The Near-iron Transporter (NEAT) Domains of the Anthrax Hemophore IsdX2 Require a Critical Glutamine to Extract Heme from Methemoglobin*

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Background: *Bacillus anthracis* secretes IsdX2 to acquire heme from methemoglobin.

Results: Heme extraction occurs when IsdX2 near-iron transporter (NEAT) domains contain a residue with an amide side chain in the fifth position of the 310-helix.

Conclusion: An amide side chain is necessary and sufficient for heme extraction.

Significance: This work advances mechanistic understanding of the critical first step in heme acquisition for possible inhibitor development.

Several Gram-positive pathogenic bacteria employ near-iron transporter (NEAT) domains to acquire heme from hemoglobin during infection. However, the structural requirements and mechanism of action for NEAT-mediated heme extraction remains unknown. *Bacillus anthracis* exhibits a rapid growth rate during systemic infection, suggesting that the bacterium expresses efficient iron acquisition systems. To understand how *B. anthracis* acquires iron from heme sources, which account for 80% of mammalian iron stores, we investigated the properties of the five-NEAT domain hemophore IsdX2. Using a combination of bioinformatics and site-directed mutagenesis, we determined that the heme extraction properties of IsdX2 are dependent on an amino acid with an amide side chain within the 310-helix of the NEAT domain. Additionally, we used a spectroscopic analysis to show that IsdX2 NEAT domains only scavenge heme from methemoglobin (metHb) and that autooxidation of oxyhemoglobin to metHb must occur prior to extraction. We also report the crystal structures of NEAT5 wild type and a Q29T mutant and present surface plasmon resonance data that indicate that the loss of this amide side chain reduces the affinity of the NEAT domain for metHb. We propose a model whereby the amide side chain is first required to drive an interaction with metHb that destabilizes heme, which is subsequently extracted and coordinated in the aliphatic heme-binding environment of the NEAT domain. Because an amino acid with an amide side chain in this position is observed in NEAT domains of several genera of Gram-positive pathogenic bacteria, these results suggest that specific targeting of this or nearby residues may be an entry point for inhibitor development aimed at blocking bacterial iron acquisition during infection.

During infection, bacterial pathogens must acquire the essential nutrient iron from the host to survive (1). However, the majority of mammalian iron is tightly coordinated by heme, which is further sequestered by hemoproteins, such as hemoglobin (Hb) (2, 3). Nevertheless, bacteria can circumvent this “nutritional immunity” by using protein systems that acquire heme from heme sources (4–6).

The past 10 years have brought numerous advances in understanding how Gram-positive bacteria acquire iron and transport it into the cell. Specifically, a conserved protein domain termed a near-iron transporter (NEAT)2 is involved in heme uptake in pathogens, such as *Bacillus anthracis, Bacillus cereus, Staphylococcus aureus*, and *Streptococcus pyogenes* (7–15). NEAT domains fold into a β-sandwich and bind the heme iron atom through a conserved tyrosine located on the eighth β-strand (16–18). The pocket is bordered by a 310-helix that is thought to be critical for NEAT function, including heme and/or hemoprotein binding (9, 12, 19, 20). NEAT-NEAT heme transfer can be facilitated by the conserved iron-coordinating tyrosine (21–24). However, the exact structural and molecular mechanisms mediating NEAT domain heme acquisition from methemoglobin (metHb) remains to be identified.

To elucidate the structural requirements for heme acquisition from metHb, we chose to study the five highly similar yet functionally distinct NEAT domains of the hemophore IsdX2 (iron-regulated surface determinant X2), a secreted *B. anthracis* protein that enhances replication in low iron environments (20, 25). Here, we present evidence that a glutamine in the 310-helix is necessary for heme extraction by the NEAT domains of IsdX2, which seems to initiate a NEAT-metHb interaction. The crystal structure of IsdX2 NEAT5 revealed the position of this residue as being solvent-exposed and may be in a primary position to interact with metHb. Unraveling the mechanism of action of bacterial NEAT proteins will aid in the development of small molecule inhibitors that block heme uptake and provide novel avenues for the development of new therapeutics.

*This work was supported, in whole or in part, by National Institutes of Health Grants AI097167 and AI069697 (to A. W. M.) and AI081161 (to C. W. G.). The atomic coordinates and structure factors (codes 4H8P and 4H8Q) have been deposited in the Protein Data Bank (http://wwpdb.org/j).†1 To whom correspondence should be addressed: Dept. of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-7369; Fax: 713-798-7375; E-mail: maresso@bcm.edu.

1 The abbreviations used are: NEAT, near-iron transporter; metHb, methemoglobin; oxyHb, oxyhemoglobin; r.m.s., root mean square.
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EXPERIMENTAL PROCEDURES

Bacterial Strains, Reagents, and Cloning—Escherichia coli strains (DH5α or BL21) were grown in Luria-Bertani (LB) supplemented with 50 μg/ml ampicillin (Fischer). The creation of pGEX2Tkgst-isdX2NEAT3 and -isdX2NEAT5 expression plasmids was described previously (13). For the creation of the NEAT point mutants, pGEX2TK plasmids containing wild type (WT) isdX2NEAT3 and isdX2NEAT5 inserts were subject to pfu-turbo (Agilent) PCR with primers containing specific nucleotide mismatches to produce the desired amino acid mutations: NEAT3 T29Q forward, 5'-CGA AAT TTC AAT GAT CAA CCA ATA TAC AAA AAG AGG-3'; NEAT3 T29Q reverse, 5'-CCT GGA CTT TTT GTA TAG TGG TTC ATC ATT GAA ATT TCG-3'; NEAT3 T29N forward, 5'-CGA AAT TTC AAT GAT CAA CCA ATA TAC AAA AAG AGG-3'; NEAT3 T29N reverse, 5'-CCT GGA CTT TTT GTA TAG TGG TTC ATC ATT GAA ATT TCG-3' and -GTG TAC ATC ATT CAT GGA ATT TCG-3'; NEAT5 Q29T forward, 5'-GAA ATT TCA ATC AGT ATA GAC ACA TAT GTT GTA AGT CCA GCA AGG-3'; NEAT5 Q29T reverse, 5'-CCT GTC TGG ACT TAC ATA GTG TAC ATC ATT CAT GGA ATT TCG-3'; NEAT5 Q29N forward, 5'-GAA ATT TCA ATC AGT ATA GAC ACA TAT GTT GTA AGT CCA GCA AGG-3'; NEAT5 Q29N reverse, 5'-CCT TGC TGG ACT TAC ATA GTG TAC ATC ATT CAT GGA ATT TCG-3'. After PCR amplification, plasmid DNA was purified using the GeneClean kit (Qiagen) and subjected to DpnI (New England Biolabs) digestion to eliminate the template. WT and mutant NEAT domains were amplified with primer sets specific for each NEAT domain and cloned into the pGEX2TK plasmid using the In-Fusion cloning kit (TaKaRa). Each mutation was transformed into DH5α E. coli, and the mutation was verified by DNA sequencing. Plasmid DNA was then transformed into chemically competent BL21 E. coli.

Protein Purification—E. coli BL21 strains harboring pGEX2TK-gst-neat, were grown in iron-depleted medium or LB supplemented with 50 μg/ml ampicillin and 25 μg/ml kanamycin (EMD Millipore). Each protein was expressed using isopropyl 1-thio-β-D-galactopyranoside (1.5 mM; Sigma) induction for 1.5 h at 37 °C or overnight at 30 °C. Cells were centrifuged (6,000 × g) and resuspended in 50 mM Tris-HCl, pH 7.0. Bacteria were lysed using a French press and centrifuged at 30,000 × g for 15 min, and supernatants were subjected to affinity chromatography with glutathione-Sepharose resin (Amersham Biosciences). After one 30-ml wash in buffer, each protein was eluted off of the column after incubation with 50 mM reduced glutathione (Calbiochem) to isolate NEAT, or with 25 mM glutathione (Calbiochem) to isolate GST-NEAT. Thrombin was removed from protein preparations using aminobenzamidine resin (Sigma). WT and mutant NEAT domains precipitated upon treatment with methyl ethyl ketone, a common procedure used to make apo forms of hemoproteins (13, 26). The use of iron-depleted medium, which is a minimal medium, reduces the amount of endogenous heme from E. coli that co-purifies with the NEAT domains, providing essentially “apo” NEAT preparations (molar heme concentration calculations showed <1 μM heme bound; data not shown). NEAT domains produced in this manner were used for all of the experiments in this report with the exception of the “qualitative” heme association results shown in Fig. 2, C and D. The concentration of recombinant NEAT was determined using the Bradford assay (Bio-Rad) (27) or by using the extinction coefficients reported previously (13). Heme concentrations were measured as described previously (13).

Measurement of NEAT Domain Heme Binding—For a qualitative measure of heme binding, E. coli expressing NEAT, were grown in 500 ml of LB for 3 h at 37 °C, followed by isopropyl 1-thio-β-D-galactopyranoside induction overnight at 30 °C. Each protein was then purified as described above, and the absorbance spectrum of the purified recombinant NEAT protein was scanned from 250 to 650 nm using a Beckman Coulter DU800 spectrophotometer.

For quantitative heme-binding analysis, each NEAT protein was purified in the apo form, as described above, titrated with hemin (4 μM; Sigma) and incubated at room temperature for 15 min. Spectral absorbances were then collected from 250 to 650 nm and compared with hemin-only controls.

Heme Scavenging from MetHb—GST-NEAT, (∼10 μM final concentration) was immobilized on 1 ml of glutathione-Sepharose resin and washed with 30 ml of Tris-HCl (50 mM, pH 7.0). The resin-NEAT complex was incubated with 1 ml (3.5 μM monomer) of bovine metHb (Sigma) or buffer alone at 25 °C. After 30 min, the supernatant (metHb) was removed, and the resin-NEAT was washed with 30 ml of buffer. Next, each GST-tagged NEAT, was eluted with 4 ml of 25 mM reduced glutathione, and relative heme content was determined by recording the intensity of the Soret band compared with an equivalent reaction of GST-NEAT, incubated with buffer only. SDS-PAGE was performed on each sample to verify that any differences in NEAT protein amounts or carryover of metHb into NEAT elutions (data not shown) (13).

Heme Scavenging from Oxyhemoglobin (OxyHb)—Experimental methods were identical to that of heme scavenging from metHb (above), with some exceptions. Here, oxyHb was used as the heme donor, instead of metHb, and 1 ml of oxyHb (20 μM tetramer) was incubated with the immobilized NEAT domains for 2 h. The resulting NEAT elutions were analyzed as reported for the metHb heme scavenging assay.

NEAT Domain Interaction with MetHb—A BIAcore 3000 biosensor (Amersham Biosciences) was used to measure the interaction of each NEAT domain with holo-metHb as described previously (13). Briefly, holo-metHb (in 50 mM Tris-HCl, pH 7.0) was covalently coupled to a CM5 sensor chip at 25 °C to a density of 3,600 response units using amine chemistry (28, 29). Each NEAT protein (1–10 μM) in HBS-N buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) was injected at 30 μl/min for 400 s at 25 °C. Data were obtained for each reaction using parallel injections of analyte flowed over a control surface and then over immobilized metHb. The kinetics and affinity constants were calculated using BLAevaluation version 4.1 software (Amersham Biosciences), and the data were fit as described (30). All fits for this kinetic analysis had a χ² value less than two. S.D. values for each NEAT protein were calculated from three different concentrations of NEAT injected from two independent experiments (n = 6).

Cloning of IsdX2 NEAT5 WT and IsdX2 NEAT5 Q29T and Protein Purification for Crystallization—IsdX2 NEAT5 WT and Q29T were cloned out of the pGEX2TK plasmids using the
TABLE 1
Crystalllography statistics for holo-IsdX2 NEAT5 WT and holo-IsdX2 NEAT5 Q29T

| Data collection, phasing, and refinement statistics | WT | Q29T |
|----------------------------------------------------|----|------|
| Space group | 1 2.3 | 1 2.3 |
| Wavelength (Å) | 0.98 | 1.0 |
| Cell dimensions | | |
| a, b, c (Å) | 109.02, 109.02, 109.02 | 109.09, 109.09, 109.09 |
| α, β, γ (degrees) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 54.51–2.05 | 38.57–1.49 |
| Completeness (%) | 99.7 (99.3) | 100 (100) |
| Unique reflections | 13571 | 35031 |
| Rmerge (%) | 14.8 (41.1) | 7.3 (15.5) |
| Mean I/σI | 8.8 (5.1) | 30.9 (3.4) |
| Redundancy | 7.4 (7.5) | 44.5 (43.2) |
| Refinement | | |
| Resolution (Å) | 34.48–2.05 | 38.57–1.7 |
| Rwork/Rfree (%) | 19.6/22.3 | 19.8/21.7 |
| No. of protein atoms | 999 | 1014 |
| No. of water molecules | 99 | 130 |
| Heme/monomer | 1 | 1 |
| r.m.s. deviation, bond lengths (Å) | 0.007 | 0.006 |
| r.m.s. deviation, bond angles (degrees) | 1.24 | 1.06 |
| Diffraction precision index (Å) | 0.129 | 0.081 |
| Ramachandran plot statistics (%) | | |
| Preferred regions | 98.3 | 99.2 |
| Allowed regions | 1.7 | 0.8 |
| Outliers | 0 | 0 |
| Protein Data Bank code | 4H8P | 4H8Q |

* Values for the highest resolution shell are shown in parentheses.
* Rmerge = Σ|I_i|/ΣI_i, where I_i is the observed intensity for reflection hkl, and I is the mean intensity.
* Rwork = Σ|Fo|−|Fc|/Σ|Fo|; Rfree is calculated in the same way with 5–10% of reflections excluded from refinement, and the same Rfree reflections were used for refinement of both WT and Q29T holo-IsdX2-NEAT5 models.
* The diffraction precision index is calculated using the formula, d(C)<br>work = N_C/n_C, where N is the number of carbon, nitrogen, and oxygen atoms, including water molecules. C is the completeness and n is the total number of independent intensities obtainable to resolution limit dwork.

following primers: forward, 5′-GGCC TATA GATG ATG GTC AC-3′; reverse, 5′-CCGC ATCC TAC TAA TTT GCA TCA AAT T-3′. The forward primer contained an NheI and the reverse a BamHI restriction endonuclease site, which were used to clone the respective DNA into a pET28a vector (Novagen). IsdX2 NEAT5 WT and IsdX2 NEAT5 Q29T were expressed and purified according to the procedure described by Ekworomadu et al. (12). pET28a harboring each gene was transformed into E. coli BL21-Gold (DE3) cells and grown at 37 °C in LB medium containing 30 μg/ml kanamycin. After 4 h, the cells were centrifuged at 6,000 × g, the supernatant was removed, and the cells were resuspended in 50 mM Tris, pH 7.4, 300 mM NaCl. Cell lysis by sonication was conducted after the addition of egg albumin lysozyme (5 mg; Sigma) with phenylmethylsulfonyl fluoride (40 μM; Sigma), and the cell lysate was centrifuged at 30,000 × g for 20 min. The supernatant was loaded onto a Ni2+-charged HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (between 100 and 250 mM imidazole). Fractions containing IsdX2 NEAT5 WT were identified by SDS-PAGE, pooled, and dialyzed into 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl2 (cleavage buffer). Cleavage of the His tag was done in cleavage buffer by adding 0.5 mM of thrombin-agarose suspension (Sigma) to the protein, followed by the removal of thrombin-agarose on a glass frit. The protein was then run over a Ni2+-charged HisTrap column to separate IsdX2 NEAT5 WT or IsdX2 NEAT5 Q29T from the cleaved His tag. Final purification was achieved by running each protein over an S75 gel filtration column equilibrated with 50 mM Tris, pH 7.4, 150 mM NaCl to yield 100% homogeneous protein.

Crystallization of IsdX2 NEAT5 WT/Q29T and Data Collection—Crystals of holo-IsdX2 NEAT5 WT were grown at 100 mg/ml by the hanging drop, vapor diffusion method against a reservoir containing 10% PEG 3000, 0.2 mM zinc acetate, and 0.1 M sodium acetate, pH 4.5, with crystallization drops containing 1 μl of protein to 1 μl of reservoir solution. Crystal growth was observed within 24 h. The crystal was passaged through a 1:1 (v/v) solution containing the reservoir solution and 40% glycerol for cryoprotection, and the crystals were harvested under cryoconditions. The diffraction data were collected at 77 K on beamline 7-1 at the Stanford Synchrotron Radiation Lightsource at 0.98 Å. Crystals of holo-IsdX2 NEAT5 Q29T were grown at 60 mg/ml by the hanging drop, vapor diffusion method against a reservoir containing 5% PEG 3000, 0.2 mM zinc acetate, and 0.1 M sodium acetate, pH 5.25, with crystallization drops containing 2 μl of protein to 1 μl of reservoir solution. Crystal harvesting and data collection were carried out in the same manner as for the IsdX2 NEAT5 WT protein. Data were collected on beamline 9-2 at the Stanford Synchrotron Radiation Lightsource at 1.0 Å.

Structure Determination for IsdX2 NEAT5 WT/Q29T—Images were indexed, integrated, and reduced using the Mosfilm suite, resulting in a nearly complete data set to 2.05 Å resolution for IsdX2 NEAT5 and a 100% complete data set at 1.49 Å resolution for IsdX2 NEAT5 Q29T. The complete crystallographic statistics can be found in Table 1. The phases were calculated using AutoMR (31) utilizing holo-IsdX1 (Protein Data Bank code 3SIK) (12) as the search model for IsdX2 NEAT5 WT and IsdX2 NEAT5 WT as the search model for IsdX2 NEAT5 Q29T. The resulting electron density map was subjected to automated and manual model building procedures through a
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domains had intense Soret signals, suggesting co-purification. As indicated in Fig. 2, the spectroscopic assay used to detect the presence of bound heme iron (7, 41), Soret absorbance at 400 nm, a well-documented spectroscopic assay, was used to analyze the stereochemistry and geometry of the models, which were found to be acceptable.

**RESULTS**

Identification of Functional Residues Using Informatics—We previously described the heme acquisition and heme transfer properties of the five NEAT domains of the anthrax hemophore, IsdX2 (13). These NEAT domains share ~70% similarity at the amino acid level, except for NEAT2 (Fig. 1). We aimed to find a position where the heme-scavenging NEAT domains (NEAT1 and NEAT5) had the same amino acid, and the non-scavenging NEAT domains (NEAT2, NEAT3, and NEAT4) had a different amino acid (13). Using these criteria, only one position was identified: the fifth amino acid in the 310-helix (the 29th amino acid overall; Fig. 1). Specifically, the heme-scavenging NEAT domains had a glutamine (Gln-29) at this position, whereas the non-heme-scavenging NEAT domains had a threonine (Thr-29). This residue was thus chosen as a potential mediator of heme scavenging.

We hypothesized that the presence of an amino acid with an amide side chain found at this site within the helix was dictating the heme extraction functions of IsdX2 NEAT1 and NEAT5. Therefore, we performed site-directed mutagenesis to swap the fifth amino acid in this region of NEAT3 with that of NEAT5. Mutation did not affect heme binding, recombinant NEAT domains expressed overnight were assayed for their ability to co-purify with endogenous heme during expression in E. coli. Each NEAT domain was purified as stated under “Experimental Procedures,” and bound heme was detected by measuring the Soret absorbance at ~400 nm, a well-documented spectroscopic assay used to detect the presence of bound heme iron (7, 12, 20, 24, 35–40). As indicated in Fig. 2, C and D, all NEAT domains had intense Soret signals, suggesting co-purification with heme. Because the wavelength of the respective Soret maxima did not change for the NEAT3 or NEAT5 mutants and the Q-bands remained at 500 and 630 nm, we concluded that the mutant domains were binding oxidized heme (hemin) in the same high spin, five-coordinate state as the WT domains (41).

To further investigate the ability of these proteins to bind heme, each NEAT domain was incubated with 4 μM hemin. In
Fig. 3 (gray lines), the 4 μM hemin-only control is low in intensity and has a characteristic broad left shoulder. In contrast, when hemin is incubated with all assayed NEAT domains, a red shift and increase in Soret band absorbance are observed, along with its narrowing, a strong indicator that heme has been bound (Fig. 3, compare black lines with gray lines). It should be noted that the wavelength for the Soret band remained at 403 nm for all NEAT3 recombinant proteins, and 404 nm for all NEAT5 recombinant proteins. This suggests that 4/H9262M hemin is completely bound by the NEAT domains; otherwise, the Soret band maxima for the reconstituted NEAT domains would shift to the left, indicative of unbound heme present in the sample.

Whereas the NEAT domains purified out of E. coli with some heme bound (Fig. 3, Soret band on NEAT-only control), the increases detected when exogenous hemin is added represent specific heme binding. Together, these data suggest that this single amino acid change in the 310-helix does not affect the ability of the mutant NEAT domains to bind heme.

**Heme Scavenging from MetHb**—One of the critical roles of the anthrax hemophore IsdX2 is to scavenge heme from metHb, the initial step of heme iron acquisition (13, 20). We therefore sought to identify the mechanism behind this function and aimed to determine the role of Gln-29 in this process. Each GST-tagged NEAT domain was immobilized on glutathione-Sepharose resin and incubated with metHb or buffer alone for 30 min at room temperature, and eluted NEAT fractions were analyzed for heme content by Soret spectroscopy. NEAT5 WT demonstrated an increase in the Soret band maxima after incubation with metHb (Fig. 4A, left), consistent with data published previously (13). Measurement of the heme levels (Table 2) indicated that NEAT5 WT incubated with metHb had more bound heme relative to the amount of endogenous heme originally bound (133% increase). In contrast, there was no increase in Soret band absorbance for NEAT3 WT (Fig. 4A, left) or an increase in heme concentration (Table 2), suggesting that this NEAT domain was incapable of scavenging heme, as reported previously (13).

When the mutant proteins were analyzed for their ability to scavenge heme, a striking difference was observed. The NEAT5 Q29T mutant did not acquire heme from metHb under the same conditions as NEAT5 WT (Fig. 4B, middle panel; compare with left panel). In contrast, NEAT3 T29Q was now able to acquire heme from metHb, compared with NEAT3 WT (Fig. 4A, middle), with a 63% increase in bound heme compared with endogenous heme bound before metHb addition (Table 2). The finding that only a single amino acid was mutated in these two NEAT domains suggests that Gln-29 in the 310-helix is important for heme extraction. In data not shown, all NEAT domains analyzed in this experiment retained heme binding function after elution from the resin, even if heme scavenging did not occur.

Based on these results, we also generated NEAT3 T29N and NEAT5 Q29N NEAT domains that were subjected to the same analysis to determine their ability to acquire heme from metHb. As shown in Fig. 4, the asparagine mutant for NEAT3 acquired heme from metHb, because an increase in Soret band absorbance was detected after incubation with metHb (Fig. 4A, right), correlating with a 63% increase in the concentration of bound heme (Table 2). Also, the NEAT5 Q29N mutant was able to scavenge heme from metHb, similar to NEAT5 WT (Fig. 4B, right), with a 109% increase in heme concentration (Table 2). Together, these data suggest that a single amino acid with
amide side chain chemistry at the fifth residue in the 3_10-helix is important for the heme extraction function of IsdX2.

**Determination of NEAT Domain Affinity for MetHb**—Previous work on IsdX2 suggested that the ability to scavenge heme could be attributed to a higher affinity between NEAT and metHb (13). Therefore, we subjected the NEAT domains to surface plasmon resonance. As indicated in Table 3, the $K_D$ for the heme-scavenging NEAT3 mutants with metHb decreased (higher affinity) 43% (T29Q) and 67% (T29N) compared with NEAT3 WT. Also, the NEAT5 Q29T, which lost the ability to extract heme, showed a 58% increase (lower affinity) in $K_D$ compared with NEAT5 WT. In contrast, NEAT5 Q29N, a mutant that retained heme-scavenging ability, showed essentially no change in affinity for metHb (Table 3). These data suggest that the presence of an amide side chain at the fifth position in the 3_10-helix aids in the association of NEAT domains with metHb.

**Heme Scavenging from OxyHb**—Previous studies analyzing NEAT domains for the ability to scavenge heme have exclusively used metHb as the heme source (5, 9–15, 19, 20, 36, 39, 42–44). We questioned if oxyHb, a biologically relevant form of Hb, could also be a source of heme for NEAT domains.

To test this possibility, each NEAT domain was incubated with oxyHb or buffer alone, separated by affinity chromatography, and analyzed for heme content by Soret spectroscopy. No heme extraction from oxyHb was detected for NEAT5 WT after 30 min of incubation (data not shown). After 2 h of incubation, however, NEAT5 WT exhibited an increase in the Soret peak, suggesting that heme scavenging had occurred (Fig. 5B, left, compare dotted and solid lines). The heme concentration...
Table 4 indicated that NEAT5 WT had more heme bound relative to the amount of heme originally present on each NEAT (72% increase). In contrast, when we performed the heme scavenging analysis on NEAT3 WT, there was no increase in Soret band intensity after a 2-h incubation with oxyHb (Fig. 5A, left), suggesting that this NEAT is unable to scavenge heme from oxyHb, which is consistent with the lack of heme scavenging seen with metHb (Fig. 4A, left).

Analysis of the mutant proteins demonstrated differences in their ability to scavenge heme from oxyHb. The NEAT5 Q29T mutant did not acquire heme from oxyHb (Fig. 5A, middle). Table 4 also indicated that there was a 67% increase in heme concentration after NEAT3 T29Q was incubated with oxyHb. Both NEAT3 T29N and NEAT5 Q29N showed an ability to extract heme from oxyHb (Fig. 5, A and B, right panels). These data suggest that the IsdX2 NEAT domains are able to acquire heme from oxyHb, which is also dependent on an amide side chain in the fifth position of the 3$_{10}$-helix. However, heme extraction only occurred after prolonged incubation (2 h), suggesting that the higher oxyHb-heme affinity is an impediment in the uptake process. Interestingly, the NEAT domains (WT and mutants) did not alter the oxidation rate of oxyHb (overall rates for each $\sim$0.036 h$^{-1}$), which indicates that differences in heme extraction among these NEAT domains is not due to NEAT-induced autoxidation.

Structures of IsdX2 NEAT5 WT and Q29T—To determine the orientation and positioning of Gln-29 in NEAT5, we solved the structure of recombinant NEAT5 WT and the Q29T mutant. IsdX2 NEAT5 WT was solved to 2.05 Å by molecular replacement using the structure of IsdX1 (Protein Data Bank code 3SIK) as the search model. The eight $\beta$-strands of IsdX2 NEAT5 are arranged in two antiparallel $\beta$-sheets, which form a $\beta$-sandwich (Fig. 6A), consistent with the immunoglobulin-like fold, as observed in multiple NEAT domain crystal structures from B. anthracis and S. aureus (8, 12, 16, 17, 35, 45, 46).

The heme binding pocket is formed by a long $\beta$-hairpin on the proximal face of the heme molecule and the 3$_{10}$-helix on the distal face. The heme iron is coordinated by the oxygen atom of Tyr-108, with the Fe–O distance of 2.0 Å (Fig. 6B). Hydrogen bonding between Tyr-112 and Tyr-108 renders the hydroxyl group on Tyr-108 more electronegative, thereby increasing the stability of the coordination bond between Tyr-108 and heme iron. Further stabilization of bound heme is achieved through hydrogen bonding between Ser-25 and the least solvent-exposed heme propionate group, together with $\pi$-stacking inter-
actions of the phenyl groups of Tyr-30 and Tyr-112 with the porphyrin ring (Fig. 6B). The side chain of Met-26 is positioned over the open distal face of the heme molecule and assumes two conformations, both of which provide non-polar and weak electrostatic interactions with the heme group (Fig. 6, B and C). The sulfur atoms of the two Met-26 conformations lie 4.2 and 5.3 Å from heme iron, whereby these distances preclude formation of a coordination bond between Met-26 and heme iron. Furthermore, the absorption spectrum of ferric IsdX2 NEAT5 WT is indicative of high spin, five-coordinate heme, further suggesting that the Met-26 does not coordinate heme iron in the ferric state (Fig. 2D). The remaining residues lining the heme binding pocket, Met-27, Asn-52, Trp-55, Val-99, Val-101, and Val-114, provide an aliphatic environment for the coordination of the nonpolar heme molecule.

The structure of the Q29T mutant of IsdX2 NEAT5 was solved to 1.49 Å by molecular replacement. The structures overlay almost completely with a r.m.s. deviation of 0.07 Å over all Cα atoms and do not feature any major differences in terms of backbone and side chain orientation (Table 1). Inspection of the heme binding pocket of IsdX2 NEAT5 WT and NEAT5 Q29T revealed no changes in the orientation of heme, the coordinating Tyr-108, and the residues surrounding the heme molecule (Fig. 6C). Furthermore, no changes in the structure of the 310-helix were observed, suggesting that the Q29T mutation impacts neither the first nor second coordination sphere of the iron. Gln-29 is not pointing into the heme binding pocket and is only indirectly part of the heme coordination environment through hydrogen bonding to the hydroxyl group of Tyr-30 and to a structural water molecule located between Gln-29 and Met-26. The latter interaction is maintained for the Q29T mutant.

A single IsdX2 NEAT5 WT monomer is present in the asymmetric unit (Fig. 7A). Two molecules of IsdX2 NEAT5 WT interact along a symmetry-related interface through distant hydrogen-bonding interactions between Tyr-30 and the backbone oxygen of Met-106 and between Gln-29 and Asn-107 of the adjacent molecules (Fig. 7A). Unlike IsdX1, the interface between IsdX2 NEAT5 monomers does not feature heme-stacking interactions (Fig. 7, compare B with A), with the heme iron atoms between crystallographic symmetry-related IsdX2 NEAT5 WT molecules being 10.2 Å apart. The significance of these results in the context of heme acquisition and heme transfer is discussed below.

DISCUSSION

The NEAT proteins IsdX1, IsdX2, and Hal from B. anthracis, full-length IsdB from S. aureus, and full-length Shr from S. pyogenes have all been proposed to extract heme from metHb (9, 11–13, 15, 19, 20, 43, 47). Heme acquisition from Hb is the first step in the multistage pathway required for the uptake of heme iron. Because the bacterial sequestration of host iron is required for the establishment and sustainability of an infection, the inhibition of this step could represent an entry point for thera-
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When the NEAT domains were analyzed for their ability to scavenge heme from metHb, the domains containing an amino acid with an amide side chain at position 29 were able to scavenge heme within 30 min (Fig. 4). This time frame was chosen so that there was no spontaneous heme dissociation from metHb subunits at room temperature (48). This suggests that heme scavenging in these experiments is an active process, with Gln-29/Asn-29 comprising a portion of the machinery needed to facilitate the process. In contrast, no heme scavenging was observed by NEAT5 WT when incubated with oxyHb for 30 min or 1 h at room temperature (data not shown). However, upon 2 h of incubation, NEAT domains with an amide 29 side chain all scavenged heme from oxyHb, and the bound heme was oxidized (Fig. 5). Additionally, when we measured the autoxidation rates of oxyHb in the presence of the NEAT domains, we observed rates similar to the natural autoxidation rate of oxyHb (49). This suggests that within the 2-h incubation step, some oxyHb may be oxidized to metHb, and the NEAT domains with an amide 29 side chain target the newly formed metHb pool for heme extraction. We therefore propose that the IsdX2 NEAT domains, and possibly other NEAT domains, preferentially target heme from metHb, which has a lower affinity for heme than oxyHb and is less stable (48).

The difference between a passive (heme is acquired after thermal dissociation from Hb) and active (heme transfer rates are accelerated by a physical interaction) process is an important distinction when considering the design of inhibitors to block bacterial heme uptake. Although we are currently devising ways to measure the kinetics of these transfer processes (which would aid in determining reaction mechanisms), the following results suggest that an active process of heme extraction is the case for IsdX2: (i) heme scavenging occurred within 30 min, which is faster than the thermal rates of heme loss from α and β metHb subunits (48); (ii) the mutations do not seem to affect the ability of the NEAT domain to associate with heme, and the crystal structures of NEAT5 WT and Q29T show no interactions between the heme and these residues or structural alterations from mutagenesis; and (iii) there is a physical interaction between the NEAT domain and metHb, with stronger affinities associated with the heme-scavenging domains. Recent data from Spirig and Clubb (46) examining two NEAT domains of IsdH indicated that there may be other mechanisms for heme removal, including NEAT-induced disruption of the tetramer, which accelerates spontaneous heme loss by creating less stable Hb dimers. This activity was dependent on the physical joining of IsdH NEAT2 and NEAT3 domains, which are spaced by an ~70-amino acid linker. There is the possibility that the NEAT domains of IsdX2 may also act synergistically to attain heme from Hb. However, the extreme sensitivity of full-length IsdX2 to proteolysis, after expression in E. coli or B. anthracis, has thus far precluded this analysis (13).

The structures of IsdX2 NEAT5 WT and NEAT5 Q29T are both similar to the characterized hemophore IsdX1 in both the holo and apo form, reflecting the same function as a hemophore (Fig. 8) (12). Although the NEAT5 Q29T mutant could not scavenge heme, both glutamine and threonine are able to receive and donate hydrogen bonds; therefore, a property beyond the ability to form hydrogen bonds may be required at...
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**FIGURE 8.** Superimposition of holo-IsdX1, apo-IsdX1, and holo-IsdX2 NEAT5 WT. A, overlay of holo-IsdX2 NEAT5 WT (pale orange), holo-IsdX1 (green), and apo-IsdX1 (purple) in a ribbon representation. B, superimposition of the heme-binding site and of the 3_{10}-helix of holo-IsdX2 NEAT5 WT (pale orange) and holo-IsdX1 (green) in a ribbon representation. The important heme binding residues are highlighted in a stick representation, where sulfur, nitrogen, oxygen, and iron atoms are colored yellow, blue, red, and orange, respectively.

**TABLE 5**
Comparison of 3_{10}-helix amino acid sequence with heme extraction function

| NEAT Domain | 3_{10}-helix | Heme Binding Function | Heme Extraction Function | References |
|-------------|--------------|-----------------------|--------------------------|-------------|
| IsdX2 NEAT1 | SMNNQY       | Yes                   | Yes                      | (13)        |
| IsdX2 NEAT2 | SMNNQY       | No                    | N/A                      | (13)        |
| IsdX2 NEAT3 | SMNNQY       | Yes                   | No                       | (13)        |
| IsdX2 NEAT4 | SMNNQY       | Yes                   | No                       | (13)        |
| IsdX2 NEAT5 | SMNNQY       | Yes                   | Yes                      | (13)        |
| IsdX1    | SMNNQY       | Yes                   | Yes                      | (12, 25)    |
| BslK     | SVASTY       | Yes                   | No                       | (36)        |
| IsdB NEAT1 | FHYYAS       | No                    | N/A                      | (43)        |
| IsdB NEAT2 | SMNNQY       | Yes                   | Unknown                  | (43)        |
| IsdH NEAT1 | YYHFFS       | No                    | N/A                      | (14)        |
| IsdH NEAT2 | FHYYAS       | No                    | N/A                      | (14)        |
| IsdH NEAT3 | SVMQGF       | Yes                   | Unknown                  | (14)        |
| llsa     | SMVQGS       | Yes                   | Unknown                  | (10)        |
| ShRET1   | SMQGGQ       | Yes                   | Unknown                  | (39, 44)    |
| ShRET2   | SMNNKA       | Yes                   | No                       | (39, 44)    |

this position for heme extraction activity. Along these lines, the side chain of glutamine is longer than threonine and could thus interact with potential binding partners at a shorter distance. Also, the presence of a guanidine group in IsdX1 Arg-54 and an amide in IsdX2 NEAT5 Gln-29 may suggest that there is a specific requirement for an amide within this location on the 3_{10}-helix for effective heme extraction (Table 5).

In addition to insights into heme acquisition and binding, the crystal packing interactions formed between IsdX2 NEAT5 WT monomers in neighboring asymmetric units provided information on how NEAT5 WT may accept heme (Fig. 7A). Like the structures of holo-IsdX1 (Fig. 7B), two monomers of IsdX2 NEAT5 WT interact with each other in the vicinity of the heme-binding site along a crystallographic symmetry interface. Recent computational NMR and cross-linking studies have shown that the crystal packing interactions reflect the solution state of NEAT-NEAT heme transfer via a “handclasp” mechanism (21–23). Therefore, the weakly interacting IsdX2 NEAT5 WT monomers may provide a picture of the initial contacts that are formed when NEAT domain monomers prepare for heme transfer (Fig. 7A). One of the hydrogen-bonding interactions formed between two IsdX2 NEAT5 WT monomers is mediated by Gln-29. In IsdX1, the equivalent residue to Gln-29 is Arg-54, which hydroxide-bonds with the more solvent-exposed propionate heme group from the adjacent IsdX1 monomer. This further suggests that Gln-29 is critical for initiating and enabling protein-protein interactions, possibly with metHb during heme scavenging. In contrast to IsdX2 NEAT5 WT, the crystal packing interactions of IsdX1 contained several interface contacts and suggested that IsdX1 efficiently transferred heme to other NEAT domains, as previously documented for both IsdX2 and IsdC (Fig. 7B) (12, 20, 25, 52). However, the fact that IsdX2 NEAT5 WT monomers have a minimal area of interaction supports previous findings that NEAT5 is unable to transfer heme to other NEAT domains, such as IsdC (13). Also, the surface charge surrounding the heme molecule of IsdX2 NEAT5 WT is more neutral compared with IsdX1, which suggests that IsdX2 NEAT5 WT may not form the electrostatic interactions with IsdC required for heme transfer. Whether IsdX2 NEAT5 transfers heme to a non-NEAT cell surface receptor, however, remains to be elucidated.

When comparing the heme scavenging data in this study with the data previously reported for other NEAT domains, we observed a correlation between Gln-29 and active NEAT heme extraction function (Table 5) as follows. (i) BslK, a B. anthracis surface protein, possesses a heme-binding NEAT domain with a threonine at the fifth helical position and is unable to scavenge heme from metHb (36). (ii) The S. aureus IsdB protein contains two NEAT domains, where only NEAT2 binds heme. However, NEAT2 possesses a threonine at the fifth helical position and has not been tested alone for heme scavenging function (43). (iii) S. aureus IsdH has three NEAT domains of which only NEAT3 binds heme and possesses a glycine at the fifth helical position. Full-length IsdH demonstrated heme acquisition rates from metHb similar to the rates of spontaneous heme loss from metHb, suggesting a passive extraction process (14). (iv) llsa of B. cereus is an essential surface protein for growth on metHb as a sole iron source, and the single NEAT domain possesses a glutamine at the fifth 3_{10}-helical position. This suggests possible heme extraction function; however, biochemical evidence of the link between llsa Gln-29 and heme extraction from metHb remains to be determined (10). (v) The S. pyogenes Shr protein possesses two NEAT domains, and the full-length protein is proposed to scavenge heme from metHb. Because Shr NEAT1 harbors a glutamine at the fourth 3_{10}-helix position, close to the IsdX2 NEAT5 Gln-29, this may indicate that Shr NEAT1 possesses the critical amide side chain amino acid...
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for active heme extraction from metHb; however, this remains to be determined biochemically (39, 44).

Krishna Kumar et al. (50) recently solved the crystal structure of IsdH NEAT1 (Hb receptor) interacting with metHb α-subunits. This NEAT domain is unable to bind or scavenge heme; therefore, the interface detected with metHb suggests that IsdH NEAT1 may perform a scaffolding function and capture metHb (14). Because NEAT domains are conserved among several bacterial species, these results may indicate that IsdX2 NEAT2, which does not bind or scavenge heme but physically interacts with metHb, functions in a similar manner as IsdH NEAT1, acting as the IsdX2-metHb scaffold (13). However, this remains to be confirmed. Once IsdX2 NEAT2 binds and “captures” metHb, IsdX2 NEAT1 and NEAT5 could interact with the heme binding pocket on metHb subunits, trigger the destabilization of heme coordination via Gln-29, and undergo a rapid heme extraction event.

In summary, we have utilized the highly similar yet functionally distinct IsdX2 NEAT domains to identify side chain chemistry and the mechanistic determinants of heme extraction from mammalian metHb. We propose that the IsdX2 NEAT5 Gln-29 residue is exposed to potential protein binding partners and initiates the heme extraction process by interacting with the metHb-heme binding pocket. Because the side chain of glutamine is polar, it can interact with other polar or charged atoms. Threonine, however, cannot undergo the same interactions; therefore, we propose that this property of glutamine, along with the presence of the nitrogen in the amide side chain, allows the Gln-29 residue to interact with a charged amino acid in the heme binding pocket of Hb. This residue may be the Hb HisE7 residue, which is not coordinated to the heme when heme is oxidized in metHb. This could be the initiating interaction between NEAT and metHb. Upon destabilization of the metHb-heme interface, Tyr-108 could coordinate the heme iron, resulting in the completion of heme extraction and coordination within the aliphatic NEAT environment. Additional interactions that facilitate NEAT heme binding include Ser-25, Met-26, hydrogen bonding of Tyr-112 with Tyr-108, and π-stacking of Tyr-30 and Tyr-112 with the heme ring (Fig. 6B).

The data presented here have implications for the development of therapeutics for bacterial infections that harbor NEAT domains. First, a small molecule could be designed to bind to the 310-helix, thereby shielding residues in this region from any possible interaction with Hb. Because an amino acid with an amide side chain at the fifth position contributes to Hb association and the loss of this amide side chain abrogates heme scavenging, the inhibitor may block heme uptake. This could slow the rate of bacterial iron acquisition, a strategy that should impede or prevent bacterial replication in infected hosts. A second potential use of these results could be to make a better vaccine against Gram-positive bacteria. There are numerous reports on the use of IsdB as a vaccine for S. aureus, a leading cause of community-acquired and nosocomial infections worldwide (53–55). Based on this success, Merck initiated clinical trials to develop this NEAT protein as a vaccine (56). However, a phase II clinical trial was halted when it was found that there was potential toxicity associated with inoculation of the antigen (51). By knowing the amino acids that dictate NEAT functions, one can envision synthesizing antigens with mutations that eliminate NEAT heme binding and scavenging activity. This may in turn reduce toxicity associated with NEAT activity in a vaccine.

Acknowledgments—We thank Drs. John S. Olson and Marian Fabian (Rice University, Houston, TX) for critical reading and evaluation of the manuscript and for the kind donation of the oxyHb used for the heme scavenging study.

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