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FOR THE RECORD

NMR experiments redefine the hemoglobin binding properties of bacterial NEAr-iron Transporter domains

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Iron is a versatile metal cofactor that is used in a wide range of essential cellular processes. During infections, many bacterial pathogens acquire iron from human hemoglobin (Hb), which contains the majority of the body's total iron content in the form of heme (iron protoporphyrin IX). Clinically important Gram-positive bacterial pathogens scavenge heme using an array of secreted and cell-wall-associated receptors that contain NEAr-iron Transporter (NEAT) domains. Experimentally defining the Hb binding properties of NEAT domains has been challenging, limiting our understanding of their function in heme uptake. Here we show that solution-state NMR spectroscopy is a powerful tool to define the Hb binding properties of NEAT domains. The utility of this method is demonstrated using the NEAT domains from *Bacillus anthracis* and *Listeria monocytogenes*. Our results are compatible with the existence of at least two types of NEAT domains that are capable of interacting with either Hb or heme. These binding properties can be predicted from their primary sequences, with Hb- and heme-binding NEAT domains being distinguished by the presence of (F/Y)YH(Y/F) and S/YXXXY motifs, respectively. The results of this work should enable the functions of a wide range of NEAT domain containing proteins in pathogenic bacteria to be reliably predicted.

Keywords: NEAT domain; hemoglobin; NMR spectroscopy; heme; bacteria; pathogen

Additional Supporting Information may be found in the online version of this article.

Significance statement: During infections, many bacterial pathogens use NEAr-iron Transporter (NEAT) domains to acquire iron, an essential nutrient that is required for microbial growth. Here we show that NMR is a powerful tool to assess the hemoglobin binding properties of these domains. Our results resolve prior discrepancies in the literature and help to define the functions of a wide range of NEAT domains based on their amino acid primary sequences.

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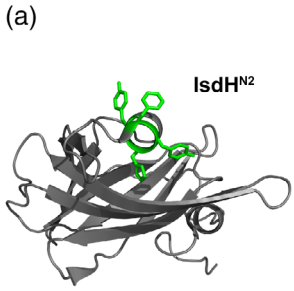
Introduction

Nearly all bacterial pathogens need to actively acquire iron from their host in order to mount an infection. Iron is frequently foraged from human hemoglobin (Hb), which contains ~60%–80% of the human body's total iron content in the form of heme (iron-protoporphyrin IX).[1–4] Since the 2000's, heme iron acquisition systems in a number of clinically important Gram-positive bacterial pathogens have been described, including: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus anthracis*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus lugdunensis*.[1–3,5–8] These systems can be complex, each employing a range of secreted and cell-wall-associated receptors that work together to scavenge heme. Elucidating the functions of these proteins is required to understand how they collectively work together to acquire heme during infections and could facilitate the development of new anti-infective strategies that work by limiting microbial access to iron.

Pathogenic Gram-positive bacteria acquire heme-using proteins that contain NEAr-iron Transporter (NEAT) domains. These modules are found in over 80 bacterial species and adopt a conserved β-stranded structure that interacts with ligands via surface exposed residues within a 3_{10}-helix (also known as the "lip-region") (Fig. 1).[3,9] The functions of individual NEAT domains within the *Staphylococcus aureus* iron-regulated surface determinant (Isd) proteins have been characterized in the greatest detail (IsdA, IsdB, IsdC, and IsdH).[3,10] IsdA and IsdC contain a single NEAT domain, while IsdB and IsdH contain two and three NEAT domains, respectively. Atomic structures and biochemical studies have shown that individual NEAT domains within these proteins exhibit monotypic ligand binding behavior, each domain binds to either Hb or heme.[11–19] The *S. aureus* IsdH and IsdB proteins contain dedicated Hb binding NEAT domains ($IsdH^{N1}$, $IsdH^{N2}$, and $IsdB^{N1}$).[17–19] Their primary sequences are distinguished by the presence of an aromatic (F/Y)YH(Y/F) motif that is located within the lip-region [colored green in Fig. 1(a,c)]. These residues in crystal structures of Hb:NEAT domain complexes contact Hb's A-helix and when mutated impair binding.[13,17,19–21] In contrast, other staphylococcal NEAT domains selectively bind to heme and do not interact with Hb. These heme-binding NEAT domains are present within IsdA and IsdC proteins, as well as in IsdB and IsdH ($IsdB^{N2}$ and $IsdH^{N3}$ domains, respectively). Their primary sequences do not contain the aromatic motif, but instead harbor conserved serine and YXXXY sequence motifs that are located in the lip-region and strand β8, respectively[14] (hereafter called S/YXXXY) [colored red in Fig. 1(b,c)]. Structures of these domains bound to heme have been determined, revealing that the first tyrosine in the conserved YXXXY element coordinates the metal, while the invariant serine interacts with heme's propionate groups.[12,14–16,22] In general, biochemical and structural studies of non-staphylococcal NEAT domains substantiate the idea that the presence of a S/YXXXY motif is a good predictor of heme binding function. However, there is some plasticity in the



(c)

		Hb binding aromatic motif	heme binding YxxxY motif	Structures of NEAT-ligand Complexes NEAT-Hb	NEAT-heme
S. aureus	IsdH-NEAT-1	YYHFFS	EETNY	3szk	--
	IsdH-NEAT-2	FYHYAS	IHEDY	4fc3	--
	IsdB-NEAT-1	FYHYAS	KEEKY	5vmm	--
	IsdH-NEAT-3	SVMDGF	YEGQY	--	2z6f
	IsdB-NEAT-2	SMMDTF	YDGQY	--	3rtl
	IsdA-NEAT	SHMDDY	YNHRY	--	2itf
	IsdC-NEAT	SIANDY	YDHHY	--	2o6p
B. anthracis	IsdX1-NEAT	SRMNRY	YNASY	--	3sik
	IsdX2-NEAT-1	SMMNQY	YHHEY	--	--
	IsdX2-NEAT-2	SKMNTY	YKQTH	--	--
	IsdX2-NEAT-3	SMMNTY	YHHFY	--	--
	IsdX2-NEAT-4	SMMNTY	YHHFY	--	--
	IsdX2-NEAT-5	SMMNQY	YHHFY	--	4h8p
	Hal-NEAT	SYATVY	YDKEF	--	4ymp
	BslK-NEAT	SVASTY	YDHKY	--	--
B. cereus	IlsA-NEAT	SMVSQY	YDHKY	--	--
L. monocytogenes	Hbp1-NEAT	SMANDY	YHHEY	--	--
	Hbp2-NEAT-1	SEADKY	YDHWY	--	--
	Hbp2-NEAT-2	SSMQNY	YKGQA	--	4myp
	Hbp2-NEAT-3	SMMESY	YDHEY	--	--
S. pyogenes	Shr-NEAT-1	SMLQGA	YDKYT	--	--
	Shr-NEAT-2	SMSNKA	KGSGL	--	--

Figure 1. Sequence alignment identifies key residues that define NEAT domain function. (a) Representative structure of a Hb binding NEAT domain with residues from the (F/Y)YH(Y/F) aromatic motif shown in green (the $IsdH^{N2}$ NEAT domain, PDB: 4FC3). (b) Representative structure of a heme-binding NEAT domain with residues from the S/YXXXY motif shown in red (the IsdA NEAT domain, PDB: 2ITF). (c) Sequence alignment showing the aromatic and X/YXXXY motifs for select NEAT domains. Conserved residues implicated in Hb and heme binding are highlighted in green and red, respectively. Columns on the right provide the PDB accession codes for structures of NEAT domains determined in complex with either heme or Hb. Domains that have been proposed to have "dual" Hb/heme-binding functions are highlighted in blue and are the subject of this investigation.

mechanism of heme recognition, since in a few heme-binding NEAT domains residues within the S/YXXXY element are not completely conserved (Hbp2^{N2}, Hal^{N}, Shr^{N1}, and Shr^{N2}).

Interestingly, several biochemical studies have led to the suggestion that pathogenic *B. anthracis* and *L. monocytogenes* bacteria express unique NEAT domains that bind to both heme and Hb. These "dual" Hb/heme-binding domains include ^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N1}, ^{Ba}IsdX2^{N3}, ^{Ba}IsdX2^{N4}, ^{Ba}IsdX2^{N5}, ^{Ba}Hal^{N}, ^{Lm}Hbp1^{N}, ^{Lm}Hbp2^{N1}, and ^{Lm}Hbp2^{N3} [indicated on Fig. 1(c) in blue].[23–26] Evidence for their ability to bind heme is strong and is based on the presence of a Soret band in their UV–vis spectra, and in some instances has been validated by X-ray crystallography.[27–29] In addition, their primary sequences contain all of the residues within the conserved heme-binding S/YXXXY sequence element; Hal^{N} is the lone exception as it lacks the second, nonmetal coordinating tyrosine residue [Fig. 1(c)]. While strong experimental and phylogenetic evidence indicates that these supposed "dual" Hb/heme-binding domains interact with heme, their ability to also interact with Hb is less convincing. This is because their primary sequences lack the conserved (F/Y)YH(Y/F) aromatic motif [Fig. 1(c)], structures of their complexes with Hb have yet to be determined and their ability to bind to Hb has only been demonstrated using immobilized Hb in either enzyme-linked immunosorbent assays (ELISAs) or surface plasmon resonance (SPR) experiments.[23–26] Thus, it remains unknown whether some bacteria produce dual Hb/heme-binding NEAT domains that are capable of binding to both Hb and heme.

In this study, we used NMR spectroscopy to probe the heme and Hb binding properties of representative NEAT domains from *S. aureus*, *B. anthracis*, and *L. monocytogenes*. Binding experiments were performed in solution using freshly purified Hb from human blood. Our results support the idea that NEAT domains are monotypic ligand binding modules—each domain binds to either heme or human Hb, but a single domain is incapable of binding to both of these ligands with strong affinity. We conclude that the aromatic Hb-binding and tyrosine heme-binding sequence motifs previously identified by phylogenetic and structural analyses are good predictors of domain function. The results of this work should enable the functions of a wide range of NEAT domain containing proteins in pathogenic bacteria to be reliably predicted.

Results and Discussion

Conclusive structural and biochemical studies have thus far revealed that there are at least two types of monotypic ligand-binding NEAT domains—modules that bind only to Hb and modules that only bind to heme. Structural analyses have revealed their mechanism of ligand binding, which occurs via residues within distinct Hb-binding (F/Y)YH(Y/F) and heme-binding S/YXXXY motifs [Fig. 1(c)].[3] Interestingly, biochemical studies have suggested that *L. monocytogenes* and *B. anthracis* bacteria scavenge heme iron using a third-type of NEAT domain that is unique because it is capable of binding to both Hb and heme [highlighted in blue, Fig. 1(c)].[23–26] The ability of these dual ligand-binding domains to interact with heme has been convincingly demonstrated by UV–vis spectroscopy and in some instances by determining their structures bound to heme. However, their ability to also bind to Hb is far less convincing, as atomic structures of their complexes with Hb have yet to be determined and their primary sequences lack the Hb-interacting (F/Y)YH(Y/F) motif. Moreover, the reliability of the experimental data used to conclude that these domains bind to Hb has been drawn into doubt, as the commercially obtained Hb employed in these *in vitro* binding experiments has recently been shown to contain numerous breakdown products.[30]

In order to clarify their functions in heme acquisition we used NMR spectroscopy to investigate the Hb and heme binding properties of several *L. monocytogenes* and *B. anthracis* NEAT domains that were previously proposed to exhibit dual Hb/heme-binding behavior. Within red blood cells, Hb exists as a tetramer of α and β globin subunits that are each bound to ferrous heme.[31] However, removed from the cell, Hb can exist in a variety of forms that differ in protein oligomerization (tetramers or dimers) and heme oxidation (ferric or ferrous). Further complicating the issue, heme can dissociate from Hb leading to the production of individual α and β globin chains. To overcome this problem, the Hb used in the NMR studies was purified "in-house" from human blood and its purity and integrity verified. Using a well-established procedure, red blood cells were lysed in a hypotonic solution and Hb was purified from hemolysate in a two-step process using cation and anion exchange chromatography.[32,33] To maintain stability throughout the purification Hb was kept in the reduced state by periodic exposure to carbon monoxide and its oxidation state was confirmed by comparing UV–vis spectral traces with published extinction coefficients. This procedure produced carbonmonoxy-Hb, a stable form of Hb in which the bound heme is present in its ferrous state and therefore less prone to dissociation.[34] The integrity of purified tetrameric Hb was verified by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography with multi-angle light scattering (SEC-MALS) [Fig. 2(a,b)]. Under the conditions used for the NMR titration experiments, the concentration of purified Hb is well above its dimer–tetramer equilibrium constant and is thus tetrameric.[35] In contrast, the commercially produced Hb used in previously reported SPR and ELISA binding studies is not well defined, existing as a mixture of oligomers that may or may not be bound to ferric heme.[23–26,28,30,36]

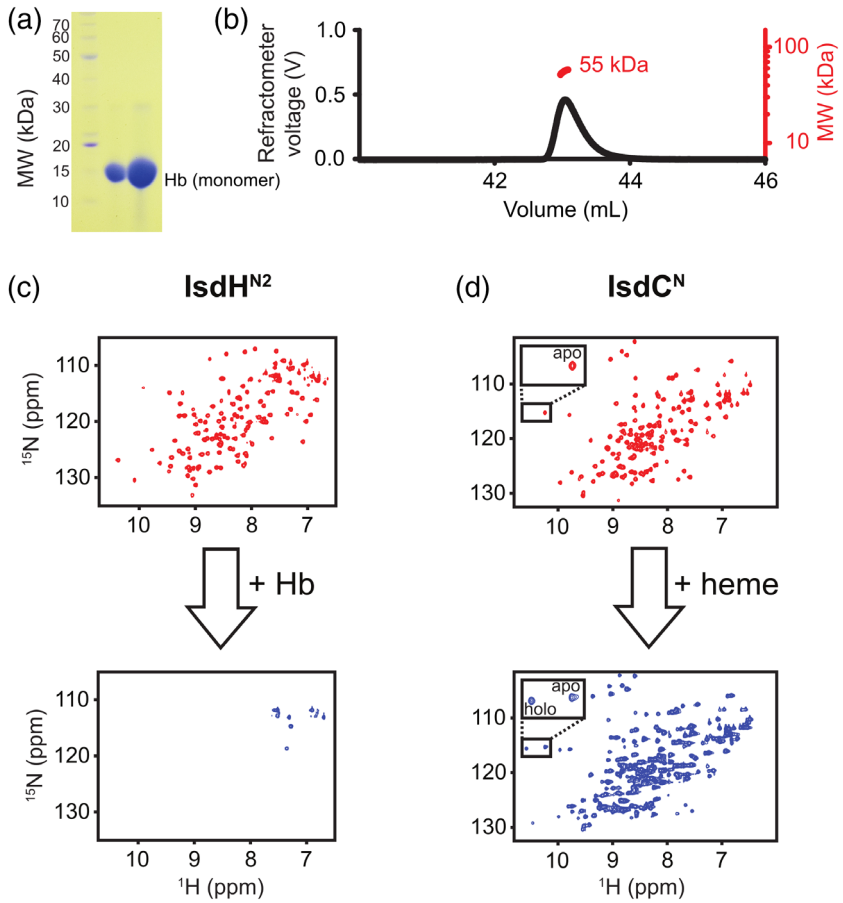


Figure 2. Characterization of purified Hb and NMR control experiments. Hb was purified from human blood and its integrity verified by (a) SDS-PAGE and (b) SEC-MALS. In the SDS-PAGE, the α and β globin chains of Hb are monomeric and appear as a single band because they have similar molecular weights. Lanes: (left) molecular weight markers, (middle) 10 μg of purified Hb, (right) 40 μg of purified Hb. As expected, in the SEC-MALS the measured molecular weight indicates that Hb is a tetramer with a mass that is slightly less than its theoretical value.[47] (c) Control Hb titration experiments using the established Hb binding [^{15}N] IsdH^{N2} domain.[18] Panels show the HSQC spectrum before and after adding one molar equivalent of Hb. Extensive signal broadening indicates that the domain binds to Hb. (d) Control heme titration experiments using the established heme-binding IsdC^{N} NEAT domain. A half molar equivalent of heme was added.[15] Binding is indicated by the appearance of a separate set of cross peaks (inset) when sub-stoichiometric amounts of heme are added.

Select NEAT domains were uniformly labeled with nitrogen-15 (^{15}N) and the effects of adding heme or Hb on their heteronuclear single quantum coherence (^{1}H-^{15}N HSQC) spectra used to ascertain binding. In all experiments described in this article, the ferric form of heme was studied (also referred to as hemin). Control NMR titration experiments were initially performed using established monotypic ligand-binding NEAT domains that selectively interact with either Hb or heme. These NEAT domains include the Hb-binding domain from IsdH (second NEAT domain from IsdH, IsdH^{N2}, residues 326–466) and the dedicated heme-binding domain from IsdC (IsdC^{N}, residue 25–150). For both domains, binding to their respective ligands has been visualized by X-ray crystallography and their primary sequences contain the aforementioned Hb- and heme-binding motifs.[15,18] In isolation, the HSQC spectra of [^{15}N]IsdH^{N2} and [^{15}N]IsdC^{N} are well resolved and their chemical shifts are consistent with previously published values.[22,37] The addition of a 1:1 equivalent of unlabeled Hb to [^{15}N]IsdH^{N2} causes the almost complete disappearance of signals originating from its backbone amide groups [compare top and bottom panels in Fig. 2(c)]. The spectral broadening is consistent with the size of the IsdH^{N2}:Hb complex (molecular weight ~130 kDa, ~4:1 stoichiometry), which substantially increases the effective rotational correlation time of the bound IsdH^{N2} protein. The data are also consistent with isothermal titration calorimetry experiments that have shown that IsdH^{N2} binds to Hb with a K_D = ~8 μM.[32] Similar Hb-dependent broadening effects have been observed for the NEAT domain from IsdB that also contains the aromatic motif.[20] The experimental NMR data also clearly identifies NEAT domain interactions with heme. This is demonstrated by adding unlabeled heme to [^{15}N]IsdC^{N}, a NEAT domain that selectively binds to this ligand.[15,22] For the NMR heme-binding experiments the heme-free (apo) form of the NEAT domain was employed. Upon adding a sub-stoichiometric amount of heme to

[^{15}N]IsdC^{N}, new chemical shifts in its HSQC spectrum emerge that are indicative of IsdC^{N}-heme complex formation [compare top and bottom panels in Fig. 2(d)]. Discrete signals for the complex are readily observable because of the small molecular weight of the heme ligand (~650 Da) and because ligand binding is in slow exchange on the chemical shift time scale. This leads to the presence of signals from both the apo- and heme-bound forms when sub-stoichiometric amounts of heme are present. A limited number of amide signals in the HSQC spectrum of the IsdC^{N}-heme complex exhibit resonance line broadening because they originate from residues that are located near the paramagnetic heme molecule. These two controls demonstrate that NMR is a powerful tool to probe both Hb and heme binding to NEAT domains.

We next used NMR to investigate the ligand binding properties of several *B. anthracis* and *L. monocytogenes* NEAT domains that had previously been proposed to exhibit dual Hb/heme-binding behavior. At present, biochemical studies have suggested that nine NEAT domains are dual Hb/heme-binding domains (^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N1}, ^{Ba}IsdX2^{N3}, ^{Ba}IsdX2^{N4}, ^{Ba}IsdX2^{N5}, ^{Ba}Hal^{N}, ^{Lm}Hbp1^{N}, ^{Lm}Hbp2^{N1}, and ^{Lm}Hbp2^{N3}). Their ability to bind heme is well accepted because they exhibit characteristic UV–vis spectra and their primary sequences harbor the conserved iron coordinating tyrosine residue. In addition, for three of the domains their atomic structures in complex with heme have been determined (^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N5}, and ^{Ba}Hal^{N}).[27–29] However, it is unclear how the nine dual domains might also engage Hb as their primary sequences lack the Hb-interacting (F/Y)YH(Y/F) aromatic motif and their binding to Hb has only been demonstrated in SPR or ELISA experiments using immobilized Hb obtained from commercial sources. We used NMR to test the Hb and heme-binding behavior of three of the nine putative dual Hb/heme-binding domains: ^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N5}, and ^{Lm}Hbp1^{N}. These domains were chosen because the heme binding properties of ^{Ba}IsdX1^{N} and ^{Ba}IsdX2^{N5} have already been verified by X-ray crystallography[27,28] and because ^{Lm}Hbp1^{N} binds to Hb with high affinity when tested by ELISA.[26]

Initially, we used NMR to determine whether ^{Lm}Hbp1^{N} binds to ferric heme and/or Hb. In order to probe the effects of heme binding we recorded HSQC spectra of [^{15}N]^{Lm}Hbp1^{N} in the presence of varying amounts of heme [heme:^{Lm}Hbp1^{N} ratios of 0:1, 1:1, 2:1, 4:1, and 8:1, Fig. S1(C)]. The apo-form of the [^{15}N]^{Lm}Hbp1^{N} NEAT domain exhibits a well-resolved spectrum [Fig. 3(a,b)]. Similar to the [^{15}N]IsdC^{N} control, addition of eightfold molar excess heme results in a distinct set of crosspeaks that correspond to the heme bound form of [^{15}N]^{Lm}Hbp1^{N} [Fig. 3(c), Fig. S1]. In the saturated heme bound form, a few crosspeaks also exhibit modest signal broadening compatible with them originating from backbone amides that are proximal to the paramagnetic heme molecule. As with the [^{15}N]IsdC^{N} control, heme binding to ^{Lm}Hbp1^{N} is in slow exchange on the NMR time scale, as evidenced by recording NMR spectra of a heme titration series [Fig. S1(C)]. In a second set of titration experiments the effects of adding in-house purified Hb to [^{15}N]^{Lm}Hbp1^{N} was investigated. Minor changes in the HSQC spectrum are observed when Hb is added, but these changes are not caused by Hb binding. Rather, they occur because heme is first released from Hb into the solvent and then bound by [^{15}N]^{Lm}Hbp1^{N}. This is evident by comparing expanded regions of the HSQC spectra of [^{15}N]^{Lm}Hbp1^{N} in its apo state [Fig. 3(b)], heme-bound form [Fig. 3(c)], and when excess Hb is present [Fig. 3(d)]. When excess Hb is present a minor set of new peaks appears, but their chemical shifts are identical to those of the heme bound form of [^{15}N]^{Lm}Hbp1^{N} [compare Fig. 3(c,d)], and the more intense crosspeaks have chemical shifts that are identical to those of the apo-form [compare Fig. 3(b,d)]. Additional evidence that the spectral changes are caused by heme released from Hb is obtained from an NMR titration series in which varying amounts of Hb is added to [^{15}N]^{Lm}Hbp1^{N} (Hb:^{Lm}Hbp1^{N} ratios of 0:1, 0.5:1, 1:1, 2:1, and 4:1). This data show that the new minor peaks corresponding to the heme bound form are also in slow exchange, and that they only appear when excess Hb is present [Fig. S1(D)]. Importantly, even when excess Hb is added, the intense crosspeaks in the spectrum of [^{15}N]^{Lm}Hbp1^{N} correspond to the apo-form, indicating that the affinity of [^{15}N]^{Lm}Hbp1^{N} for Hb is extremely weak (K_D > 10 m*M*). Collectively, the NMR data indicate [^{15}N]^{Lm}Hbp1^{N} binds to heme, but that it does not bind to Hb with high affinity. Moreover, they highlight the importance of comparing both Hb and heme NMR titration data, since even if the NEAT domain does not bind Hb, spectral changes can occur from heme that is released into the solvent by Hb.

NMR heme and Hb titration experiments were also performed using the ^{Ba}IsdX1^{N} and ^{Ba}IsdX2^{N5} NEAT domains from *B. anthracis*, which have been proposed to be dual Hb/heme-binding domains.[27,28] Similar to the results obtained for ^{Lm}Hbp1^{N}, NMR titration data indicate that these domains bind to heme, but reveal that they do not interact with Hb with high affinity (Fig. 4). Heme binding is supported by the appearance of a second set of cross peaks in their NMR spectra when this ligand is added [^{Ba}IsdX2^{N5}, Fig. 4(a); ^{Ba}IsdX1^{N}, Fig. 3(c)], as well as by heme-dependent spectral changes in their UV–vis spectra (data not shown). However, the NMR Hb titration data clearly show that ^{Ba}IsdX2^{N5} [Fig. 4(a)] and [^{15}N]^{Ba}IsdX1^{N} [Fig. 4(b)] do not interact with Hb with strong affinity, as its addition causes only minimal changes in their HSQC spectra. As observed for ^{Lm}Hbp1^{N} (Fig. 3), a few minor peaks do appear in the spectra of ^{Ba}IsdX2^{N5} and ^{Ba}IsdX1^{N} when Hb is present at excess, but these new signals correspond to

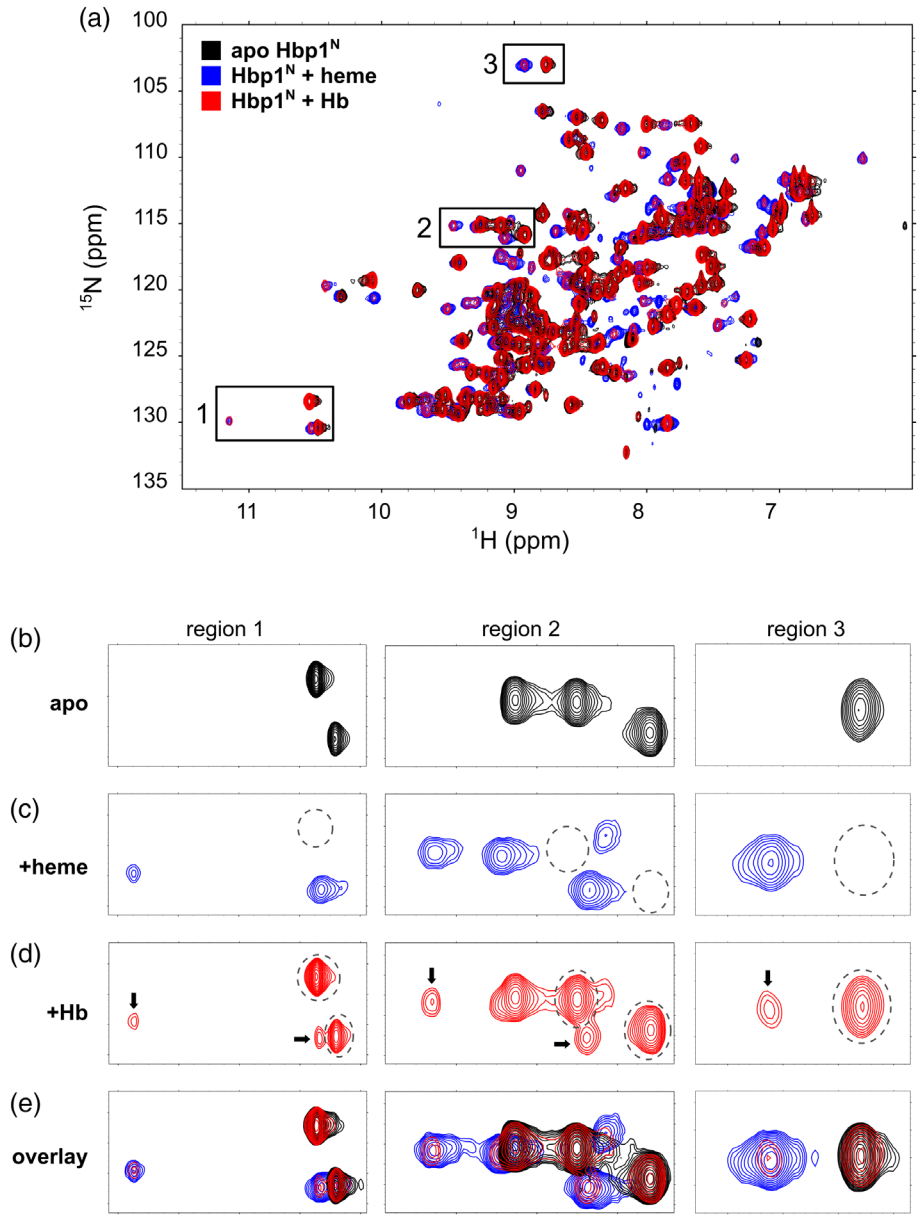


Figure 3. NMR spectra of ^{Lm}Hbp1^{N} in the presence and absence of Hb and hemin. (a) 2-D ^{15}N-^{1}H HSQC spectra overlay of ^{Lm}Hbp1^{N} in its apo-, heme added, and Hb added forms. Three regions of interest are highlighted and expanded in the panels shown beneath. Expanded view of each region is shown for: (b) the apo-form of ^{Lm}Hbp1^{N}, (c) ^{Lm}Hbp1^{N} in the presence of eightfold molar excess hemin, (d) ^{Lm}Hbp1^{N} in the presence of fourfold molar excess Hb, and (e) overlay of the spectrum of apo-^{Lm}Hbp1^{N} and spectra obtained in the presence of excess heme and Hb.

the heme bound form of each NEAT domain and are not caused by Hb interactions. To determine if the source of Hb was important for binding, NMR titration experiments were performed again using Hb obtained from a commercial source (Fig. S2). Commercially obtained Hb does not bind to the ^{Lm}Hbp1^{N}, ^{Ba}IsdX2^{N5}, and ^{Ba}IsdX1^{N} NEAT domains when tested by NMR. However, consistent with it containing breakdown products such as free heme, minor peaks corresponding to the heme bound form of the NEAT domain appeared when small amounts of commercially obtained Hb were added. Thus, contrary to previously reported findings, the ^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N5}, and ^{Lm}Hbp1^{N} domains only bind to heme and do not interact with Hb with significant affinity.

The NMR data are in conflict with results of previously reported ELISA and SPR experiments that concluded that the ^{Ba}IsdX1^{N}, ^{Ba}IsdX2^{N5}, and ^{Lm}Hbp1^{N} domains bind to Hb.[26–28] A potential explanation for this discrepancy is that prior biochemical experiments used Hb obtained from a commercial source that was subsequently shown to contain breakdown products. To investigate this issue, we used ELISA to investigate binding of ^{Ba}IsdX1^{N} to either freshly purified or commercially obtained Hb. The ELISA experiments were performed as previously described for ^{Lm}Hbp1.[26]

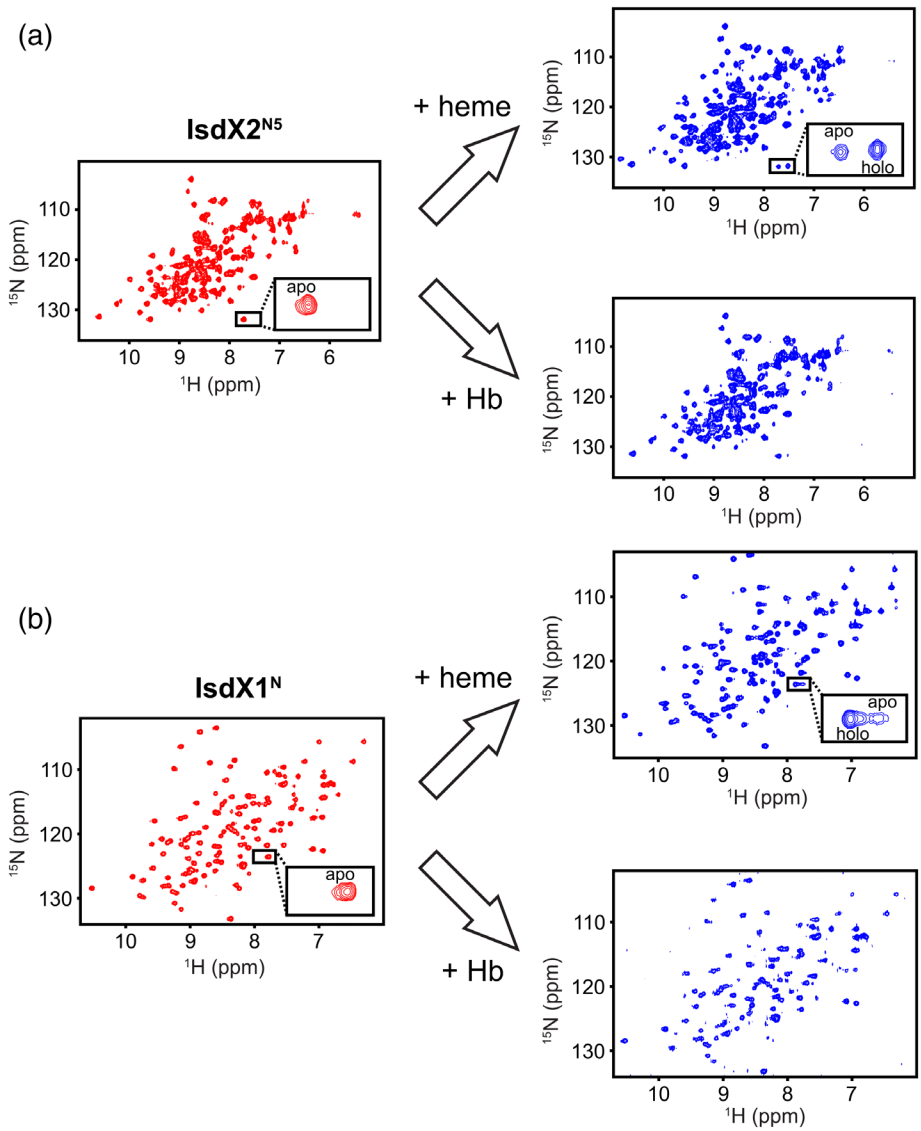


Figure 4. Putative dual Hb/Heme-binding NEAT domains do not interact with Hb with strong affinity. (a) ^{15}N-^{1}H HSQC spectrum of the *B. anthracis* [^{15}N]IsdX2^{N5} NEAT domain before (left) and after adding heme (top right) or Hb (bottom right). No significant resonance broadening is observed when Hb is added at a 20-fold molar excess, indicating that this NEAT domain does not bind to Hb with strong affinity. However, IsdX2^{N5} does bind to heme based on the appearance of a second set of NMR resonances when this ligand is added. (b) Similar experimental data for the *B. anthracis* IsdX1^{N} NEAT domain. These putative dual binding domains do not interact with Hb with strong affinity, but they do bind to heme.

Wells were coated with Hb (purified or commercially obtained), and washed and blocked microplate wells containing adhered Hb were then incubated with varying amounts of ^{Ba}IsdX1^{N} that was fused to a His6-SUMO tag. After washing, the amount of His6-SUMO-^{Ba}IsdX1^{N} retained in each well was determined using an immunoassay. Consistent with previously reported SPR binding data, His6-SUMO-^{Ba}IsdX1^{N} binds to commercially sourced Hb when tested using ELISA (Fig. 5).[27] However, consistent with the NMR results (Fig. 3), ^{Ba}IsdX1^{N} does not significantly interact with freshly purified Hb when tested by ELISA! We conclude that ELISA binding experiments that use commercially sourced Hb can lead to false positive results. Conceivably, when commercially produced Hb is used immobilization of its breakdown products in the ELISA experiment creates a platform for ^{Ba}IsdX1^{N} binding that is absent when homogenous, freshly purified Hb is immobilized. The specific breakdown product or impurity that leads to ^{Ba}IsdX1^{N} binding is not known, but it is unlikely to be free heme alone, as wells coated with this molecule do not bind ^{Ba}IsdX1^{N} (Fig. 5). SPR has also been used to probe Hb binding by NEAT domains.[27,28] The results obtained from these studies may need to be reconsidered, as they also employed commercially sourced Hb that was immobilized. Other possible explanations for the discrepancies observed between the biochemical and NMR data include the use of bovine, instead of human Hb in

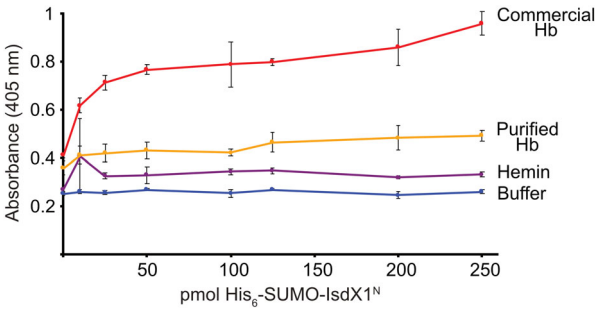


Figure 5. IsdX1^{N} binds commercial Hb, but not to freshly purified Hb. The results of an ELISA experiment showing His$_6$-SUMO-IsdX1^{N} binding to wells coated with commercial-sourced Hb (sigma) (red trace). Little or no binding occurs to wells coated with freshly purified Hb (yellow trace), heme alone (purple trace) or buffer (blue trace). Error bars represent SD from three separate experiments.

several of the biochemical experiments,[23,24] as well as differences in the oxidation state of the heme molecule bound to Hb. However, the latter difference would seem unlikely to impact NEAT domain binding as the ferric and ferrous forms adopt very similar atomic structures[38] and both forms have been shown to interact with aromatic motif containing NEAT domains.[11,17,20,39]

Six other NEAT domains have also been shown to interact with commercially produced Hb using SPR/ELISA experiments (^{Ba}IsdX2^{N1}, ^{Ba}IsdX2^{N3}, ^{Ba}IsdX2^{N4}, ^{Ba}Hal^{N}, ^{Lm}Hbp2^{N1}, and ^{Lm}Hbp2^{N3}).[24–26] Although not experimentally tested here, our NMR results suggest that they are unlikely to bind Hb with high affinity as these domains also lack the conserved Hb-interacting aromatic motif. However, this issue needs to be explored further as it is possible that other differences in the experimental conditions and reagents used in these studies led to discrepancies between the NMR and SPR/ELISA data. It is also noteworthy that ELISA based experiments have suggested that the IsdA NEAT domain is capable of binding to an array of human proteins (fetuin, asialofetuin, fibrinogen, fibronectin, loricrin, involucrin, and cytokeratin K10).[40,41] It is conceivable that other features within NEAT domains enable them to interact with other proteins, but the mechanism of this binding remains unknown. In light of the solution NMR results, and the fact that only the structure of the IsdA-heme complex has been reported, the affinity and biological significance of these protein–protein interactions need to be substantiated.

The NMR results are consistent with previous structural studies that have thus far revealed only two types of NEAT domains in pathogenic bacteria: Hb-binding domains that contain the (F/Y)YH(Y/F) aromatic motif, and heme-binding domains that contain all or some of the residues within the S/YXXXY motif. Our results shed new light on the mechanism of heme acquisition by *B. anthracis* and *L. monocytogenes*. In the well-studied *S. aureus* Isd-system, the IsdH and IsdB surface receptors engage Hb via NEAT domains that contain the aromatic motifs (IsdH^{N1} and IsdH^{N2} in IsdH and IsdB^{N1} in IsdB) and engage heme via NEAT domains that contain the S/YXXXY motifs (IsdH^{N3} in IsdH and IsdB^{N2} in IsdB). Within functional receptors (IsdH^{N2N3} and IsdB^{N1N2}) these functionally distinct NEAT domains work synergistically to structurally perturb Hb's heme-binding pocket and accelerate its heme release.[18,19,42,43] In contrast, *B. anthracis* and *L. monocytogenes* secrete and display NEAT containing proteins whose primary function appears to be to bind heme. Thus, they likely function as hemophores that are reminiscent to those found in Gram-negative bacteria that appear to passively capture heme from Hb either after it is spontaneous released or after Hb is proteolytically degraded.[4] Our results help define the biochemical functions of NEAT domain containing proteins in several microbial heme uptake systems and highlight the utility of NMR approaches to elucidate their ligand binding properties.

Materials and Methods

Protein expression and purification

Plasmids pRM213 (43) and pRM251[26] encoding residues 326–466 in IsdH (IsdH^{N2}) and 31–149 in Hbp1 (Hbp1^{N}), respectively, were transformed into *Escherichia coli* BL21(DE3) cells (New England BioLabs) and grown in M9 medium supplemented with $^{15}NH_4Cl$. Expression was induced with 1 m*M* isopropyl-*β*-D-thiogalactoside and preceded overnight at 25°C. Cells were harvested by centrifugation and resuspended in lysis buffer containing 50 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.0, 300 m*M* NaCl with phenylmethylsulfonyl fluoride (Sigma), and protease inhibitor cocktail (Calbiochem). Cells were lysed by sonication and the lysate was clarified by centrifugation. The supernatant was then applied to a Co^{2+}-chelating column (ThermoFisher Scientific). Unbound proteins were removed by washing with lysis buffer (5 column volume equivalents). Nonspecifically bound proteins were then removed by applying 5 CVs of wash buffer (lysis buffer + 10 m*M* imidazole) followed by another 5 CVs of lysis buffer. The N-terminal His$_6$-SUMO tag was cleaved with the ULP1 protease and the NEAT domain was eluted by washing the column with lysis buffer.

The plasmid encoding residues (675–797) from IsdX2 (IsdX2^{N5}) with an N-terminal GST tag was a generous gift of the Maresso Laboratory.[24] The plasmid was transformed into *Escherichia coli* BL21(DE3) cells (New England BioLabs). Cells were grown in M9 medium supplemented with $^{15}NH_4Cl$ and the protein was expressed overnight at 25°C by adding 1 m*M* isopropyl-*β*-D-thiogalactoside. Cells were harvested by centrifugation. The pellet was resuspended in 1× phosphate buffered saline (PBS) buffer (10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, 137 m*M* NaCl, 2.7 m*M* KCl, pH 7.4) with phenylmethylsulfonyl fluoride (Sigma) and protease inhibitor cocktail (Calbiochem). Cells were lysed

by sonication and the lysate was clarified by centrifugation. The supernatant was then applied to glutathione resin (GenScript) and washed with PBS buffer. $CaCl_2$ was added to a final concentration of 2.5 m*M*. 100 U of thrombin protease were added to cleave the GST tag. Cleaved $IsdX2^{N5}$ was eluted by washing the resin with 1× PBS. Fractions containing $IsdX2^{N5}$ were pooled and applied to an SEC column. Purity was assessed by SDS-PAGE. Prior to binding studies by NMR, heme-binding NEAT domains were stripped of heme using the MEK extraction method.[44] Human Hb in the carbonmonoxy-state (HbCO) was purified as described previously from the blood of a healthy donor provided by the CFAR Virology Core Laboratory at the UCLA AIDS Institute.[32]

NMR spectroscopy

NMR experiments were performed at 298 K on Bruker DRX 500, DRX 600 MHz, and AVANCE 800 MHz spectrometers equipped with triple resonance cryogenic probes. For initial Hb binding studies, 100 μ*M* samples of each ^{15}N-labeled NEAT domain were prepared in NMR buffer at pH 6.8 (50 m*M* NaH_2PO_4/Na_2HPO_4 pH 6.8, 100 m*M* NaCl, 0.01% NaN_3) and an HSQC was recorded. Then a 20-fold excess of buffer-matched Hb (heme basis) was added to the sample and another HSQC was recorded. To compensate for sample dilution upon addition of Hb, the number of scans was increased according to the relation S/N ∼ sqrt(ns), wherein the signal to noise is proportional to the square root of the number of scans. For initial heme binding studies, 200 μ*M* samples of each NEAT domain were prepared in the NMR buffer at pH 6.0 (50 m*M* $NaPO_4$ pH 6.0, 100 m*M* NaCl, 0.01% NaN_3) and an HSQC was recorded. A 20 m*M* stock of heme (Frontier Scientific) was dissolved in 0.1*M* NaOH. This stock was centrifuged to remove insoluble heme. After addition of heme at a 0.5:1 ratio of heme:NEAT, the pH of the sample was checked and an HSQC was then recorded. For $^{Lm}Hbp1^N$ titrations with heme and Hb, a 200 μ*M* sample of ^{15}N-labeled $^{Lm}Hbp1^N$ was used. After recording a spectrum of $^{Lm}Hbp1^N$ in its apo state, heme prepared as described above was added to the sample to achieve final molar ratios of heme:Hbp1 of: 0.25:1, 0.5:1, 1:1, 2:1, 4:1, and 8:1. 2-D ^{15}N-^{1}H HSQC spectra were recorded at each titration point. This process was repeated using freshly purified HbCO (described above). In this titration the final molar ratios of Hb:Hbp1 were 0.5:1, 1:1, 2:1, and 4:1, and HSQC spectra were recorded at each titration point. NMR spectra were processed using NMRPipe[45] and Topspin 3.5 (Bruker BioSpin, Inc.) and analyzed using Sparky 3 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) and NMRFAM-SPARKY.[46]

ELISA experiments

Experimental protocols are similar to those previously described for the $^{Lm}Hbp1^N$ NEAT domain.[26] Briefly, Corning Costar EIA/RIA Medium Binding 96-well plates were used, with adhesive plate seals applied during binding steps to prevent evaporation. Wells were coated with 100 μL of either: (i) 620 n*M* (10 μg/mL) human Hb purified from human blood, (ii) 620 n*M* (10 μg/mL) human Hb obtained from a commercial source (Sigma) or (iii) 620 n*M* heme dissolved in PBS, pH 7.4. Plates were incubated on an orbital shaker at 4°C for 36 h to ensure maximal binding. Wells were washed with 3 × 200 μL PBS, pH 7.4, 0.05% Tween 20 (PBST pH 7.4). Blocking of nonspecific sites was achieved by applying 200 μL 1% w/v bovine serum albumin in PBST pH 7.4 followed by incubating on an orbital shaker at room temperature for 1 h. Wells were washed with 3 × 200 μL PBST pH 7.4. Fifty microliters of serially diluted His6-SUMO-$IsdX1^N$ (2–50 μ*M*) in PBST pH 7.4 was applied to each well and plates were incubated on an orbital shaker at room temperature for 1 h. After washing 3 × 200 μL with PBST pH 7.4, 50 μL of monoclonal mouse IgG antibody against the His_6-tag (Invitrogen) was applied at a 1:1000 dilution in PBST pH 7.4. Plates were incubated on an orbital shaker at room temperature for 1 h. Wells were washed with 3 × 200 μL PBST pH 9.0. Fifty microliters of monoclonal goat IgG antibody against mouse IgG conjugated to alkaline phosphatase (Sigma) was added at 1:10,000 dilution in PBST pH 9.0. Plates were incubated on an orbital shaker at room temperature for 1 h. Wells were washed with 3 × 200 μL PBST pH 9.0 and tapped until dry. Fifty microliters 1-Step PNPP substrate solution (Thermo Scientific) was applied to each well and incubated on an orbital shaker at room temperature for 14 h to allow signal to develop. Absorbance reads were measured at 405 nm on a Tecan M1000 Plate Reader.

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