Differential Function of Lip Residues in the Mechanism and Biology of an Anthrax Hemophore

MarCia T. Ekworomadu1,*, Catherine B. Poor2,*, Cedric P. Owens3, Miriam A. Balderas1, Marian Fabian4, John S. Olson4, Frank Murphy5, Erol Balkabasi1, Erin S. Honsa1, Chuan He2, Celia W. Goulding3, Anthony W. Maresso1*

1 Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, United States of America, 2 Department of Chemistry, University of Chicago, Chicago, Illinois, United States of America, 3 Departments of Molecular Biology and Biochemistry, University of California-Irvine, Irvine, California, United States of America, 4 Department of Biochemistry and Cell Biology, Rice University, Houston, Texas, United States of America, 5 Northeastern Collaborative Access Team, Argonne National Laboratory, Argonne, Illinois, United States of America

Abstract

To replicate in mammalian hosts, bacterial pathogens must acquire iron. The majority of iron is coordinated to the protoporphyrin ring of heme, which is further bound to hemoglobin. Pathogenic bacteria utilize secreted hemophores to acquire heme from heme sources such as hemoglobin. Bacillus anthracis, the causative agent of anthrax disease, secretes two hemophores, IsdX1 and IsdX2, to acquire heme from host hemoglobin and enhance bacterial replication in iron-starved environments. Both proteins contain NEAr-iron Transporter (NEAT) domains, a conserved protein module that functions in heme acquisition in Gram-positive pathogens. Here, we report the structure of IsdX1, the first of a Gram-positive hemophore, with and without bound heme. Overall, IsdX1 forms an immunoglobulin-like fold that contains, similar to other NEAT proteins, a 3_10-helix near the heme-binding site. Because the mechanistic function of this helix in NEAT proteins is not yet defined, we focused on the contribution of this region to hemophore and NEAT protein activity, both biochemically and biologically in cultured cells. Site-directed mutagenesis of amino acids in and adjacent to the helix identified residues important for heme and hemoglobin association, with some mutations affecting both properties and other mutations affecting only heme stabilization. IsdX1 with mutations that reduced the ability to associate with hemoglobin and bind heme failed to restore the growth of a hemophore-deficient strain of B. anthracis on hemoglobin as the sole iron source. These data indicate that not only is the 3_10-helix important for NEAT protein biology, but also that the processes of hemoglobin and heme binding can be both separate as well as coupled, the latter function being necessary for maximal heme-scavenging activity. These studies enhance our understanding of NEAT domain and hemophore function and set the stage for structure-based inhibitor design to block NEAT domain interaction with upstream ligands.

Introduction

An important determinant in the outcome of a bacterial infection is how well the invading pathogen can acquire host iron. Hosts with high levels of free iron are more susceptible to infection, and deletion of iron acquisition systems in a wide range of bacterial species generally attenuates virulence [1]. The low free iron concentration in host tissues (10^{-18}–24 M) likely acts as a barrier to efficient bacterial replication [2]. However, pathogenic bacteria have evolved at least two distinct uptake systems to attain iron. One such mechanism is to secrete siderophores, small molecules that chelate ferric iron with very high affinity [3]. The iron-bound siderophore binds to the bacterial surface and specific ferric iron receptors next deliver the iron or iron-siderophore complex into the cell [4]. The genetic deletion of biosynthetic systems that make siderophores, or the surface receptors that recognize siderophores, decreases the virulence of several pathogens, including the Gram-positive bacteria B. anthracis [5] and S. aureus [6].

The second system bacteria employ to attain host iron targets iron-protoporphyrin IX, or heme. Although heme constitutes up to 80% of the bodily iron reserves, free heme is rare. Most heme is tightly bound to hemoproteins such as hemoglobin. Hemoglobin’s important role as an oxygen carrier protein means it is in high abundance and thus a target for bacterial iron uptake [7–9]. Bacterial proteins that acquire heme from hemoglobin are called hemophores [10–13]. Hemophores are generally secreted into the external milieu where they extract heme, via an undefined mechanism, from heme sources such as hemoglobin [14]. The heme-bound (holo) hemophore then delivers its bound iron-protoporphyrin to a cognate receptor on the bacterial surface, which leads to heme import into the bacterial cell [15,16]. Heme from
Mechanism of a NEAT Hemophore

Author Summary

Pathogenic bacteria need to acquire host iron to replicate during infection. Approximately 80% of mammalian iron is associated with a small molecule termed heme, most of which is bound to circulating hemoglobin and involved in O2 transport in red cells. Bacteria secrete proteins, termed hemophores, to acquire the heme from hemoglobin, a process thought to accelerate delivery of the heme to the bacterial surface for iron import into the cell. The mechanisms by which hemophores extract host heme from hemoglobin are not known. Here, we report that the IsdX1 hemophore from B. anthracis, the causative agent of anthrax disease, uses a conserved structural feature to link hemoglobin association with heme binding and extraction, thereby facilitating bacterial growth in low-iron environments. Such “molecular coupling” suggests that specific inhibition of the hemophore-hemoglobin interaction for this class of proteins may serve as a starting point for new anti-infective therapeutics aimed at short-circuiting iron uptake networks in bacterial pathogens.

hemoglobin can also be attained at the bacterial surface through similar mechanisms involving receptors on the cell wall (Gram positive) or outer membrane (Gram negative). Heme import by bacterial pathogens is important for the establishment or maintenance of infections caused by Bordetella [17], Haemophilus [18], Brucella [19], Vibrio [20], Streptococcus [21], and Staphylococcus [22] species. Further, more recent studies suggest heme is a major determinant in the promotion and severity of bacterial sepsis [23]. Collectively, these studies highlight the important role of heme acquisition during infection of mammalian hosts and support the contention that the inhibition of iron uptake systems is a promising direction for the development of new therapeutics. However, despite this knowledge, no clinically-used antibiotics have been developed for new anti-infective therapeutics aimed at short-circuiting iron uptake networks in bacterial pathogens.

To gain an appreciation of the residues in IsdX1 necessary for the hemophore activity of this protein, we solved the crystal structure of apo-IsdX1 to 1.8 Å resolution (Table 1). Overall, the backbone structure of IsdX1 is similar to the solved structures of NEAT domains from S. aureus, albeit with a unique surface charge distribution close to the heme binding pocket (Figure S1.) [40–44]. The structure consists of an immunoglobulin-like fold with eight β-strands arranged in two antiparallel β-sheets of a β-sandwich (Figure 1A).

The heme-binding pocket is enclosed primarily by a 310-helix (residues Arg-54 to Tyr-58) on one side of the heme and a long β-hairpin (β7–β8) on the other. The 310-helix is sometimes referred to as the “lip” because it seems to protrude over the heme-binding pocket [42]. The backbone of the 310-helix in IsdX1 is fairly well-ordered even in the absence of heme. The integrity of the 310-helix without heme could be due to the hydrogen bonding network between Ser-52, Ser-53, Arg-54 and Asn-56 and Met-55 from the helix making van der Waals contacts with residues in β4, β7, and β8.

The heme bound form of IsdX1 was solved to 2.15 Å by molecular replacement using the structure of apo-IsdX1 as the search model. Heme-iron coordination is achieved through Tyr-136, which is conserved among all heme-binding NEAT domain proteins (Figure S2, upper panel). The distance between the iron atom and the tyrosine oxygen ligand is 2.3 Å, which is typical for an Fe-O bond of NEAT domain proteins [40–45]. The coordination bond to iron is stabilized by the conserved residue Tyr-140, which forms a hydrogen bond with the phenolate oxygen of Tyr-136. The aromatic ring of Tyr-140 further stabilizes the pyrrole ring through π-stacking. Both of these residues lie on a β-hairpin region, a conserved region in bacterial NEAT proteins that provides a structural platform for the heme [40,42]. The least solvent exposed heme propionate forms a hydrogen bond with the hydroxyl group of Ser-53, as well as the backbone nitrogen of Arg-54. Additionally, the side chain NH1 group of Arg-54 from chain A also hydrogen bonds to the least solvent exposed heme propionate, and also to the hydroxyl group of Ser-53 (Figure 2A, B). However, this hydrogen-bonding network is not observed for the side chain of Arg-54 from chain B. The heme molecule is further stabilized by Tyr-58, which weakly π-stacks with the buried heme pyrrole ring. The other residues lining the heme-binding pocket are Arg-54, Met-55, Phe-59, Ile-84, Val-127, Ile-129, Ile-131, Ile-142 and Phe-144, which mainly form an aliphatic
environment to accommodate the hydrophobic regions of the heme and its side chains.

The role of the 3\textsubscript{10}-helix in heme binding

The location of the 3\textsubscript{10}-helix in the structures of the NEAT domains suggests this region is important for NEAT protein function, including heme binding, hemoglobin association, heme extraction and NEAT-NEAT heme transfer [41–43,45]. There is some conservation in this region, as noted by Pilpa et al, with aromatic residues common in the equivalent positions of amino acids 54 and 58 for IsdX1 [46]. Interestingly, a serine residue (Ser-53 in IsdX1), which is immediately adjacent to the first residue of the helix (Arg-54), is well conserved in these NEATs, including every NEAT domain from \textit{B. anthracis} (Figure 3, bold) [47]. To determine the role of this and adjacent residues in the ability of IsdX1 to bind heme, each amino acid in 52-SSRM-55 was changed to alanine and mutant proteins purified from \textit{E. coli}.

Whereas wild-type IsdX1 co-purified with a significant amount of endogenous heme from \textit{E. coli}, IsdX1(S5R-AAAA) bound approximately 10-fold less heme after purification (Figure 4A, quantitated in 4\textsuperscript{C}). Removal of the bound heme by organic extraction (Figure 4B) and quantitation of the Soret band intensity after titration of the apo protein with hemin confirmed the heme-binding defect of the mutant protein (Figure 4D).

To determine the residues responsible for this defect, single substitution changes in 52-SSRM-55 were generated and each purified mutant assessed for heme binding activity. As demonstrated in Figure 4C and D, mutation of Ser-52, Ser-53, or Met-55 decreased the ability of each of these mutant proteins to either co-purify with heme (Figure 4C) or bind exogenously added hemin (Figure 4D). The raw spectra for these mutants are illustrated in Figure S3. These effects are not due to gross disruption of IsdX1 secondary structure since the mutant proteins retained a similar overall \textbeta-sheet content as the wild-type protein when assessed by far-UV circular dichroism (Figure S4). Further, the heme binding site seems to be somewhat intolerant of even small structural

---

Table 1. Crystallography statistics.

<table>
<thead>
<tr>
<th>Data Collection, Phasing, and Refinement Statistics</th>
<th>Apo</th>
<th>SeMet</th>
<th>Holo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection/parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
<td>P1</td>
<td>P4 3</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97929</td>
<td>0.97940</td>
<td>1.0</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>36.6, 43.7, 47.4</td>
<td>37.0, 43.9, 47.7</td>
<td>64.98×64.98×74.2</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>46.1-1.80</td>
<td>34.5-2.11</td>
<td>50.0-2.15</td>
</tr>
<tr>
<td>Completeness (%)*</td>
<td>97.2 (94.4)</td>
<td>97.0 (96.3)</td>
<td>99.1 (100.0)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>24540 (2336)</td>
<td>24211 (250118)</td>
<td>16738 (251543)</td>
</tr>
<tr>
<td>R\textsubscript{merge} (%)*</td>
<td>4.5 (35.9)</td>
<td>7.4 (55.1)</td>
<td>4.4 (33.1)</td>
</tr>
<tr>
<td>I/σ*</td>
<td>18.1 (1.7)</td>
<td>10.9 (2.3)</td>
<td>43.79 (7.23)</td>
</tr>
<tr>
<td>Redundancy*</td>
<td>2.0 (1.9)</td>
<td>3.9 (3.6)</td>
<td>15.0 (15.1)</td>
</tr>
<tr>
<td><strong>SAD Phasing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>34.5-2.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenium sites (#)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure of merit (before/after density modification)</td>
<td>0.32/0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>25.57-1.80</td>
<td>32.49-2.15</td>
<td></td>
</tr>
<tr>
<td>R\textsubscript{work}/R\textsubscript{free} (%)</td>
<td>20.9/23.4</td>
<td>19.6/23.4</td>
<td></td>
</tr>
<tr>
<td># of protein atoms</td>
<td>1907</td>
<td></td>
<td>1948</td>
</tr>
<tr>
<td># of water molecules</td>
<td>168</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td>hemes/monomer</td>
<td>0</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>R\textsubscript{msd} bond lengths (Å)</td>
<td>0.002</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>R\textsubscript{msd} bond angles (°)</td>
<td>0.566</td>
<td></td>
<td>1.192</td>
</tr>
<tr>
<td>PDB ID</td>
<td>3SZ6</td>
<td></td>
<td>3SIK</td>
</tr>
<tr>
<td><strong>Ramachandran plot statistics (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preferred regions</td>
<td>210 (95.0)</td>
<td>238 (98.8)</td>
<td></td>
</tr>
<tr>
<td>Allowed regions</td>
<td>11 (5.0)</td>
<td>3 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Outliers</td>
<td>0 (0)</td>
<td></td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Values for the highest resolution shell are shown in parentheses.

\[ R\text{merge} = \frac{\sum |I_{hlk} - \langle |I_{hlk} | \rangle|}{\sum |I_{hlk}|} \] where \( I \) is the observed intensity for reflection hkl, and \( \langle |I| \rangle \) is the mean intensity.

\[ R\text{work} = \frac{\sum |F_{o}(hkl)| - |F_{c}(hkl)|}{\sum |F_{o}(hkl)|} \] and \( R\text{free} \) is calculated in the same way with 5–10% of reflections excluded from refinement.

doi:10.1371/journal.ppat.1002559.t001
Figure 1. Apo and holo IsdX1 crystal structures. (A) Ribbon representation of apo-IsdX1 where β-strands and helices are colored pink and cyan, respectively. Important heme-binding residues are represented in stick model with carbon, sulfur, nitrogen, and oxygen atoms colored white, yellow, blue and red, respectively. (B) Cartoon representation of holo-IsdX1 where β-strands and helices are colored gold and blue. Heme (purple) is depicted in stick model with carbon (purple), nitrogen (blue), oxygen (red) and Fe (orange sphere). (C) Heme-binding pocket with Fo−Fc omit map contoured at 3.0 σ (dark blue mesh). Hydrogen bonds are indicated by black dashed lines.

doi:10.1371/journal.ppat.1002559.g001
changes, since substitution of Ser-53 to a threonine, which differs only in the length of the side chain (extra methyl group), also abolished the interaction with heme (Figure 4 C,D). Interestingly, mutation of Arg-54 led to a purified IsdX1 preparation with a Soret band approximately three times greater than wild-type protein (Figure 4C). The high heme content in this sample was confirmed using the pyridine hemochrome method, which demonstrated the molar amounts of heme, on average, were 75–90% the molar concentration of protein, suggesting binding was stoichiometric for this preparation (data not shown). However, upon removal of the heme and incubation of R54A with hemin, very little heme bound the protein, despite a far-UV spectrum indistinguishable from wild-type protein (Figure 4D, Figure S4). In the structure of apo and holo-IsdX1, the side chain of Arg-54 shifts 2.5–2.8 Å to accommodate the heme (Figure 2B). This suggests placement of a less bulky residue (alanine) in place of Arg-54 may allow heme access to the heme-binding site, but potentially only during translation of the protein when partially unfolded. Regardless of the exact reason for this, these results demonstrate residues in and around the 310-helix are involved in the binding of heme to IsdX1.

Figure 2. Superimposition of apo-IsdX1 and holo-IsdX1. (A) Ribbon representation of superimposition of apo-IsdX1 (pink) and holo-IsdX1 (grey). (B) Heme-binding pocket with stick representation of heme (carbon, blue and Fe, orange sphere), apo-IsdX1 residues (carbon, pink) and holo-IsdX1 residues (carbon, grey), and sulfur (yellow), nitrogen (blue) and oxygen (red). Arg-54 in the holo structure had two conformations and the alternate conformation has cyan carbon atoms.

doi:10.1371/journal.ppat.1002559.g002
In an attempt to provide a more quantitative assessment of the importance of each residue in the 310-helix to the stabilization of bound heme, we measured the rates of heme dissociation from wild-type and mutant IsdX1 proteins. Each protein was purified from *E. coli* as described in the Materials and Methods, reconstituted with hemin, and holo-protein purified away from unbound hemin by gel filtration chromatography. The rate of heme dissociation was then assessed by mixing holo-IsdX1 preparations with excess H64Y/V68Y apo-myoglobin (Mb), a mutant globin with a high heme affinity (Kd, 10⁻¹² M) and very low rate of heme dissociation [30,48,49]. The dissociation rate constant of heme loss from IsdX1 can be determined by measuring the spectral changes that occur with time as released heme is scavenged passively by the apo-Mb reagent.

As observed in Figure 5, IsdX1 containing mutations in Ser-53, Arg-54, and Met-55 all lose heme significantly faster than wild-type IsdX1, with S53A showing rates of heme loss that were greater than 400 times faster than the wild-type IsdX1 (see Table 2 for rates). Interestingly, S52A showed comparable rates of heme dissociation to that of wild-type, despite its apparent poor heme binding ability at equilibrium (Figure 4D). The best explanation for this is that while its rate of heme loss may be unaffected, its rate of heme association in the absence of hemoglobin may be poor. Taken together, the data are consistent with Ser-53 and Arg-54 of the 310-helix playing a substantial role in stabilizing the bound heme in IsdX1.

The role of the 310-helix in hemoglobin association

The position of 52-SSRM-55 extending over the heme-binding site implies these residues may also be involved in the direct interaction with hemoglobin. To test this hypothesis, the association of each mutant with holo-hemoglobin was investigated by surface plasmon resonance spectroscopy. Apo forms of wild-type or mutant IsdX1 proteins, which were purified from *E. coli* as described in the Materials and Methods, were reconstituted with hemin, and protein purified away from unbound hemin by gel filtration chromatography. The rate of heme dissociation was then assessed by mixing holo-IsdX1 preparations with excess H64Y/V68Y apo-myoglobin (Mb), a mutant globin with a high heme affinity (Kd, 10⁻¹² M) and very low rate of heme dissociation [30,48,49]. The dissociation rate constant of heme loss from IsdX1 can be determined by measuring the spectral changes that occur with time as released heme is scavenged passively by the apo-Mb reagent.

As observed in Figure 5, IsdX1 containing mutations in Ser-53, Arg-54, and Met-55 all lose heme significantly faster than wild-type IsdX1, with S53A showing rates of heme loss that were greater than 400 times faster than the wild-type IsdX1 (see Table 2 for rates). Interestingly, S52A showed comparable rates of heme dissociation to that of wild-type, despite its apparent poor heme binding ability at equilibrium (Figure 4D). The best explanation for this is that while its rate of heme loss may be unaffected, its rate of heme association in the absence of hemoglobin may be poor. Taken together, the data are consistent with Ser-53 and Arg-54 of the 310-helix playing a substantial role in stabilizing the bound heme in IsdX1.

The role of the 310-helix in hemophore biology

IsdX1 and IsdX2 promote the growth of *B. anthracis* on hemoglobin as the sole source of iron [26]. To determine the functional contribution of the 52-SSRM-55 helix towards heme scavenging activity, we tested the ability of wild-type and mutant proteins to rescue a hemophore-dependent growth defect of a *B. anthracis* strain (ΔIsdX1, ΔIsdX2) lacking both hemophores grown in iron-deficient media with or without hemoglobin. Although little
growth is observed in the absence of hemophore, the addition of wild-type IsdX1 to cultures with hemoglobin led to a 3 to 8-fold increase in growth (Figure 7, compare 7 to 8, from 4 to 8 hrs). This enhancement of growth is not due to heme or iron contamination of the protein preparation since only a marginal increase in growth was observed in the absence of hemoglobin (Figure 7, compare 8 to 1,2). Interestingly, whereas ΔisdX1, ΔisdX2 B. anthracis supplemented with S52A or M55A IsdX1 provided intermediate growth (Figure 7, compare 9,12 to 3,6 at 8 hrs), the level of replication in the S53A and R54A IsdX1 supplemented cultures was similar to the S53A/R54A-only controls, suggesting these proteins are unable to rescue a ΔisdX1, ΔisdX2-dependent growth defect on hemoglobin as the sole iron source (Figure 7, compare 10,11 to 4,5 at 8 hrs). Taken together, these results indicate Ser-53 and Arg-54-mediated association of IsdX1 with hemoglobin is important for heme scavenging in iron-limiting environments and provides the first experimental demonstration of the biologic function of this dynamic region in NEAT proteins in growing cells.

Figure 4. Functional role of the 310-helix and adjacent residues: heme binding. (A) Ser-52, Ser-53, Arg-54, and Met-55 of IsdX1, designated SSRM, were each substituted to alanine and recombinant protein purified from E. coli as described in the Experimental Procedures. The absorbance properties immediately after purification from E. coli of wild-type (black) and SSRM (grey) IsdX1 were analyzed from 260–560 nm. (B) Recombinant IsdX1 was treated with low pH to remove co-purifying heme and the absorbance (250–500 nm) compared to the same preparation that was not acid treated. (C, D) Wild-type IsdX1, IsdX1-SSRM, or IsdX1 harboring mutations in Ser-52, Ser-53, Arg-54, or Met-55 were purified from E. coli and the heme content assessed by determining the ratio of the heme (399 nm) to protein (280 nm) absorbance (referred to as “bound heme”). In (C), the relative amount of associated heme is recorded following the purification of each IsdX1 variant from E. coli. In (D), all endogenous heme was removed from the preparations as described in (B) and apo-proteins incubated with 5 μM heme for 10 minutes at 25°C, followed by absorbance measurements. The absorbance value of a heme-only control (5 μM) was subtracted from all IsdX1 plus heme reaction readings. The values in (C) and (D) represent the mean and standard deviation of three independent experiments. The asterisk (*) means the differences were significant (p<0.05).

doi:10.1371/journal.ppat.1002559.g004
Here, we report (i) the crystal structure of the apo and holo forms of a Gram-positive hemophore, (ii) the structure of a non-staphylococcal NEAT protein, (iii) that residues in and adjacent to the $\beta_10$-helix contribute to the binding of the heme-iron in this hemophore, (iv) that the processes of heme binding and hemoglobin association can be delineated, and (v) that both optimal heme and hemoglobin binding are necessary for full hemophore activity for growing bacilli. Thus, these studies extend our knowledge of the molecular mechanism of NEAT protein function and provide evidence that abolishing a functional interface between the NEAT domain and hemoglobin can slow bacterial growth in iron-limiting environments.

Research into bacteria hemophores is a growing field, and several hemophores have been discovered [10,16,50,51]. The most well-documented secreted hemophore is that of HasA from the Gram-negative pathogen \textit{S. marscescens} [52]. Although functionally similar, it is likely that IsdX1 and HasA resulted from convergent evolution, as there is little structural similarity between the two proteins. Whereas HasA obtains heme from hemoglobin through a passive mechanism that does not seem to require a physical interaction, IsdX1 binds hemoglobin directly, an event that likely facilitates heme transfer [15,24,26]. Both proteins transfer the bound heme to their cognate cell surface receptors (IsdC for IsdX1 and HasR for HasA) through direct engagement. However, the IsdX1-IsdC interaction is dependent on the hemophore being heme loaded and is transient [30].

**Table 2.** Rate constants for heme dissociation from IsdX1 variants.

<table>
<thead>
<tr>
<th>IsdX1 variant</th>
<th>Rate constant (min$^{-1}$)</th>
<th>Halftime (min)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$k = 0.0034$</td>
<td>190</td>
</tr>
<tr>
<td>S52A</td>
<td>$k = 0.0034$</td>
<td>190</td>
</tr>
<tr>
<td>S53A$^b$</td>
<td>$k_1 = 1.4 \text{ (70%), } k_2 = 0.03 \text{ (30%)}$</td>
<td>1</td>
</tr>
<tr>
<td>R54A</td>
<td>$k_1 = 0.042 \text{ (30%), } k_2 = 0.0035 \text{ (70%)}$</td>
<td>110</td>
</tr>
<tr>
<td>M55A</td>
<td>$k_1 = 0.077 \text{ (20%), } k_2 = 0.01 \text{ (80%)}$</td>
<td>47</td>
</tr>
</tbody>
</table>

*$^a$The halftime is defined as the amount of minutes for one-half of the heme to dissociate from IsdX1.

$^b$For S53A and R54A, the dissociation curves are best described by two phases, each with a single rate constant. The percentages indicate the proportion of the total population giving that particular rate.

doi:10.1371/journal.ppat.1002559.t002
contrasts with the HasA-HasR interaction, where both the apo and holo forms of HasA bind with similar affinities and to the same site on HasR [15,53]. More recently, several secreted hemophores have been reported. HmuY, from P. gingivalis, binds heme and may deliver its heme to the surface receptor HmuR [54,55]. Also in P. gingivalis, the recently described HusA is a heme-binding protein that is needed for growth under conditions of heme limitation [12]. A putative secreted hemophore from Mycobacterium tuberculosis has been characterized with a proposed heme-binding site consisting of one Tyr and two His, which has a similar heme-binding structural motif to that of HasA [56]. However, the overall fold of the Mt hemophore is structurally diverse in comparison with both HasA and IsdX1 folds, and it was postulated that the Mt hemophore may also be a product of convergent evolution [56].

Recent work has shed new insights into how Gram-positive bacteria acquire heme from mammalian hosts [57,58]. The central structural unit is the NEAT domain, a protein module that mediates heme acquisition and import at the bacterial surface [26,29,31,41,57–61]. If NEAT proteins are to be targets for the development of anti-infectives, the molecular determinants of their mechanism of action need to be defined. In this context, the study of IsdX1 is particularly appropriate because this protein contains the only known NEAT domain that contains all the activities associated with hemophore activity (heme and hemoglobin association, heme extraction from hemoglobin, and heme transfer to receptors) [26]. Thus, the study of IsdX1 allows for insights into the relationship between heme coordination, hemoglobin binding, and heme extraction from mammalian globins.

To gain insights into these activities, we solved the 3-dimensional structure of apo and holo IsdX1. The overall structure is similar to the structures of the staphylococcal NEAT domains of IsdH (NEAT 1/3) [40,44], IsdA [41], IsdC [42,43],
and IsdB (NEAT 2) [62], with three common features: (i) an immunoglobulin-like fold arranged into eight $\beta$-sheets, (ii) a small 4–5 residue 3 10-helix extending over the heme-binding pocket, and (iii) two anti-parallel $\beta$-sheets that house a conserved tyrosine (Tyr-136 in IsdX1) which coordinates the heme-iron (Figure S2A). However, surface charge calculations indicate a charge distribution on IsdX1 quite distinct from the other NEAT domains, including a net positively charged region near the 310-helix of the heme pocket (Figure S1). It is not known if this difference relates to the fact that IsdX1 acts extracellularly after secretion, as opposed to the staphylococcal NEAT proteins that are covalently anchored to the cell wall. Of note is the finding that the staphylococcal homolog of the putative receptor for IsdX1 (IsdC) contains overall anionic character in this region, a feature which leads one to postulate that opposing charges may partially dictate the association between these two proteins upon heme transfer.  

The structures of apo-IsdX1 and holo-IsdX1 are highly similar with RMSD of 0.51 Å. However, superimposition of both apo and holo structures highlight subtle residue sidechain conformational differences surrounding the heme binding site. This finding suggests residues in the 310-helix may partially stabilize the heme-free form (discussed below), a requirement of this hemophore immediately after secretion, as opposed to the staphylococcal NEAT proteins that are covalently anchored to the cell wall. Of note is the finding that the staphylococcal homolog of the putative receptor for IsdX1 (IsdC) contains overall anionic character in this region, a feature which leads one to postulate that opposing charges may partially dictate the association between these two proteins upon heme transfer.  

Figure 7. Functional role of the 310-helix and adjacent residues: hemophore and NEAT-domain biology. Purified wild-type or mutant IsdX1 were added to a final concentration of 1 μM to hemophore-deficient ($\Delta$isdX1, $\Delta$isdX2) B. anthracis Sterne 34F2 grown in iron-chelated RPMI with or without hemoglobin (10 μM) and the OD$_{600}$ recorded at 2, 4, 6, and 8 hours. The results represent the mean and standard deviation of three independent experiments. The asterisk (*) means the differences were significant (p<0.05). Hb = hemoglobin. doi:10.1371/journal.ppat.1002559.g007
With respect to the residues in and around the 3_10-helix of IsdX1, several interesting properties can be gleaned from this study. First, amino acids in this region contribute to the stabilization of heme, as demonstrated by the fact that the substitution of Ser-52, Ser-53, and Met-55 to alanine abrogated the ability of IsdX1 to attain heme from E. coli lysates as well as bind pure hemin when incubated with the apo protein. Mutation of Arg-54 led to significantly higher amounts of heme co-purifying with IsdX1. However, removal of this heme and assessment of heme binding yielded a protein unable to subsequently coordinate heme. We cannot definitively explain this result, other than to propose that it is possible that while being synthesized in E. coli, the partially unfolded IsdX1 binds the heme and then folds thereby enclosing upon the iron-porphyrin. Upon heme removal, the resulting apo protein, potentially highly ordered, now becomes restricted and does not allow heme to access the binding pocket.

Interestingly, this was not observed in an IsdA variant harboring an alanine substitution in the equivalent position (His-83) [41]. It was proposed this position in IsdA and IsdC (Ile-48) sterically hindered access to the sixth coordination position of the heme-iron. Indeed, cyanide and azide, two ligands often used to probe accessibility to the sixth position, did not bind IsdA or IsdC [42,65]. The ability of the E. coli form of R54A to associate with more heme while expressed in E. coli may provide experimental support for this hypothesis, with Arg-54 sterically blocking access to Try-136 in the wild-type protein.

Second, mutation of this region decreases hemoglobin association; however, differential effects are observed. Substitution of Ser-55 and Arg-54 with alanine significantly reduced binding to hemoglobin, with R34A yielding the largest effect (greater than 300-fold). However, mutation of Ser-52 and Met-55, the two residues flanking Ser-53 and Met-54, produced hemoglobin-binding affinities similar to the wild-type protein. These findings support a model by which Arg-54, being rather forward in its location over the heme-binding site, provides initial contact with hemoglobin, perhaps stabilizing the initial interaction. The engagement of Arg-54 with hemoglobin may “peel” the side chain of this residue away from the heme-binding site, thus removing the steric block observed for residues in this position for other NEAT proteins. Further support for this hypothesis is that the hemoglobin-binding NEAT domain, IsdB-N2, has a Met in the same position as Arg-54, and the crystal structure of IsdB showed an alternate conformation of Met coordinated with heme-iron, whereby the authors suggest that this Met might be involved in heme transfer. If alternate conformations of Arg-54 are sampled, one conformation has the NH1 group of Arg-54 being sterically blocked access to Try-136 in the wild-type protein.

Once Ser-53 engages the heme, Tyr-136 of IsdX1, from the opposite side of the heme, can now displace hemoglobin’s His-iron coordination to become the fifth axial ligand of IsdX1, with Tyr-140 providing additional strength for this coordination by H-bonding to the phenolate of Tyr-136. In addition to Tyr-136, the heme is also secured in the binding pocket by Ser-53 and Arg-54, since these residues show the highest rates of heme loss when mutated. Ser-52, whose mutation does not lead to greater heme dissociation, may also assist in drawing the heme into the heme-binding pocket but once the heme is in, does not contribute much to its stabilization. This hypothesis aligns well with the observation that the side chain of this residue sticks out into the solvent while the side chain of its neighbor, Ser-53, points into the heme-binding pocket.

During review of our manuscript, Kumar et al described the first ever co-crystal of a NEAT protein (the first NEAT domain of IsdH from S. aureus) in complex with the alpha-chain of hemoglobin [67]. The structure reveals several stabilizing contacts between the 3_10-helix of IsdH and hemoglobin, thereby confirming our predictions for the role of this helix in direct association with hemoglobin and heme scavenging. Perhaps most important is a hydrogen bond between a serine in IsdH NEAT 1 (Ser-130) with Lys-11 on hemoglobin. This serine is just 5 residues downstream of what would be the position of Ser-53 in IsdX1, leading us to speculate that the lack of functional interaction with hemoglobin observed upon mutation of this residue is because a key hydrogen bond is severed in the IsdX1-Hb interaction.

Whether or not the function of the 3_10-helix is confined only to heme acquisition from hemoglobin remains to be determined. For example, Grigg et al found the conserved coordinating tyrosine in the first NEAT domain of IsdA (the equivalent of Tyr-136 in IsdX1), and not residues in the 3_10-helix, played a role in NEAT to heme transfer [63]. In contrast, Villaruel et al found that mutation of a single 3_10-helix residue in S. aureus IsdA did affect NEAT to heme transfer; however, this mutation was coupled to another mutation elsewhere in the protein that was also believed to mediate NEAT-NEAT binding [64]. Thus, more work is required to determine if the 3_10-helix also functions in downstream heme transfer processes.

Although this model of hemoglobin and heme binding is consistent with available data, we cannot rule out that there are additional residues outside of the 3_10-helix that participate in this process. Indeed, as demonstrated by Pilpa et al [46] for one of the staphylococcal hemoglobin receptors (NEAT 1 of IsdH) [68,69], amino acids distal to this position (on the β3-β4 loop), also seem to be important for hemoglobin association. However, NEAT 1 of IsdH does not bind heme, and instead requires a third NEAT domain (NEAT 3), to acquire and stably bind the heme from the IsdH NEAT 1-hemoglobin complex. Thus, the data suggests residues in and around the 3_10-helix in IsdX1 evolved to bind both heme and hemoglobin, as well as those that are not interdependent, in order to provide several functionalities (heme binding coupled to heme extraction) within a single NEAT domain.

Finally, the exogenous addition of recombinant IsdX1 to culture restored the growth of a hemophore-deficient strain of B. anthracis on hemoglobin, suggesting all the information necessary for hemophore activity is contained within its amino acid sequence. The partial restoration of growth observed by S52A and M55A may be due to the ability of these mutants to still bind heme, albeit poorly, after association with hemoglobin. Although S33A and R54A bind heme at similar levels as S52A and M55A, it would seem their much lower affinity for hemoglobin precludes them from acquiring any heme in this assay (or alternatively, does not promote the release of enough heme from hemoglobin), meaning no or little free heme is available for transfer (or sequestration) at the bacterial surface. Because mutation of Ser-52, Ser-53, Arg-54, and Met-55 results in poor heme-binding activity, it is difficult to biochemically determine the exact role of these residues in the ability of IsdX1 to extract heme from hemoglobin. However, it is clear from the SPR and growth studies that mutations that affect the association with hemoglobin significantly compromises hemophore activity. Performing an identical experiment with a
single mutant (ΔisdX1) strain did not yield a significant enough phenotype to evaluate these mutants for function (data not shown), likely because IsdX1 and IsdX2 are functionally redundant [26]. Taken as a whole, these experiments highlight the biological role of the 310-helix in NEAT protein function and hemophore biology and provide direct evidence that the mechanistic extraction and binding of heme from hemoglobin are important for B. anthracis replication in low-iron environments.

Although attempts to develop a universal inhibitor of heme uptake is likely to be challenging, the seemingly conserved functional properties shared by distinct NEAT domains does offer the prospect of targeting these processes in select Gram-positive bacteria. As we make advances in our understanding of NEAT mechanism of action, the elucidation of additional structures, an understanding of side chain chemistry in transfer reactions, and the identification of factors that drive ligand binding specificity, will all be required for the creation of novel anti-infectives that prevent iron-porphyrin uptake in these pathogenic bacteria.

Materials and Methods

Bacterial strains, reagents, and mutagenesis

DNA encoding for amino acids 26–146 of IsdX1 were amplified from the genome of B. anthracis strain Sterne 34F2 using PCR and primers containing EcoRI and BamHI restriction sites [70]. Amplified DNA was digested, ligated into pGEX2TK, and pGEX-IsdX1 transformed into E. coli BL21 for the expression of IsdX1 as a glutathione-S-transferase (GST) fusion protein as described [26,29]. For the purification of IsdX1 used to generate the holo-protein crystals, DNA encoding residues 27–152 was cloned into pET28a (Novagen) encoding a fusion protein of IsdX1 with a His6-tag using NheI and NotI restriction enzyme sites. The 26–146 (apo) and 27–152 (holo) protein constructs were chosen because they yielded crystals that diffractioned with the highest resolution. Site-directed mutagenesis of IsdX1 was performed on the 26–146 IsdX1 construct using QuikChange (Stratagene, Santa Clara, CA) according to the manufacturer’s instructions. After DpnI digestion of reaction mixtures, DNA was transformed into E. coli BL21 and the resultant plasmid clones sequenced to confirm the presence of the mutation [71,72]. All E. coli strains were grown in Luria-broth (LB) supplemented with 50 μg/mL ampicillin (Fisher Scientific, Waltham, MA) except for the pET28a construct that was supplemented with 30 μg/mL kanamycin (Fisher).

Purification of proteins for activity studies

Wild-type and mutant IsdX1 proteins were purified by GST-affinity chromatography as previously described [26,29]. Brieﬂy, 50-mL of overnight cultures of E. coli BL21 containing wild-type or mutant pgaA-isdX1 were inoculated into 2-L of LB with ampicillin and rotated at 250 rpm at 37°C. After 2 hours, isopropyl-β-D-thiogalactoside (IPTG - 1.5 m) was added and cultures grown for an additional 2 hours. Bacteria were centrifuged at 6,000 × g for 8 min, resuspended in phosphate buffered saline (PBS - 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4), and cells lysed by French press. The supernatant was obtained by centrifugation at 30,000 × g for 15 min and ﬁltered through a 0.45-μm pore-size cellulose filter. Lysates were next applied to 1-mL of glutathione-sepharose (GE Healthcare, Piscataway, NJ), washed with 40-mL of PBS, and bound protein incubated with thrombin (100 units, GE Healthcare) for 3 hours at 25°C. GST-free IsdX1 preparations were next incubated with 200-μL of aminobenzamidine sepharose (Sigma, St. Louis, MO) to remove thrombin. To remove endogenous heme, preparations were treated with HCl (final pH of 2.0) and methyl ethyl ketone was added to separate heme (organic layer) from IsdX1 (aqueous layer) as described [73]. Protein concentrations were either determined by UV/vis spectroscopy, the bicinchoninic acid method (Pierce, Rockford, IL), or by SDS-PAGE [74]. All protein preparations were stored at −20°C.

Purification of proteins for crystallography

apo-IsdX1 - To obtain an IsdX1 preparation for crystal seeding, IsdX1 was expressed and bound to glutathione-sepharose as described above [26,29]. Bound protein was then cleaved from the column with Factor-XT (10 units, Amersham Biosciences) in 1 mM CaCl2, 100 mM NaCl, 50 mM Tris pH 7.9 for 16 hours. Heme was removed as described above and IsdX1 further puriﬁed by cation exchange chromatography using a Mono S column (GE Healthcare) and gel filtration chromatography using a Superdex 200 column (GE Healthcare). Selenomethionine-substituted protein was produced by inhibiting methionine biosynthesis and puriﬁed as above.

Holo-IsdX1 - IsdX1 was transformed into BL21-Gold (DE3) cells and grown at 37°C in LB medium containing 30 μg/mL kanamycin. Protein expression was induced when cells reached OD600nm of 0.8 by the addition of 1 mM IPTG and cells harvested after 4 hours by centrifugation at 5, 100 rpm for 20 minutes, followed by resuspension in 50 mM Tris, pH 7.4, and 350 mM NaCl. Cells were next lysed by sonication after addition of egg hen lysozyme (5 mg, Sigma) with phenylmethylsulfonyl fluoride (40 μM, Sigma) and the cell lysate centrifuged at 14,000 rpm for 20 minutes. After addition of 400 μL Proteoblock protease inhibitor cocktail (Fermentas), the supernatant was loaded onto a Ni2+-charged HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (between 100–250 mM imidazole). Fractions containing IsdX1 were identiﬁed by SDS-PAGE, pooled and concentrated using a Centricron centrifugal concentrator (Millipore). Further puriﬁcation of IsdX1 was achieved by running the protein over an S75 gel ﬁltration column (GE Healthcare) equilibrated with 50 mM Tris pH 7.4, 150 mM NaCl, which yielded nearly 100% homogeneous protein. Cleavage of the His6-tag was conducted in cleavage buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM CaCl2) by adding 1 mL of thrombin-agarose suspension (Sigma) to the protein, followed by removal of thrombin-agarose on a glass frit. IsdX1 was then run over an S75 gel ﬁltration column equilibrated with 50 mM Tris pH 7.4, 150 mM NaCl to separate IsdX1 from the His6-tag.

Crystallization and structure determination

apo-IsdX1 - Puriﬁed IsdX1 in buffer A [10 mM MES pH 6.6, 200 mM NaCl] crystallized at room temperature using the hanging drop vapor diffusion method and a reservoir solution of 0.1 M citric acid pH 3.5 and 2 M NaCl. Crystals were frozen in N2 (l) following cryoprotection with the reservoir solution containing 16% glycerol. Data were collected to 1.8 Å at the Structural Biology Center beamline 19-BM at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), and processed using HKL2000 [75]. SeMet-IsdX1 crystallized with a reservoir solution of 0.1 M citric acid pH 3.5 and 3.3 M NaCl and was frozen as above. Data were collected to 2.1 Å at the Life Sciences Collaborative Access Team (LS-CAT) beamline 21-ID-D at the APS, and processed with XDS and scaled using SCALA (Table 1) [26]. The structure was determined by the single-wavelength anomalous dispersion method using the anomalous scattering from two selenium atoms in the asymmetric unit cell with the program PHENIX [77]. The model was built
at 399 nm and Abs399/Abs280 ratios greater than 3 were pooled. The UV/vis absorbance spectrum for incubation at room temperature, excess heme was removed using maximum from 399 nm to a shorter wavelength. After 1-hour absorption spectrum was recorded after each addition of heme and heme to IsdX1 preparations in small increments. The UV/vis m was incubated with hemin chloride (5 μM) mixed with apo-Mb (26 μM) at 25°C in PBS, pH 7.4. Dissociation rates are determined by measuring the increase in absorbance at 419 nm (apo-Mb) versus a reference, control wavelength (380 nm).

Hemoglobin-binding analysis
The interaction of wild-type and mutant IsdX1 proteins with hemoglobin was measured using a BLAcore 3000 biosensor (Amersham Biosciences, Piscataway, NJ) [30]. Briefly, 100 μL of holo- or apo-hemoglobin (Sigma-H2500, 10 μM in 50 mM Tris-HCl, pH 7.0) was covalently coupled to a CM5 sensor chip at 25°C to a density of 4000 response units (RU) using amine chemistry as previously described [85,86]. Wild-type or mutant IsdX1 proteins (100, 200, 300, 350, or 500 nM) in HBS-N (0.01 M HEPES, 0.15 M NaCl, pH 7.4) were injected at 20 μL/min for 300 s at 25°C and response curves followed for a total of 800 seconds. A parallel injection of IsdX1 over a blank CM5 surface (no hemoglobin) was used to control for non-specific binding. Injections at each concentration were performed in triplicate, and the data from the 300 nM injection used to calculate the dissociation constants, which were determined using BLAevaluation 4.1 software (Amersham Biosciences) after fitting the data to a 1:1 Langmuir binding model with dR/dt = kₐ[R]ₘₐₓ – R – kₐ[R], where R is the SPR signal (in response units), kₐ is the association rate constant (in M⁻¹ s⁻¹), kₐ 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 dR/dt is the rate of change of the SPR signal [87].

B. anthracis growth experiments
Purified IsdX1 (wild-type or mutant proteins) were treated with HCl and methyl ethyl ketone to remove any endogenous heme [88]. Preparations were then dialyzed against 2-L of PBS (pH 7.4) and treated with 100 mg/mL Chelex-100 (Sigma) to remove any contaminating free iron. B. anthracis Sterne strain (34F2) harboring deletions in both isdX1 and isdX2 [26] were subcultured into 3-mL of LB plus kanamycin at 30°C. After 12-hours, cells were washed 2× with PBS and 5-μL of washed, normalized cells inoculated into 500-μL of RPMI (iron chelated by treatment with 100 mg/mL Chelex-100 for 12 hours) with or without hemoglobin (10 μM) in a 48-well Costar tissue-culture plate. Wild-type or mutant proteins (1 μM) were next added to each well and OD₅₆₀ recorded at 2, 4, 6, and 8 hours at 37°C using a Tecan 200 Pro microplate reader. The results represent the mean and standard deviation of three independent experiments.

Circular Dichroism Spectroscopy
Circular dichroism spectra of apo forms of wild-type and mutant IsdX1 were obtained using a JASCO-B15 CD spectropolarimeter at 25°C [89,90]. Protein samples were resuspended in PBS buffer, pH 7.4 at a concentration of approximately 50 μM. Far-UV spectra were recorded from 200–260 nm using a 1-mm path length at a scanning speed of 50 nm/min, with a bandwidth of 2 nm. Raw spectra are shown and represent the average accumulation of six scans.
Accession numbers

The NCBI accession number for *isdX1* is NC_005945.1 (*B. anthracis* Sterne gene ID: 2851614).

Supporting Information

Figure S1 Comparison of surface charge distribution of *S. aureus* (Sa) and *E. coli* (Ec) NEAT domain structures. Molecular surface representation with electrostatic potential as shown from −70 eV (negative, red) to +70 eV (positive, blue). Heme is represented by stick model and Fe as an orange sphere, with carbon, oxygen and nitrogen atoms colored in purple, red and blue, respectively. The PDB codes are Ba-IsdX1 (3SIK), Sa-IsdX1 NEAT domain (4FZH), Sa-IsdX1 NEAT domain (3RTL).

Figure S2 Comparison of NEAT domain structures. Upper panel, Comparison of the heme-binding pocket of the IsdX1 NEAT domain to those of *S. aureus* (Sa) NEAT proteins. Heme and residues in close proximity are represented by stick model with heme carbon atoms in purple and Fe as an orange sphere (all oxygen, nitrogen, and sulfur atoms colored red, blue, and yellow, respectively). Lower panel, An alternative conformation of Arg-54 coordinated to heme-iron is shown.

Figure S3 Spectral properties of wild-type and mutant IsdX1. Wild-type (black) or S52A, S53A, R54A, or M55A (grey) IsdX1 were purified from *E. coli* and the absorbance properties from 250–650 nm analyzed immediately after purification.

Figure S4 Far-UV CD analysis of wild-type and mutant IsdX1. Spectra of apo forms of wild-type and mutant IsdX1 (50 μM) were obtained using a JASCO-815 CD spectropolarimeter at 25°C. Raw spectra are shown and represent the average accumulation of six scans.

References


Author Contributions

Conceived and designed the experiments: AWM CWG ME CBP CPO EB CH MB MFJO. Performed the experiments: AWM ME CBP CPO EB CH MB MFJO. Contributed reagents/materials/analysis tools: AWM CWG ME CBP CPO EB CH MB MFJO. Wrote the paper: AWM CWG ME CPO JO.
Mechanism of a NEAT Hemophore


