A *Bacillus anthracis* S-Layer Homology Protein That Binds Heme and Mediates Heme Delivery to IsdC

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The sequestration of iron by mammalian hosts represents a significant obstacle to the establishment of a bacterial infection. In response, pathogenic bacteria have evolved mechanisms to acquire iron from host heme. *Bacillus anthracis*, the causative agent of anthrax, utilizes secreted hemophores to scavenge heme from host hemoglobin, thereby facilitating iron acquisition from extracellular heme pools and delivery to iron-regulated surface determinant (Isd) proteins covalently attached to the cell wall. However, several Gram-positive pathogens, including *B. anthracis*, contain genes that encode near iron transporter (NEAT) proteins that are genomically distant from the genetically linked Isd locus. NEAT domains are protein modules that partake in several functions related to heme transport, including binding heme and hemoglobin. This finding raises interesting questions concerning the relative role of these NEAT proteins, relative to hemophores and the Isd system, in iron uptake. Here, we present evidence that a *B. anthracis* S-layer homology (SLH) protein harboring a NEAT domain binds and directionally transfers heme to the Isd system via the cell wall protein IsdC. This finding suggests that the Isd system can receive heme from multiple inputs and may reflect an adaptation of *B. anthracis* to changing iron reservoirs during an infection. Understanding the mechanism of heme uptake in pathogenic bacteria is important for the development of novel therapeutics to prevent and treat bacterial infections.

Pathogenic bacteria need to acquire iron to survive in mammalian hosts (12). However, the host sequesters most iron in the porphyrin heme, and heme itself is often bound to proteins such as hemoglobin (14, 28, 85). Circulating hemoglobin can serve as a source of heme-iron for replicating bacteria in infected hosts, but the precise mechanisms of heme extraction, transport, and assimilation remain unclear (25, 46, 79, 86). An understanding of how bacterial pathogens import heme will lead to the development of new anti-infectives that inhibit heme uptake, thereby preventing or treating infections caused by these bacteria (47, 68).

The mechanisms of transport of biological molecules into a bacterial cell are influenced by the compositional, structural, and topological makeup of the cell envelope. Gram-negative bacteria utilize specific proteins to transport heme through the outer membrane, periplasm, and inner membrane (83, 84). Instead of an outer membrane and periplasm, Gram-positive bacteria contain a thick cell wall (59, 60). Proteins covalently anchored to the cell wall provide a functional link between extracellular heme reservoirs and intracellular iron utilization pathways (46). In addition, several Gram-positive and Gram-negative bacterial genera also contain an outermost structure termed the S (surface)-layer (75). The S-layer is a crystalline array of protein that surrounds the bacterial cell and may serve a multitude of functions, including maintenance of cell architecture and protection from host immune components (6, 7, 18, 19, 56). In bacterial pathogens that manifest an S-layer, the “force field” function of this structure raises questions concerning how small molecules such as heme can be successfully passed from the extracellular milieu to cell wall proteins for delivery into the cell cytoplasm.

*Bacillus anthracis* is a Gram-positive, spore-forming bacterium that is the etiological agent of anthrax disease (30, 33). The life cycle of *B. anthracis* begins after a phagocytosed spore germinates into a vegetative cell inside a mammalian host (2, 40, 69, 78). Virulence determinants produced by the vegetative cells facilitate bacterial growth, dissemination to major organ systems, and eventually host death (76–78). The release of aerosolized spores into areas with large concentrations of people is a serious public health concern (30).

Heme acquisition in *B. anthracis* is mediated by the action of IsdX1 and IsdX2, two extracellular hemophores that extract heme from host hemoglobin and deliver the iron-porphyrin to cell wall-localized IsdC (21, 45). Both IsdX1 and IsdX2 harbor near iron transporter domains (NEATs), a conserved protein module found in Gram-positive bacteria that mediates heme uptake from hemoglobin and contributes to bacterial pathogenesis upon infection (3, 8, 21, 31, 44, 46, 49, 50, 67, 81, 86). Hypothesizing that *B. anthracis* may contain additional mechanisms for heme transport, we provide evidence that *B. anthracis* S-layer protein K (BslK), an S-layer homology (SLH) and NEAT protein (32, 43), is surface localized and binds and transfers heme to IsdC in a rapid, contact-dependent manner. These results suggest that the Isd system is not a self-contained...
conduit for heme trafficking and imply that there is functional cross talk between differentially localized NEAT proteins to promote heme uptakes during infection.

MATERIALS AND METHODS

**Protein purification.** IsdX1 and IsdC were purified as previously described (44, 45). BslK was purified as a glutathione S-transferase (GST) fusion protein. Briefly, DNA encoding the NEAT domain (amino acids 46 to 165) of bslk (bslK) was amplified from B. anthracis genome DNA (45) using primers NEATforward (5' GATCGATGGATCTGATGAGGGCGTTCTCTCTC 3') and NEATreverse (5' GATCGATCGATTCTGATGAGGGCGTTCTCTCTC 3'). The underlined sequence refers to BamHI and EcoRI restriction sites, respectively. Following amplification, DNA was ligated into the BamHI/EcoRI restriction sites of pGEX2T to create pgst-bslK and transformed into Escherichia coli XL1-Blue.

**Holo-BSlK purification.** Purification of holo-BSlK was propagated in 100 ml of LB with ampicillin (0.1 mg/ml) overnight at 37°C. Bacteria were next inoculated into 1.5 liters of fresh LB + ampicillin and rotated at 250 rpm at 37°C. After 2 h of incubation, 1.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added and cultures were incubated for an additional 2 h. Bacteria were sedimented by centrifugation at 6,000 x g for 8 min and suspended in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2.5 mM sodium phosphate monobasic, pH 7.4), and cells were broken with two passes through a French press at 14,000 lb/in². Bacterial lysates were centrifuged at 30,000 x g for 15 min, and soluble protein in supernatants was passed through a 0.45-μm-pore-size cellulose filter. Filtrates were subjected to affinity chromatography on 2 ml glutathione-Sepharose (GE Healthcare, Piscataway, NJ), and the column was preequilibrated with 20 ml of PBS. Columns were washed with 40 ml PBS, and target proteins were eluted with 2 ml PBS containing 25 mM reduced glutathione.

GST-BslK was dialyzed against 4 liters of PBS for 24 h to remove the glutathione. When necessary, the GST tag was cleaved away from GST-BslK by thrombin digestion according to the manufacturer's instructions (GE Healthcare). Endogenous bound heme was removed from BslK preparations by lowering the pH to 2.0 with HCl and extracting free heme with methyl ethyl ketone (MEK), as described previously (44). Approximately 50% of the recombinant BslK was observed to precipitate following MEK treatment. Protein concentrations were determined by either the bichinchoninic acid method (Pierce, Rockford, IL) or SDS-PAGE (36). All protein preparations were stored at −20°C. This method yields 1 to 2 mg/ml of GST-free apo-BslK, that is greater than 90% homogeneous, as judged by SDS-PAGE.

**Holo forms of BslK.** Holo-BslK were prepared by passing 50 ml of a solution of 10 μM hemin (solubilized in 0.1 M NaOH) over GST-BslK (~2 mg) overnight on glutathione-Sepharose, followed by a wash with 50 ml of PBS, pH 7.4. Bound protein was treated with either thrombin (to release holo-BslK) or 25 mM reduced glutathione [to elute GST-(holo)BslKN], and heme content was measured by the A632/A270 ratio. This procedure results in approximately 8-fold increase in bound heme over that in untreated controls.

**BslK localization.** To create BslK harboring a hexa-histidyl C-terminal tag (BslKH6), DNA encoding full-length bslk was amplified from B. anthracis genome DNA (45) using primers bslKforward (5' GATCGATCTCTAGAGAACTCTTTGCACTTCTCTGTTTCATGGCG 3') and reverse (5' GATCGATCTCTAGAGAACTCTTTGCACTTCTCTGTTTCATGGCG 3'). The underlined and italicized sequences refer to BamHI and EcoRI restriction sites and the hexa-histidyl tag, respectively. DNA encoding pLM5-gst-bslKN (XL1-Blue harboring pLM5-gst-bslKN XL1-Blue) was digested with BamHI and EcoRI and NEATforward (5' GATCGATCGAATTCTTAGTGCCCAAGTTGGTA 3') and NEATreverse (5' GATCGATCGAATTCTTAGTGCCCAAGTTGGTA 3') and ligated into the BamHI/EcoRI restriction sites of pGEX2T to create pgst-bslKH6 and transformed into Escherichia coli BL21 (DE3). The underlined sequence refers to XbaI/KpnI restriction sites and the hexa-histidyl tag, respectively. DNA encoding the NEAT domain (amino acids 46 to 165) of bslK (bslK) was amplified from B. anthracis genome DNA (45) using primers NEATforward (5' GATCGATGGATCTGATGAGGGCGTTCTCTCTC 3') and NEATreverse (5' GATCGATCGATTCTGATGAGGGCGTTCTCTCTC 3'). The underlined sequence refers to BamHI and EcoRI restriction sites, respectively. Following amplification, DNA was ligated into the BamHI/EcoRI restriction sites of pGEX2T to create pgst-bslKH6 and transformed into Escherichia coli XL1-Blue.

**Hemo transfer from BslK to IsdC.** Hemo recipients (apo forms of IsdC, IsdX1, or bovine serum albumin [BSA]) were either purified as described above or commercially purchased (BSA from Sigma). Each recipient (150 μl of a 10 μM solution) was incubated with GST-(holo)BslK (~10 μM) coupled to glutathione-Sepharose for 30 min at 25°C. Reactions were centrifuged to generate sediment (donor) and supernatant (recipient) fractions, the sediment was washed three times with 1 ml PBS, and the donor was eluted with 150 μl of 25 mM reduced glutathione in PBS, pH 7.4. Both fractions were subjected to absorbance spectroscopy at 403 nm to quantitate heme content.

**Spectrometry-based studies.** The kinetics of transfer from holo-BslK to apo-IsdC was measured using an RSM-1000 (OLIS, Bolgart, GA) stopped-flow spectrophotometer (1,000 spectra per second). Briefly, 200 μl of holo-BslK (4 μM) was mixed with 200 μl of apo-IsdC (5 μM) in PBS at 20°C, and the absorbance changes at 410 and 380 nm were obtained (relative to the Amax) from spectra accumulated over 2 s. The dead time of the instrument is ~3 ms, and the light path is 20 nm. The spectral changes at 380 nm are specific for holo-BSlK, while those at 410 nm are specific for IsdC. The rates of heme loss from BslK were determined by incubating purified holo-BslK (~4 μM) with the apo form of a mutant (H64Y/V68F) sperm whale myoglobin (40 μM) heme-scavenging agent (27), and spectral changes at 403 nm relative to the control wavelength (433 nm) were monitored over 4 h at 25°C using an HP8453 spectrophotometer (Agilent, Santa Clara, CA). For the spectral scan demonstrated in Fig. 5C, recombinant GST-free BslK (~4 μM) purified from E. coli was analyzed via a wavelength scan using an HP8453 spectrophotometer.

**Results.** The interaction of holo-BslK with IsdC. The interaction of holo-BslK with apo-IsdC was analyzed by surface plasmon resonance (SPR) spectroscopy using a BiACore 3000 biosensor (GE Healthcare, Sweden) (54, 58). Apo-IsdC in HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl) was immobilized on a maleimide-covalently coupled to a carboxy-methyl (CM) sensor chip at 25°C to 5,650 response units (RU) using amine chemistry as described previously (29, 55). Holo-BslK (0.5 to 10 μM) in HBS was injected at 20 μl/min for 300 s, and dissociation was monitored after HBS injection for another 300 s at 25°C. Affinity constants were estimated by fitting the data to a simple 1:1 binding model (15, 21) and analyzed using dθ/δt = kθ(Rmax − R) − kRδ where R is the SPR signal (in response units), kθ is the association rate constant (in M−1 s−1), kR is the dissociation rate constant (in s−1), C is the concentration of holo-IsdX1 (in M), Rmax is the maximum holo-IsdX1 binding capacity (in response units), and dθ/δt is the rate of change of the SPR signal. The equilibrium binding constant (Kθ) of the complex was determined as the ratio of kθ/kR. All reported constants represent the mean of results from nine separate injections of holo-BslK, varying in concentration from 0.5 to 10 μM. Data were prepared by “double referencing,” where parallel injections of analyte and buffer are flowed over a control dextran surface and immobilized apo-IsdC. Sensograms were fit using BIAevaluation 4.1 software (GE Healthcare).

RESULTS

**BslK localizes to the cell surface.** Reasoning that B. anthracis may contain multiple mechanisms or systems for heme uptake, an *in silico* search of the B. anthracis genome for genes predicted to encode NEAT domain-containing proteins yielded the recently

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described Isd hemophore system (isdC, isdX1, and isdX2) (44, 45), a gene encoding a putative surface protein and virulence factor (BAS0520) (8), and bslK (BAS1021). Interestingly, bslK is also annotated as encoding three SLH domains (Fig. 1) and is conserved in the Bacillus cereus group (B. anthracis, B. cereus, and B. thuringiensis) of Gram-positive bacteria (32). The SLH domain has been demonstrated to mediate protein binding to cell wall structures (23, 38, 39, 43, 53, 62). Examination of the DNA immediately upstream of the transcriptional start site of bslK indicates a sequence (GACATTGATAATCATTATC) that is similar (three mismatches) to the consensus sequence for the binding of the ferric uptake repressor (fur), suggesting that bslK is regulated by fur and expressed under conditions of iron limitation (similar to the Isd proteins) (9, 45, 50). Collectively, this information led to the hypothesis that in B. anthracis and related bacteria, BslK is a surface protein that participates in heme acquisition.

To characterize the localization of BslK, the open reading frame of full-length bslK was fused to DNA encoding a hexahistidyl tag and cloned into pLM5 such that the recombinant gene is under the control of an IPTG-inducible spac promoter (pLM5-bslKH6) (48). B. anthracis harboring pLM5 (vector control) or pLM5-bslKH6 was grown in the presence or absence of IPTG, and cultures were separated into cell-associated (bacterial sediment [SD]) and secreted (culture supernatant [Sec]) fractions. Samples of each fraction were subjected to SDS-PAGE, and BslKH6 was detected by αHis6 Western analysis. As indicated in Fig. 2A, a single immunoreactive species of approximately 37 kDa was observed in the cell-associated fraction of cultures that had been incubated with IPTG. This species was not observed in the absence of IPTG or in the vector (pLM5) control samples, consistent with the observed species being BslK166.

To determine if cell-associated BslK166 is a surface protein, we treated BslK166-expressing cells with guanidine-HCl (GN), a chaotropic agent known to induce the release of noncovalently attached cell envelope proteins such as S-layer polypeptides from the surface of bacteria (42, 63, 70). B. anthracis cells were grown in the presence of IPTG, and bacterial sediments were treated with 4 M guanidine for 5 min. Following centrifugation, guanidine-extractable proteins (ES) and guanidine-resistant sediments (SD) were subjected to αHis Western blotting or proteins were stained with Coomassie blue. The arrowhead in panel C indicates the migration of Sap, a known S-layer protein (20, 32). The results represent one of three independent determinations.
envelope (Fig. 2C) (20, 32). In similar studies, guanidine treatment did not lead to the release of the membrane protein sortase B, indicating that the cell membrane remained intact in these experiments (data not shown). Collectively, these results provide evidence that BslK is noncovalently associated with the cell envelope of B. anthracis, a notion consistent with the presence of its three SLH domains.

BslK binds heme. To determine if BslK is a heme-binding protein, DNA encoding the NEAT domain (amino acids 46 to 165 termed BslKN) was fused to DNA encoding glutathione S-transferase (GST) in the plasmid pGEX2TK to create pgst-bslkN. Following transformation into E. coli, an approximately 42-kDa protein (theoretical mass of GST-BslKN/H11005 40,322 Da) was overexpressed when cells were incubated with IPTG (Fig. 3A). The addition of lysates to glutathione-Sepharose imparted a brown tint to the Sepharose resin, even after extensive washing (Bound), and following elution with glutathione (Elute). (C) SDS-PAGE analysis of eluted proteins (Elute), glutathione-Sepharose after thrombin cleavage of bound GST-BslKN (Sediment), and released BslKN (Soluble). (D) Apo-BslKN (1 μM) was incubated with increasing amounts of hemin (0.1 to 1.6 μM), and data were quantitated after subtraction of the A403 from a hemin-only (control) reference cuvette at each concentration assayed. The graph represents one of three independent determinations.

Western blotting (data not shown), confirming the 42-kDa protein to be GST-BslKN and the 26-kDa protein to be free GST. Thrombin treatment of glutathione-Sepharose-bound GST-BslKN isolated the approximately 18-kDa NEAT NEAT domain of BslK from GST (Fig. 3C, Soluble), whereas GST remained bound to the glutathione-Sepharose (Fig. 3C, Sediment).

After removal of endogenous iron-porphyrin (4), purified apo-BslKN was incubated with increasing amounts of hemin and the spectral absorbance was monitored. As indicated in Fig. 3D, BslKN bound hemin in a dose-dependent and saturable manner with an apparent dissociation constant (Kd) of less than 0.1 M. Taken together, these data suggest that BslK is a heme-binding protein in B. anthracis.

BslKN can transfer heme to IsdC. The finding that BslKN is a surface and heme-binding protein prompted us to examine if holo-BslKN could transfer heme to the cell wall protein IsdC. To test this, we used an assay in which purified heme donors fused to GST are incubated with potential heme recipients and reaction mixtures are separated by GST-affinity chromatography (21). GST-(holo)BslKN, coupled to glutathione-Sepharose, was incubated with equimolar amounts (Ct = 10 μM) of apo proteins 2 to 4 from the procedure shown in panel A, mixtures were separated by centrifugation, and heme content in the sediment and supernatant fractions was assessed by measuring the absorbance at ~403 nm. The mean and standard deviation of three independent experiments are shown. (C) Ten microliters of each sample was subjected to SDS-PAGE, and the resultant gel was stained with Coomassie blue.
quantitating the absorbance of each fraction at 403 nm (Fig. 4A). As demonstrated in Fig. 4B, nearly 60% of the heme originally loaded onto GST-(holo)BslKN was transferred to apo-IsdC. In contrast, less than 10% of the heme was transferred to BSA or apo-IsdX1 (Fig. 4B). The latter result suggests that heme exchange in this experiment is not due to free or nonspecifically bound heme being present in the GST-(holo)BslKN preparations. Heme signals for each fraction were also not due to differences in either the donor or recipient protein amounts (Fig. 4C). Collectively, these results suggest that holo-BslK$_N$ can transfer its heme to apo-IsdC in a directional and specific manner at equilibrium.

**The transfer of heme from holo-BslK$_N$ to apo-IsdC is rapid.** A determination of the mechanism of heme exchange between hemoproteins in bacteria is necessary to understand the biological function of heme uptake during infection. To gain insights into the mechanism of heme transfer from BslK$_N$ to IsdC, we measured the rates of heme dissociation from holo-BslK$_N$ and transfer to apo-IsdC. Because the absorbance in the Soret region (~400 nm) acts as a diagnostic “fingerprint” for the individual hemoproteins, transfer kinetics can be determined by measuring spectral changes in this region over time. The loss of heme from holo-BslK$_N$ was measured after incubation with the heme-scavenging agent, apo-myoglobin (apo-Mb) (21). As indicated in Fig. 5A and quantitated in Fig. 6A, no spectral changes were observed in 24 h, indicating that holo-BslK$_N$ retained its heme over the course of the experiment, a result that suggests that heme dissociation is very slow ($\leq 10^{-5}$ s$^{-1}$) (27). However, incubation of holo-BslK$_N$ with apo-IsdC resulted in pronounced spectral changes compared to the result with a holo-BslK$_N$-only control. A decrease (loss of heme) in the BslK$_N$-specific absorbance peak at $A_{380}$ and an increase (gain of heme) in the IsdC-specific peak at $A_{410}$ were observed (Fig. 5B). As measured by stopped-flow spectrophotometry, the time courses for these changes were rapid (<1 s) with pseudo-first-order rate constants of $-6$ s$^{-1}$ at both wavelengths (Fig. 6B). These data indicate that the transfer of heme from BslK to IsdC is much faster ($>10^5$ s) than the thermal dissociation of heme from BslK into solution. The “broad” shoulder observed from 350 to 380 nm is characteristic of holo-BslK$_N$ preparations and is also observed for recombinant BslK$_N$ purified directly from *E. coli* (Fig. 5C). The molecular basis of these spectra is not understood.

**Heme transfer from BslK$_N$ to IsdC is mediated by protein-protein interactions.** The rapid kinetics of heme transfer from BslK$_N$ to IsdC suggests that this process may be facilitated by a protein-protein interaction mechanism. To test this hypothesis, we used surface plasmon resonance (SPR) spectroscopy, a sensitive technique that allows for the determination of the kinetics, specificity, and strength of molecular interactions in real time (54, 58). Apo-IsdC was covalently coupled to a carboxy-methyl (CM) chip. Following infusion of increasing amounts of holo-BslK$_N$, a dose-dependent increase in response units was observed (Fig. 7). Fitting the data to a 1:1 binding interaction allowed for the determination of the $K_a$ (1.53 M$^{-1}$), $K_d$ (336 M$^{-1}$ s$^{-1}$), and $K_d$ ($5.14 \times 10^{-4}$ s$^{-1}$). No response was observed when holo-BslK$_N$ was infused over apo-IsdX1 or when BslK$_N$ lacked heme (data not shown), consistent with the lack of transfer to IsdX1 observed in Fig. 4. Collectively, these data indicate that holo-BslK$_N$ associates with apo-IsdC with low-micromolar affinity and suggest that *B. anthracis* contains multiple, diverse proteins for heme transfer through its envelope structure.

**DISCUSSION**

Targeting iron uptake systems to prevent the establishment of bacterial infections represents a promising direction in the development of novel antibacterial agents (47, 68). The generation of effective therapeutics with *in vivo* efficacy will require basic knowledge of the mechanism of action of iron acquisition systems on a molecular and cellular level. Central to this concept is an understanding of the mode of iron transport through bacterial surface structures that differ in their structure and composition.

The metallo-porphyrin heme is an abundant source of iron...
for bacterial pathogens, as this molecule constitutes up to 80% of circulating iron inside mammalian hosts. As an indirect form of iron "nutritional immunity," host heme is tightly bound by the oxygen carrier protein hemoglobin, which itself is sequestered inside erythrocytes (14, 28, 74, 85). To access and utilize host hemoglobin as an iron source, three general processes must occur. First, erythrocytes must be lysed by specific bacterial lipases, thereby releasing hemoglobin into plasma. Second, heme must be extracted from circulating hemoglobin and delivered to the bacterial cell envelope. Finally, heme must be transported through the envelope into the cell for iron-porphyrin degradation.

Several bacterial pathogens also produce an additional surface structure called the S-layer (6, 7, 18, 19, 56, 75). This raises questions concerning whether S-layers, in addition to their proposed structural and protective roles, also function in the transport of small molecules, including heme-iron. Biochemical and microscopic evidence indicates that \textit{B. anthracis} is capable of forming an S-layer that is principally composed of two proteins: EA1 (extractable antigen 1) and Sap (surface array protein) (5, 20, 53). A common feature of EA1 and Sap is that they each harbor three SLH domains, a conserved structural module that may mediate both the intramolecular assembly of S-layer proteins and intermolecular association with carbohydrates on the cell wall (23, 38, 39, 43, 53, 62). Indeed, the genome of \textit{B. anthracis} harbors 22 SLH proteins, including BslK (32). The finding that BslK also contains a NEAT domain prompted an investigation into whether this potential surface protein partakes in heme binding and transfer. Treatment of \textit{B. anthracis} cells expressing a His-tagged form of BslK with guanidine led to the release of BslK H6 into the supernatant, a finding consistent with the extraction of noncovalently associated S-layer proteins from the bacterial surface as reported by others (32, 42, 53, 63, 70). Immunofluorescence microscopy using Texas Red-conjugated anti-hexahistidyl antibodies also indicated that BslKH6 localized to the bacterial surface (Y. Tarlovsky and A. W. Maresso, unpublished observations). Taken together, these data suggest that BslK may function in heme trafficking at the cell envelope.

An attempt to purify BslK that lacks the signal peptide but contains the SLH and NEAT domains was compromised by a high degree of insolubility of the recombinant protein (Tarlovsky and Maresso, unpublished observations). Fusion of the NEAT domain of BslK (BslKN) to GST allowed for the purification of a soluble protein with bound iron-porphyrin, suggesting that BslK is a heme-binding protein. Indeed, GST-free BslKN binds pure hemin with an estimated dissociation constant that is lower than 10^{-7} M, consistent with the finding that no appreciable loss of heme from holo-BslK N is observed, compared to holo-IsdX1 (21), following incubation with apo-Mb (Fig. 6A). Since the \Delta A_{403} begins to saturate at hemin concentrations between 0.5 and 1.0 \mu M, it appears that interaction of BslKN (1 \mu M) with hemin follows a 1:1 binding stoichiometry.

Work over the past 5 years has provided insights into how NEAT domain proteins modulate the movement of heme from extracellular sources such as hemoglobin to proteins localized on the bacterial envelope. In \textit{Staphylococcus aureus}, the Isd system provides for coordinated heme import with proteins
covalently anchored to the cell wall (50, 80). IsdH and IsdB likely function as hemoglobin receptors and partake in heme extraction from hemoglobin (16, 17, 65, 81). Heme is next passed to IsdA and IsdC, two additional cell wall-anchored proteins, which subsequently transfer the heme to the membrane protein IsdE (26, 41, 50, 57, 66, 67, 80, 82, 86). Finally, IsdE likely passes heme to the monooxygenase IsdG, where enzymatic degradation of the heme yields free iron and biliverdin (72, 73).

However, in *B. anthracis*, heme acquisition from hemoglobin seems to be mediated by the action of two secreted hemophores, the NEAT proteins IsdX1 and IsdX2 (45), which transfer their bound heme directly and rapidly to IsdC (21), a cell wall-anchored protein (24a, 44). Since IsdC has been proposed to be the central conduit for NEAT-mediated heme trafficking in Gram-positive bacteria (41, 58, 71, 86), we hypothesized that BslK may transfer its bound heme directly to apo-IsdC. Indeed, holo-BslK directionally and rapidly transferred heme to apo-IsdC and with kinetics similar to those observed for IsdX1/IsdX2 → IsdC transfers (21). Further, SPR data indicate that holo-BslK associates with apo-IsdC, a finding also observed for holo-IsdX1/apo-IsdC mixtures. The low rate of heme loss from several characterized NEAT proteins, combined with the growing evidence of NEAT-NEAT interactions observed for heme donor/recipient pairs, suggests that heme transfer between NEAT proteins is mediated by protein-protein interactions in Gram-positive bacteria (21, 41, 57, 61, 86). The finding that *B. anthracis* contains a noncovalently associated surface protein with heme-binding properties adds to the diverse mechanisms of iron acquisition in *B. anthracis*, which includes iron scavenging by siderophores (1, 10, 24, 35, 37, 64).

The characterization of BslK generates interesting questions concerning the dynamic interplay of multiple iron-uptake proteins or systems during infection with *B. anthracis*. For example, the notion that BslK and IsdC form a heme-relay pair indicates that heme can be shuttled to the Isd system by NEAT proteins whose corresponding genes lie outside the Isd locus. This property may confer two functionally distinct advantages for *B. anthracis* and related Gram-positive bacteria during infection. First, it potentially allows for the differential regulation of NEAT protein expression at the transcriptional level, thereby modulating the amount or timing of NEAT production in response to changes in host iron or heme availability. Second, the presence of multiple NEAT proteins may confer a type of “heme permissiveness” that ensures heme uptake in different tissue niches (a change in the host environment) or in response to changes in the topology of the bacterial envelope.

For example, BslK, by virtue of its SLH domains, may localize to S-layer structures and thereby provide for heme acquisition at a stage during infection when a functional S-layer is present (11, 52). These concepts imply that heme uptake in *B. anthracis* is more complicated than the simple diffusion of released hemoglobin, or heme-bound IsdX1 and/or IsdX2, to the cell wall. *B. anthracis* seems to have evolved several mechanisms to ensure that the integrity of heme uptake is maintained despite changes in the host environment and/or the bacterial surface that would otherwise hinder access and transport of limiting nutrients that are required for constant growth and replication of the bacterial cell. Finally, heme transfer between multiple NEAT proteins in vivo will also be influenced by the physical location of each hemoprotein in the cell wall and may be modulated by unknown factors. A test of these biological concepts will require a thorough phenotypic analysis of NEAT mutant strains for their properties of heme extraction, uptake, and utilization in both cell culture and animal models of disease. These studies are currently ongoing in the laboratory.

The findings of this report require an expansion of the model of heme acquisition in *B. anthracis* (Fig. 8) (21, 44, 45). Under conditions of iron starvation, *B. anthracis* secretes the hemophores IsdX1 and IsdX2, which scavenge heme from host hemoglobin. The heme-bound hemophores form an active complex with IsdC that facilitates heme transfer to the cell envelope. Alternatively, *B. anthracis* may utilize BslK, which is noncovalently bound to the external surface of the cell wall via its SLH domains (22, 51), for heme transfer in a rapid manner to IsdC via protein-protein interactions. Holo-IsdC next passes the iron-porphyrin to membrane transporters for delivery into the cell cytoplasm. Iron is then liberated via oxidative degradation of the heme by IsdG (72). Our results provide direct evidence that non-Isd NEAT proteins such as BslK may funnel heme into the Isd system, thereby supporting a model recently proposed by Daou and coworkers for the NEAT protein IlsA (13). Future studies will elucidate the source of heme for BslK and the functional interplay among multiple NEAT proteins at the cell envelope of *B. anthracis* and will provide mechanistic insights into the processes of heme extraction, transport, and utilization in Gram-positive pathogenic bacteria.

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