extracellular matrix; GAS, Group A Streptococcus; NTD, N-terminal domain; metHb, methemoglobin.

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A second tyrosine near the axial ligand helps stabilizing the heme binding [22,23]. A unique tyrosine-methionine hexacoordination of the iron is used by the NEAT2 domain of IsdB [24]. In addition to the Isd proteins, other receptors contain NEAT domain(s) often near leucine-rich repeats (LRR) and other protein modules [25–27]. Alternative non-NEAT heme-binding domains were recently described in heme receptors from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*[28,29].

Despite the common immunoglobulin-like fold shared by the NEAT modules, individual domains exhibit major differences in preference for various ligands and partner proteins. Some NEAT domains interact only with host hemoproteins, such as for example IsdH NEAT1 and 2 and IsdB NEAT1 [8,9,30,31], whereas others only bind heme, such as IsdH NEAT3, IsdB NEAT2 and IsdC [10,11,30,32]. A few domains, as the single NEAT of IsdA [33], are able to interact with additional ligands including plasma and extracellular matrix (ECM) proteins. This complex functional diversity is well illustrated within the five NEAT domains of IsdX2 hemophore [18]. While both NEAT1 and NEAT5 in IsdX2 scavenge heme from hemoglobin, only NEAT1 transfers its heme to IsdC. NEAT3 and NEAT4 bind free heme and can transfer it to IsdC, but both are unable to obtain heme from hemoglobin. Finally, NEAT2 does not bind heme, but interacts with hemoglobin.

Group A Streptococcus (GAS) is a common human pathogen capable of producing a spectrum of clinical manifestations including invasive, life-threatening episodes and disabling post-infection sequelae [34]. A 10-gene operon named *sia*, for streptococcal iron acquisition, encodes a heme relay system in GAS [35,36]. This machinery consists of the two NEAT-type receptors, Shr and Shp, which are anchored to the cell membrane and not to the peptidoglycan. Shr is a protective antigen important for heme uptake, adherence, and virulence in GAS [37]. The functional domains in Shr include a unique N-terminal domain (NTD) that interacts with methemoglobin (metHb) and two distinctive heme-binding NEATs separated by a series of LRRs. While NEAT1 binds only heme, NEAT2 also binds extracellular matrix components and exhibits novel iron-reduction ability [25]. GAS NEAT domains do not have the axial ligand or the accessory tyrosine residue that are found in heme-binding NEAT domains of the Isd superfamily. For heme ligation, the NEAT-like domain in Shp uses bis-methionyl [38]; the mechanisms used by Shr NEAT1 and NEAT2 to acquire and transfer heme remain unknown. Other than GAS NEAT domains, the NEAT domain of HalA from *B. anthracis* is the only one known to bind heme without an accessory tyrosine residue [27].

Shr obtains heme from metHb (or solution) and transfers it to a NEAT-like module in Shp [39,40]. This transfer is through a biphasic process and is partial, as only about 63% of Shr heme is transferred to Shp [39]. From Shp, heme is transferred rapidly to SiaA through the formation of an activated protein complex [41]. The role of individual Shr NEAT domains in heme transport and the mechanism involved were not previously characterized. Here, we study the heme transfer kinetics by stopped-flow spectroscopy using isolated Shr NEAT domains. We demonstrate the first intramolecular heme transfer in isolated bacterial heme receptors, and show that only NEAT1 is capable to actively donate heme to Shp. Based on these observations we propose a modification of the existing model for heme flow on the streptococcal surface.

Experimental procedures

Bacterial strains, media and antibiotics

Escherichia coli cells were grown aerobically in Luria–Bertani (LB) medium at 37 °C supplemented with 100 μg/mL ampicillin. The *E. coli* strains used in this study are listed in Table 1.

Recombinant Shr and Shp proteins

The plasmids and primers used in this study are listed in Tables 1 and 2. The cloning of the recombinant Shr proteins, NTD-N1 (pEB11) and NEAT2 (pHSL2), each expressed as N-terminal fusion to the Strep-Xpress® epitope was previously described [25]. Shr NEAT1 fragment is insoluble when expressed as an isolated domain [25]. MBP was previously shown to help increase the solubility of recombinant proteins; therefore, an N-terminal fusion of Shr NEAT1 to His-MBP was constructed. Cloning was performed using the Gateway® technology (Invitrogen) according to the manufacturer's protocol. In brief, the NEAT1 region was amplified from the NZ131 chromosome with the ZE353/354 primer set and was cloned into the entry vector pDONR™221 by BP Clonase™ II. The resulting entry clone, pYSH5, which carries the NEAT1 fragment flanked by the *att*L sites, was then allowed to interact with the destination vector pDEST-His tag-MBP. The resulting plasmid, pYSH6, expresses a His-tag-MBP-NEAT1 fusion protein from the TAC promoter. The construction of Shp-His expression vector was accomplished by TOPO® directional cloning according to the manufacturer's instructions (Invitrogen, K101-01). The *shp* ORF was amplified from the NZ131 chromosome using the ZE406/ZE407 primer set and introduced into the pET101/D-TOPO vector. The resulting plasmid pOM101 codes for an Shp-His-tagged fusion protein expressed from the T7 RNA polymerase promoter.

Proteins expression and purification

NTD-N1 and NEAT2 were prepared as previously described [25]. Expression of His-tagged MBP-NEAT1 and His-tagged Shp was induced overnight at 27 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested and resuspended in 20 mM Tris-Cl, 100 mM NaCl, 0.1% Triton X-100, pH 8.0, with the addition of 0.5 mg/mL lysozyme and Complete mini-EDTA-free protease inhibitor cocktail tablets (Roche), then lysed by sonication. The cell pellet was centrifuged and the cleared lysate was then applied to a 5 mL HisTrap HP affinity column (nickel column) and purified using FPLC. Purified proteins were dialyzed in 10 mM sodium phosphate, 100 mM NaCl, pH 7.4, prior to their use for experiments. Western blot analysis of recombinant Shp was carried out using anti-His antibodies from mouse (Sigma).

Preparation of apoproteins, measurement of heme and protein concentrations

Preparation of apoproteins and total protein measurements were performed as previously described [25]. Heme concentration was determined by the absorbance at 410 nm using Beer's law: $A = \varepsilon bc$, where ε is the molar extinction coefficient at 410 nm of the corresponding heme-bound protein determined by the hemochromogen method [42], b is the path length of the sample and c is the molar concentration of heme in the sample.

Stopped-flow spectrophotometric analysis

Rapid kinetics was carried out on a Hi-Tech SF-61 stopped-flow spectrophotometer. The reacting apoproteins were mixed individually in 10 mM sodium phosphate, 100 mM NaCl, pH 7.4 and 25 °C with different concentrations of holoprotein. All concentrations of heme-loaded proteins were based on the absorbance of the heme bound to the protein. All concentrations used maintained pseudo-first order conditions. Absorbance data were collected over the 340–700 nm wavelength range of the diode-array detector. Alternatively, time-courses of the reaction were collected at the wavelength of maximal change with a monochromator and a photomultiplier detector. The optical path length for all the stopped-

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