Staphylococcus aureus Uses a Novel Multidomain Receptor to Break Apart Human Hemoglobin and Steal Its Heme*

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Background: During infections, Staphylococcus aureus acquires heme-iron from human hemoglobin using the receptor proteins IsdH and IsdB.

Results: A conserved multidomain unit in IsdH and IsdB synergistically captures heme and destabilizes the hemoglobin tetramer.

Conclusion: Receptor domain synergy and hemoglobin dissociation allow efficient heme uptake by S. aureus.

Significance: IsdH and IsdB may represent novel targets for antibiotics that limit microbial access to iron.

Staphylococcus aureus is a leading cause of life-threatening infections in the United States. It requires iron to grow, which must be actively procured from its host to successfully mount an infection. Heme-iron within hemoglobin (Hb) is the most abundant source of iron in the human body and is captured by S. aureus using two closely related receptors, IsdH and IsdB. Here we demonstrate that each receptor captures heme using two conserved near iron transporter (NEAT) domains that function synergistically. NMR studies of the 39-kDa conserved unit from IsdH (IsdH$^{N2N3}$, Ala$^{326}$–Asp$^{660}$) reveals that it adopts an elongated dumbbell-shaped structure in which its NEAT domains are properly positioned by a helical linker domain, whose three-dimensional structure is determined here in detail. Electrospray ionization mass spectrometry and heme transfer measurements indicate that IsdH$^{N2N3}$ extracts heme from Hb via an ordered process in which the receptor promotes heme release by inducing steric strain that dissociates the Hb tetramer. Other clinically significant Gram-positive pathogens capture Hb using receptors that contain multiple NEAT domains, suggesting that they use a conserved mechanism.

Staphylococcus aureus is a leading cause of lethal hospital- and community-acquired infections in the United States. These infections result in a range of life-threatening diseases, such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. Highly virulent methicillin-resistant strains of S. aureus are now common, which annually cause more deaths in the United States (over 18,500) than any other single infectious agent (1). Iron is an essential nutrient required for S. aureus growth and is actively procured from its host during infections. As an innate defense mechanism, humans and other vertebrates exploit this dependence by sequestering the majority of the body’s total iron within cells and by binding extracellular iron to transferrin and lactoferrin glycoproteins (2, 3). Iron-protoporphyrin IX (heme), found in the oxygen transport protein hemoglobin (Hb), contains ~75% of the body’s total iron. As a result, S. aureus and other microbial pathogens have developed elaborate heme acquisition systems to exploit this rich nutrient source.

S. aureus captures heme-iron from human Hb using nine iron-regulated surface determinant (Isd) proteins (4–7). Four Isd proteins are attached to the cell wall and capture Hb, extract its heme, and pass it across the peptidoglycan to the membrane. These proteins include IsdA, IsdB, and IsdH (also known as HarA), which are attached to the cell wall by the SrtA sortase enzyme (8–11), and IsdC, which is attached to the cell wall by the SrtB sortase (12). Biochemical and cellular localization studies indicate that heme capture is mediated by an ordered set of heme transfer reactions. This process is initiated when the IsdH and IsdB proteins exposed on the cell surface bind Hb and remove its heme (8, 11). Heme is then transferred to IsdA, which, in turn, relays it to the IsdC protein buried within the cell wall (11, 13, 14). Holohistidine then passes heme to the IsdE-IsdF complex, a transporter that pumps heme across the membrane.

The abbreviations used are: Isd, iron-regulated surface determinant; NEAT, near iron transporter; SUMO, small ubiquitin-like modifier; ESI, electrospray ionization; HSQC, heteronuclear single quantum coherence; TOCSY, total correlation spectroscopy; RDC, residual dipolar coupling.

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into the cytoplasm. In the cytoplasm, the heme oxygenase IsdG or its paralog, IsdI, degrades the tetrapyrrole ring to release free iron for use by the bacterium (15). A molecular level understanding of the Isd system could facilitate the development of new anti-infective agents that work by disrupting heme uptake, because several studies have shown that its components are required for S. aureus virulence (12, 16–19), and related systems are present in a number of other important pathogens, including *Listeria monocytogenes* (20, 21), *Bacillus anthracis* (22), and *Streptococcus pyogenes* (23–25).

In the Isd system, both Hb and heme are captured by near iron transporter (NEAT) domains that are located within the IsdA, IsdB, IsdC, and IsdH proteins. These conserved binding modules are ~125 residues in length and are named for the location of their genes, which are proximal to putative Fe$^{3+}$ siderophore transporter genes (26). Biochemical studies of isolated NEAT domains indicate that they have distinct binding specificities that enable interactions with one or more distinct ligands, including heme, Hb, haptoglobin, and other host proteins. The atomic structures of several isolated NEAT domains have now been determined, revealing the mechanism of heme and Hb binding (27–29). In addition, recent studies have shown that heme transfer from IsdA to IsdC occurs when their NEAT domains transiently associate via an ultra-low affinity hand clasp complex (30, 31).

The first step in heme acquisition is the capture of Hb and the extraction of its heme molecules by IsdH and IsdB. Both receptors are potential targets for the development of novel antibiotics because *isdH* and *isdB* mutant strains of *S. aureus* are reduced in their ability to infect mice (16, 19, 32, 33), and purified antibodies against IsdH and IsdB confer protection from staphylococcal infections in animal models (33). IsdH and IsdB share a significant degree of primary sequence homology, and, unlike other components of the Isd system, they contain multiple NEAT domains. IsdB has been shown to bind Hb and capture its heme at least 150 times faster than the rate at which Hb spontaneously releases heme into the solvent, suggesting that the receptors capture heme via an activated receptor-Hb complex (14, 34). Here we demonstrate that heme capture by IsdB and IsdH is mediated by a conserved structured unit that contains two NEAT domains that are connected by an α-helical linker domain. We show, based on absorbance spectroscopy and electrospray ionization mass spectrometry (ESI-MS) measurements, that the linker domain in IsdH forms a three-helix bundle structure that is essential for efficient heme capture. NMR studies of a 39-kDa polypeptide containing the conserved unit from IsdH indicate that it adopts an extended but ordered structure. A model of the heme extraction process is presented in which IsdH dissociates the Hb tetramer to promote heme release.

**EXPERIMENTAL PROCEDURES**

Cloning, Protein Expression, and Purification—Plasmids were generated encoding IsdH and IsdB receptor constructs as small hexahistidine-ubiquitin-like modifier (SUMO)-tagged proteins under control of an inducible promoter: pRM208 coding for amino acids 326–660 in IsdH (IsdH<sub>linker-N2</sub>), pRM213 coding for amino acids 326–466 in IsdH (IsdH<sub>linker</sub>), and pRM219 coding for amino acids 467–543 in IsdH (IsdH<sub>linker-N2</sub>), and pRM221 coding for amino acids 544–600 in IsdH (IsdH<sub>N3</sub>). Briefly, the DNA was amplified from the *S. aureus* RN4220 genome by polymerase chain reaction (PCR) and cloned into the vector pHis-SUMO using BamHI and XhoI restriction enzymes (35). pRM233 coding for amino acids 326–660 (IsdH<sub>N2-GS-N3</sub>) was generated from pRM208 using two-step PCR, such that the linker was replaced with a nine-amino acid artificial linker (GSGSGSGSG). The sequence of all plasmids was verified by DNA sequencing. Generation of the plasmid pRM216 coding for amino acids 326–660 in IsdH with an alanine substitution of Tyr<sup>462</sup> (IsdH<sub>N2N3(Y642A)</sub>) has been described earlier (36). Protein expression in *Escherichia coli* BL21(DE3) cells (New England BioLabs) transformed with the overexpression plasmids in LB/kanamycin (50 µg/ml) was induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37 °C. For production of isotopically labeled [13C<sup>14</sup>N]protein, the cells were grown in M9 minimal medium containing [15NH<sub>4</sub>]Cl and [13C]glucose (Cambridge Isotope Laboratories). The bacterial cells were harvested by centrifugation, resuspended in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 7.0, and ruptured by sonication. The cell debris was removed by centrifugation, and the supernatant containing the SUMO-tagged proteins was purified using a Co<sup>2+</sup>-chelating column (Thermo Scientific). After cleavage of the fusion proteins with ULP1 protease for 2 h at 4 °C, they were reapplied to the Co<sup>2+</sup> chelating column to remove the protease and cleaved SUMO-affinity tag. The receptor proteins were further purified by gel filtration on a Superdex 75 column (GE Healthcare) equilibrated with 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM NaCl, pH 6.0. Heme contents of holo- and apoproteins were determined by the pyridine hemochrome assay, and homogeneous apo forms of the heme-binding proteins were generated by extraction with methyl ethyl ketone (37). Expression and purification of [U-2H,13C,15N]IsdH<sub>N2N3(Y642A)</sub> was performed according to a previously published protocol (36).

**Preparation of Human Hemoglobin**—Human blood (30–40 ml) was collected with heparin anticoagulant by a health practitioner following appropriate institutional protocols. Red blood cells were collected by centrifugation at 700 ∗ g for 10 min at 4 °C. The cells were washed three times with 0.9% NaCl and bubbled with carbon monoxide (CO) for 5 min. The cells were then collected by centrifugation and lysed by resuspension in five volumes of water, followed by incubation on ice for 30 min. NaCl was added to a final concentration of 0.9%, resulting in aggregation of the membrane fractions into a gelatinous phase, which was removed by centrifugation for 15 min at 9500 ∗ g. The supernatant containing Hb was supplemented with 1 mM EDTA and bubbled with CO for 5 min. After adjusting the pH to 6.9, the hemolsate was applied to an SP Sepharose Fast Flow column (GE Healthcare) equilibrated with 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM DTT, pH 6.9, and Hb was eluted with 10 mM Tris-HCl, pH 8.5. The fractions containing Hb were pooled, supplemented with 1 mM EDTA, and bubbled with CO for 5 min. Subsequently, the sample was applied to a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with 10 mM Tris-HCl, pH 8.5. Pure Hb was eluted with 30 mM NaH<sub>2</sub>PO<sub>4</sub>,
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pH 6.9, with a yield of 100 mg of protein/ml of blood. Hb concentrations were determined using Drabkin’s reagent (Sigma).

Electrospray Ionization Mass Spectrometry and Circular Dichroism Spectroscopy—Purified human Hb and IsdH proteins were prepared in 10 mM ammonium acetate buffer at pH 6.9, subsequently mixed to concentrations of 10 and 20 μM, respectively, and incubated at 25 °C for 1 h. MS measurements of protein samples were performed on a Waters Synapt G1 QTOF mass spectrometer (Waters Corp., Milford, MA) (35). The protein solutions were electrosprayed using Proxeon glass capillary nanoelectrospray emitters at flow rates between 30 and 50 nl/min. Quantification of Hb and Hb-receptor complexes was performed based on the Waters Synapt data by comparing summed peak heights. Higher resolution mass spectrometry experiments were performed using a 15-tesla Fourier transform ion cyclotron resonance instrument (SolarIX hybrid Qq-FTMS, Bruker Daltonics, Billerica, MA). Circular dichroism spectra of 0.2 mg/ml IsdB linker and IsdH linker in 10 mM NaH2PO4, 50 mM NaF, pH 6.8, were recorded on a JASCO J-715 spectropolarimeter (JASCO Corp.) at 25 °C with a scan rate of 20 nm/min.

Heme Transfer Kinetics and Affinity Measurements—Heme transfer reactions from Hb to IsdBN1N2 and various IsdH protein constructs were monitored by following absorbance changes using a conventional spectrophotometer (Shimadzu UV-1700 PharmaSpec), as described previously (14). Human hemoglobin was purchased from Sigma and dissolved in 20 mM NaPO4, pH 7.5, 150 mM NaCl. Briefly, 1 μM holo-Hb (expressed in tetrameric units) was mixed with 10 μM apo-receptor protein in 20 mM NaPO4, pH 7.5, 150 mM NaCl. Entire absorbance spectra were recorded for heme transfer from holo-Hb to apo-IsdH N2N3 over time. To compare the heme transfer rates from Hb to the various acceptor proteins, changes in absorbance at 371 and 406 nm were recorded over time at 25 °C for up to 2 h. Apparent rate constants for the heme transfer reactions were determined by fluorescence spectroscopy as described (38).

RESULTS

A Conserved Unit in IsdB and IsdH Containing Two NEAT Domains Rapidly Captures Heme from Hb—The S. aureus Hb receptors IsdB and IsdH contain two and three NEAT domains, respectively (Fig. 1A). Isolated domains from these receptors have been characterized in vitro and bind to either Hb or heme; the IsdH N1, IsdH N2, and IsdB N1 NEAT domains bind to Hb, whereas the C-terminal NEAT domains in both proteins interact with heme (IsdH N3 and IsdB N2) (32, 38, 50–54). Interestingly, a sequence alignment reveals that IsdB and IsdH share 64% primary sequence identity with one another over a region that encodes two NEAT domains (Figs. 1A and 2). This conserved unit contains two NEAT domains that are joined by a ~70-amino acid segment, hereafter referred to as the “linker.” In IsdH, the unit corresponds to the N2 and N3 domains, which are homologous to the N1 and N2 domains in IsdB, respectively (Fig. 1A, enclosed in a dashed box). In vitro, full-length IsdB rapidly captures heme from Hb (14). To determine if the conserved unit within IsdB and IsdH is responsible for efficient heme capture, UV-visible absorption spectroscopy was used to measure the rate of heme transfer from heme-loaded methemoglobin (MetHb) to either IsdB N1N2 (residues Thr121–Asn458, containing the N1 and N2 domains in IsdB) or IsdH N2N3 (Ala326–Asp660, containing the N2 and N3 domains in IsdH). All studies were performed under oxidizing conditions, in which heme is in its ferric form. Upon mixing of MetHb with apo-IsdH N2N3, a rapid shift of the UV absorbance spectrum of Hb to the heme bound spectrum of IsdH N2N3 is observed (Fig. 1B). This spectral change is indicative of heme transfer to IsdH and is most pronounced at 371 and 406 nm where the absorb-
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A homolog bidomain unit for heme capture in IsdH and IsdB. A, schematic of the NEAT domains present in *S. aureus* IsdH and IsdB. NEAT domains binding to Hb or heme are colored in black/white or gray, respectively. Sequence identities of functionally homologous NEAT domains as well as the linker connecting them are indicated. B, spectral shifts as a function of time after mixing for the reaction of 1 μM holo-Hb with 10 μM apo-IsdH<sup>N2N3</sup>. Arrows indicate the increase and decrease in absorbance over time at 371 and 406 nm, respectively. C, time courses of ΔA<sub>406-371</sub> for heme transfer from Hb to IsdH<sup>N2N3</sup> or IsdH<sup>N1N2</sup>. The symbols and curves represent the observed data and the single-exponential fitting curves, respectively, yielding heme transfer rates of 0.048 ± 0.001 s<sup>−1</sup> and 0.055 ± 0.001 s<sup>−1</sup> for IsdH<sup>N2N3</sup> and IsdB<sup>N1N2</sup>, respectively.

**FIGURE 1.** A homolog bidomain unit for heme capture in IsdH and IsdB. A, schematic of the NEAT domains present in *S. aureus* IsdH and IsdB. NEAT domains binding to Hb or heme are colored in black/white or gray, respectively. Sequence identities of functionally homologous NEAT domains as well as the linker connecting them are indicated. B, spectral shifts as a function of time after mixing for the reaction of 1 μM holo-Hb with 10 μM apo-IsdH<sup>N2N3</sup>. Arrows indicate the increase and decrease in absorbance over time at 371 and 406 nm, respectively. C, time courses of ΔA<sub>406-371</sub> for heme transfer from Hb to IsdH<sup>N2N3</sup> or IsdH<sup>N1N2</sup>. The symbols and curves represent the observed data and the single-exponential fitting curves, respectively, yielding heme transfer rates of 0.048 ± 0.001 s<sup>−1</sup> and 0.055 ± 0.001 s<sup>−1</sup> for IsdH<sup>N2N3</sup> and IsdB<sup>N1N2</sup>, respectively.

The NEAT Domains within IsdH and IsdB Are Connected by a Functionally Important Helical Linker—The ~70-amino acid linker segments that connect the NEAT domains in IsdB and IsdH share 70% sequence identity (Fig. 2). To investigate their structure, we purified polypeptides containing this segment from IsdB (IsdB<sup>linker</sup>, Ser<sup>263</sup>–Ser<sup>361</sup>) and IsdH (IsdH<sup>linker</sup>, Pro<sup>466</sup>–Val<sup>564</sup>). Their circular dichroism (CD) spectra indicate that IsdB<sup>linker</sup> and IsdH<sup>linker</sup> adopt a helical conformation, which is evident by negative bands in their CD spectra at 222 and 208 nm and a positive band at 193 nm (Fig. 5A). This is consistent with secondary structure predictions, which propose that amino acids in this region form several α-helices. To explore the functional role of the linker domain in IsdH, UV-visible absorbance spectroscopy was used to follow heme capture from MetHb. IsdH<sup>linker</sup> was unable to acquire heme from MetHb (Fig. 3B). Moreover, the presence of IsdH<sup>linker</sup> and IsdB<sup>N2</sup> did not accelerate the rate at which IsdH<sup>N3</sup> captures heme from MetHb (Fig. 3C, N2 + linker + N3). This indicates that the isolated components of the conserved unit in IsdH are unable to associate with one another via non-covalent interactions to form a fully functioning receptor. To further investigate the function of the linker, polypeptides in which the linker was fused to either the N2 (IsdH<sup>N2-linker</sup>, Ala<sup>326</sup>–Gln<sup>543</sup>) or N3 (IsdH<sup>N2-linker</sup>, Ala<sup>326</sup>–Gln<sup>543</sup>) domains were studied. Slow transfer from MetHb to the isolated N3 domain was observed when IsdH<sup>N2-linker</sup> was added in *trans*, indicating that MetHb binding by IsdH<sup>N2-linker</sup> did not significantly promote heme release and subsequent capture by IsdH<sup>N3</sup>. Similarly, IsdH<sup>N2-linker</sup> captures heme slowly from MetHb in either the presence or absence of IsdH<sup>N2</sup>, indicating that the presence of the helical linker does not influence the N3 domain’s ability to scavenge heme (Fig. 3C). To determine if the structure of the linker is important for function, we studied IsdH<sup>N2-GS-N3</sup>, which replaces the linker with a nine-residue glycine- and serine-rich polypeptide (GSGSGSGSG). Spectroscopic measurements reveal that IsdH<sup>N2-GS-N3</sup> captures heme slowly from MetHb at a rate that is similar to that of the isolated N3 domain.
Combined, these data indicate that the NEAT domains in IsdB and IsdH are connected by a helical linker, whose primary function is to properly position the domains so as to specifically facilitate heme transfer from MetHb to the N3 domain.

IsdH Destabilizes Hb to Promote Heme Release—We used ESI-mass spectrometry to investigate the mechanism through which IsdH accelerates heme release from Hb. ESI-MS allows the quantification of different Hb oligomers in the presence and absence of IsdH (55). Hb consists of $\gamma_{2}$ and $\beta$-globin chains each bound to a heme. The globins assemble into a noncovalently bound $\gamma_{2}\beta$ tetramer that dissociates into $\gamma_{2}\beta$ dimers with a dissociation constant ($K_d$) of 2 $\mu$M (56). This is evident from the ESI-MS spectrum of a 10 $\mu$M solution of Hb; from the ratio of the signal from the dimer and tetramer ions, the dimer/tetramer ratio is 1:1.4. This is consistent with previously reported studies (57, 58) and validates the use of ESI-MS to estimate the relative abundances of Hb species. ESI-MS spectra of Hb were acquired in the presence or absence of either wild-type IsdH, IsdH(Y642A), which contains a Y642A mutation in the N3 domain that disrupts heme binding (Fig. 6, A–D). In all of the experiments, the receptors were present at a 2-fold molar excess relative to Hb (expressed in tetrameric units). The ESI-MS data are summarized in Fig. 6E, which shows a histogram plot of the relative abundances of the various forms of Hb (the sum of the monomeric $\gamma$- or $\beta$-globins ($M$); $\gamma_{2}\beta$ dimer ($D$); and $\gamma_{2}\beta$ tetramer ($T$), as well as receptor-bound forms of the $\gamma_{2}\beta$ tetramer ($T-R$), and $\gamma_{2}\beta$ dimer ($D-R$). Incubation of IsdH$^{N2N3}$ with Hb substantially reduces the amount of dimeric and tetrameric Hb, which is converted into monomeric globins. This is consistent with previous studies that have shown that Hb dissociates into its component globins upon heme removal (58, 59). Because substochiometric amounts of the receptor were used, Hb dissociation is not complete, leaving mostly a mixture of dimeric Hb and the $\gamma_{2}\beta$ dimer-receptor complex. Importantly, after the IsdH$^{N2N3}$ addition, most of the Hb tetramer disappears, and very little $\gamma_{2}\beta$ tetramer-receptor complex is formed. This indicates that receptor binding and/or heme removal significantly destabilizes the tetramer.

FIGURE 2. Alignment of IsdH and IsdB. A primary sequence alignment of IsdH (Q99TD3) and IsdB (Q7A656) was generated using ClustalW (69). Conserved residues are indicated by gray boxes. The predicted NEAT domains IsdH$^{N1}$, IsdH$^{N2}$/IsdB$^{N1}$, and IsdH$^{N3}$/IsdB$^{N2}$ are highlighted by yellow, red, and green boxes, respectively. The IsdH and IsdB linker domains are indicated by blue boxes.

(Fig. 3, compare B and C). Combined, these data indicate that the NEAT domains in IsdB and IsdH are connected by a helical linker, whose primary function is to properly position the domains so as to specifically facilitate heme transfer from MetHb to the N3 domain.

IsdH Destabilizes Hb to Promote Heme Release—We used ESI-mass spectrometry to investigate the mechanism through which IsdH accelerates heme release from Hb. ESI-MS allows the quantification of different Hb oligomers in the presence and absence of IsdH (55). Hb consists of $\alpha$- and $\beta$-globin chains each bound to a heme. The globins assemble into a noncovalently bound ($\alpha\beta$)$_2$ tetramer that dissociates into ($\alpha\beta$) dimers with a dissociation constant ($K_d$) of 2 $\mu$M (56). This is evident from the ESI-MS spectrum of a 10 $\mu$M solution of Hb; from the ratio of the signal from the dimer and tetramer ions, the dimer/tetramer ratio is 1:1.4. This is consistent with previously reported studies (57, 58) and validates the use of ESI-MS to estimate the relative abundances of Hb species. ESI-MS spectra of Hb were acquired in the presence or absence of either wild-type IsdH$^{N2N3}$, IsdH$^{N2}$-GS$^{-N3}$, or IsdH$^{N2N3}$Y642A, which contains a Y642A mutation in the N3 domain that disrupts heme binding (Fig. 6, A–D). In all of the experiments, the receptors were present at a 2-fold molar excess relative to Hb (expressed in tetrameric units). The ESI-MS data are summarized in Fig. 6E, which shows a histogram plot of the relative abundances of the various forms of Hb (the sum of the monomeric $\alpha$- or $\beta$-globins ($M$); ($\alpha\beta$) dimer ($D$); and ($\alpha\beta$)$_2$ tetramer ($T$), as well as receptor-bound forms of the ($\alpha\beta$)$_2$ tetramer ($T-R$), and ($\alpha\beta$) dimer ($D-R$). Incubation of IsdH$^{N2N3}$ with Hb substantially reduces the amount of dimeric and tetrameric Hb, which is converted into monomeric globins. This is consistent with previous studies that have shown that Hb dissociates into its component globins upon heme removal (58, 59). Because substochiometric amounts of the receptor were used, Hb dissociation is not complete, leaving mostly a mixture of dimeric Hb and the ($\alpha\beta$) dimer-receptor complex. Importantly, after the IsdH$^{N2N3}$ addition, most of the Hb tetramer disappears, and very little ($\alpha\beta$)$_2$ tetramer-receptor complex is formed. This indicates that receptor binding and/or heme removal significantly destabilizes the tetramer.
To gain insight into the role of the linker and heme binding in the acquisition process, ESI-MS spectra of Hb in the presence of IsdHN2-GS-N3 or IsdHN2-N3(Y642A) were acquired. Unlike the wild-type receptor, when the IsdHN2-GS-N3 linker mutant is incubated with Hb, the majority of the receptor binds to the (H9251/H9252)2 tetramer to form a (H9251/H9252)2-IsdHN2-GS-N3 complex, and a significant fraction of the tetramer remains intact (Fig. 6E). Moreover, smaller amounts of Hb are converted to its monomeric globins, whereas roughly similar amounts of (H9251/H9252)2 dimer and (H9251/H9252)3 dimer-receptor complex are present. The absence of monomeric globins is compatible with the kinetic data that showed that IsdHN2-GS-N3 extracted heme from Hb inefficiently. The fact that the linker mutant does not disrupt the tetramer suggests that it adopts a unique structure as compared with the wild-type protein, such that the mutant receptor can no longer impart sufficient structural strain to rupture the tetramer. To determine if the receptor needs to bind heme in order to dissociate the Hb tetramer, we studied IsdHN2N3(Y642A), which contains a Y642A mutation in the N3 domain that disrupts heme binding. When Hb is incubated with IsdHN2N3(Y642A), the amount of tetrameric Hb is significantly reduced as a result of its conversion into the (H9251/H9252)2-IsdHN2N3(Y642A) complex. However, only small amounts of monomeric globin are produced. This suggests that heme removal from the tetramer by the receptor is not required to dissociate it into its dimeric state. However, heme removal appears to be required to convert Hb into its monomeric units. A working model of the extraction process is presented under “Discussion.”

Structure of the Linker Domain—To gain a better understanding of the molecular basis of heme capture, we determined the NMR solution structure of IsdHlinker (Protein Data Bank accession code 2LHR). The NMR spectra of IsdHlinker are well resolved, enabling nearly complete 1H, 13C, and 15N resonance assignments (Fig. 5B). A total of 1793 experimentally derived restraints were used to determine the structure, including 1469 interproton distance restraints, 118 dihedral angle restraints, 54 JHN/H9251 restraints, and 15213C secondary shift restraints. An ensemble of 20 conformers representing the structure of IsdHlinker is displayed in Fig. 7A. The structure is well defined by the NMR data; the backbone and heavy atom coordinates of the structured residues Val470–Val531 can be superimposed with a root mean square deviation of 0.42 ± 0.10 Å and 0.87 ± 0.07 Å, respectively (experimental and structural parameters are presented in Table 1).

The linker forms a three-helix bundle that is composed of helices α1 (Asp571–Lys486), α2 (Leu490–Lys505), and α3
(Glu<sup>506</sup>–Ala<sup>530</sup>) (Fig. 7B). In the bundle, the long axes of the helices are co-linear and are connected by short reverse turns. The structure is stabilized by a hydrophobic core that is formed by nine leucine and tyrosine residues (Leu<sup>477</sup>, Leu<sup>480</sup>, Leu<sup>481</sup>, Tyr<sup>484</sup>, Leu<sup>500</sup>, Leu<sup>504</sup>, Tyr<sup>508</sup>, and Tyr<sup>512</sup>; Fig. 7C). Although each helix contributes residues to the hydrophobic core, helix α3 is longer than the other helices, such that its C-terminus projects from the bundle. This region and residues immediately following it presumably facilitate interactions with the N3 domain in the intact receptor (see below). \[1H\]15N heteronuclear NOE measurements are compatible with the structure because residues Val<sup>470</sup>–Val<sup>531</sup>, whose coordinates are precisely defined in the ensemble, exhibit large magnitude NOE values, indicating that they are immobile on the picosecond time scale (Fig. 5C).

**IsdHN<sub>2N3</sub> Adopts an Extended but Ordered Multidomain Structure**—We used NMR to investigate the structure and dynamics of IsdHN<sub>2N3(Y642A)</sub>. It is structurally identical to the wild-type protein based on its HSQC spectrum but is reduced in its ability to bind heme. Previously, we sequence-specifically assigned the chemical shifts of its backbone atoms (36). To learn whether the domains form a rigid unit within IsdHN<sub>2N3(Y642A)</sub>, we measured \[1H\]15N heteronuclear NOE relaxation parameters. As shown in Fig. 8A, residues spanning the N2, linker, and N3 domains exhibit positive and mostly uniform \[1H\]15N heteronuclear NOEs, which indicates that they are structurally ordered. Notably, residues that connect the domains also exhibit positive NOEs. Because some of these residues are unstructured in the isolated linker polypeptide (Fig. 5C), this suggests that in the context of IsdHN<sub>2N3(Y642A)</sub>, they form stabilizing interactions with residues located in the N2 and/or N3 domains and that the domains form a single structured unit.

Although the structure of the full receptor is unknown, the structures of the isolated linker and N3 domains are known, and the structure of the N2 domain can be accurately modeled using the previously determined NMR and crystal structures of IsdHN<sub>1</sub>, which shares 54% sequence identity with N2 (50, 51, 53). To determine whether the domains undergo major structural changes upon incorporation into IsdHN<sub>2N3</sub>, we measured \[15N\]-1H residual dipolar couplings (1DN<sub>H</sub>) in a sample of IsdHN<sub>2N3</sub> partially aligned in pentaethylene glycol monododecyl ether (C<sub>12</sub>E<sub>5</sub> PEG)/hexanol. The 1DN<sub>H</sub> data provide information about the angle of each backbone N–H bond relative to an alignment tensor. The compatibility of the individual domain structures with the RDC data was evaluated by plotting the back-calculated versus experimental 1DN<sub>H</sub> values (Fig. 9). There is good agreement between the experimental data and the individual structures of the N2, linker, and N3 domains, which have calculated Q-factors of 0.28, 0.10, and 0.23, respectively. This indicates that incorporation of the domains into IsdHN<sub>2N3</sub> does not significantly alter their structure and is consistent with our previously reported Cα and Cβ backbone secondary chemical shifts of IsdHN<sub>2N3</sub>, which suggested that the domains have similar secondary structures in isolation and when incorporated into IsdHN<sub>2N3</sub> (36).

The chemical shifts of IsdHN<sub>2N3(Y642A)</sub> and polypeptides containing its isolated domains were compared with the aim of

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**FIGURE 5. The IsdH and IsdB linkers fold into soluble α-helical domains.** A, far-UV CD spectra of the linker domains of IsdH (solid) and IsdB (dashed). The strongly negative CD signals with minima around 208 and 222 nm are indicative of α-helical proteins. B, 1H-15N HSQC spectrum of 1.1 mM 15N-labeled IsdH<sub>linker</sub> acquired at 800 MHz. The amino acid assignment for each cross-peak is indicated with one-letter code and residue number. Amide NH<sub>2</sub> of Asn and NH<sub>2</sub> of Gln are connected by lines and denoted as sc for side chain. An asterisk indicates the cross-peak from the NH of Arg<sup>520</sup> which was folded in the 15N dimension. C, \([1H\]15N\) heteronuclear NOE data of the IsdH<sub>linker</sub>. The peak intensity ratio between spectra with and without 1H saturation as a function of the residue number is shown. The average heteronuclear NOE values and S.D. values of two experiments are displayed, and the location of the three α-helices is indicated at the top.
learning if the domains interact with one another in the context of IsdH<sup>N2</sup>N<sup>3</sup>(Y642A). Fig. 8B shows an overlay of the secondary chemical shifts of IsdH<sup>N2</sup>N<sup>3</sup>(Y642A) and IsdH<sub>linker</sub>. Similar secondary chemical shifts were observed for the structured part of the linker, suggesting that its conformation is preserved in IsdH<sup>N2</sup>N<sup>3</sup>(Y642A). Average chemical shift differences of the backbone amide signals of isolated linker and the corresponding residues in IsdH<sup>N2</sup>N<sup>3</sup> are displayed in Fig. 8C. In general, small chemical shift differences were observed for residues in the core helices of the linker, indicating that they do not form a molecular surface that interacts with the N2 or the N3 domains. However, significant chemical shift differences in the linker occur for residues located at the beginning of helix α2 (Leu<sup>490</sup>–Arg<sup>492</sup>) and at its N terminus (Asp<sup>468</sup>–Glu<sup>472</sup>, Thr<sup>474</sup>–Tyr<sup>475</sup>) and C terminus (Gln<sup>526</sup>–Ser<sup>529</sup>, Val<sup>531</sup>–Thr<sup>538</sup>, Thr<sup>540</sup>–Gln<sup>543</sup>). Mapping these changes onto the NMR structure of the linker reveals that they reside at distinct ends of the domain (Fig. 8D). This is consistent with residues at the beginning of helix α2 and the N terminus of the linker contacting the N2 domain, while residues at the C-terminal end interact with the...
N3 domain. Interestingly, comparison of the secondary chemical shifts suggests that helix α3 in the linker domain is lengthened at its C terminus when it is incorporated into IsdH\(^{N2N3}\) (Fig. 8B). Moreover, residues immediately following this segment, based on their secondary chemical shifts, do not participate in regular secondary structure when located in IsdH\(^{N2N3}\) but are nevertheless highly ordered, based on the heteronuclear NOE data (Fig. 8A). To further ascertain whether the domains in IsdH\(^{N2N3}\) might be significantly interacting with one another in IsdH\(^{N2N3}\), we produced \(^{15}\)N samples of IsdH\(^{N2}\) and IsdH\(^{N3}\). The \(^{1}\)H-\(^{15}\)N HSQC spectrum of IsdH\(^{N2}\) is well resolved and, when overlaid with the spectrum of IsdH\(^{N2N3}\), reveals very similar chemical shifts (data not shown). This suggests that, in the context of IsdH\(^{N2N3}\), the N2 domain does not contain a large contact surface that interacts with the remainder of the protein. A similar analysis using \(^{15}\)N-labeled IsdH\(^{N3}\) was also attempted but did not prove fruitful because the cross-peaks in its spectrum are partially broadened, presumably because of protein aggregation. Combined, the absence of extensive interaction surfaces in the linker and N2 domains suggests that, while ordered, IsdH\(^{N2N3}\) does not adopt a compact structure.

**DISCUSSION**

To successfully mount an infection, *S. aureus* and other pathogens acquire the essential nutrient iron from human Hb. Two surface-displayed *S. aureus* receptors capture Hb on the cell surface, IsdB and IsdH. The receptors share a high degree of sequence homology over a region that contains two NEAT domains that are separated by a ~70-amino acid “ linker” segment (Figs. 1A and 2). The NEAT domains in the conserved units have distinct functions; in each protein, the N-terminal domain binds to Hb, and the C-terminal domain interacts with heme (8, 38). Interestingly, the NEAT domains in IsdB appear to function synergistically, because Lei and colleagues (14) have shown that IsdB captures heme from Hb ~28–250 times faster than proteins that contain only a single NEAT domain. To gain insight into the molecular basis of this synergy, we studied the conserved bi-NEAT domain unit located within IsdH (IsdH\(^{N2N3}\)). UV-visible spectroscopy measurements of heme transfer from Hb indicate that IsdH\(^{N2N3}\) rapidly acquires the heme of Hb at a rate that is 110–580 times faster than the rate at which Hb spontaneously releases heme into the solvent (IsdH\(^{N2N3}\) acquires heme at a rate of 0.048 ± 0.001 s\(^{-1}\))

**TABLE 1**

Structural statistics for the solution structure of IsdH linker domain

| Root mean square. deviations | (SA)\(^{a}\) | (SA) |
|-----------------------------|------------|------|
| NOE interproton distance restraints (Å) (1469) | 0.046 ± 0.002 | 0.051 |
| Dihedral angle restraints (degrees) (118) | 0.072 ± 0.099 | 0.306 |
| \(^{15}\)C\(^{1}\) coupling constants (Hz) (54) | 0.532 ± 0.018 | 0.543 |
| Secondary \(^{13}\)C shifts (ppm) | 1.212 ± 0.206 | 1.267 |
| Deviations from idealized covalent geometry | 0.0044 ± 0.0002 | 0.0174 |
| Bonds (Å) | 0.623 ± 0.028 | 1.538 |
| Angles (degrees) | 0.492 ± 0.031 | 1.181 |
| Improper angles (degrees) | 0.42 ± 0.10 | 0.538 |
| Coordinate precision (Å) | 0.36 ± 0.07 | 0.108 |

\^a None of the structures exhibits distance violations greater than 0.5 Å, dihedral angle violations greater than 5°, or coupling constant violations greater than 2 Hz.

\(^{b}\) Experimental dihedral angle restraints comprised 48 ϕ, 48 χ, and 16 χ2 angles.

\(^{c}\) PROCHECK-NMR data include residues Val\(^{770}\)–Val\(^{551}\) of the linker domain.

\(^{d}\) The coordinate precision is defined as the average atomic root mean square deviation of the 20 individual simulated annealing structures and their mean coordinates. The reported values are for residues Val\(^{770}\)–Val\(^{551}\) of the linker domain.
that connects the domains is required for efficient heme capture; an IsdH\(^{\text{N2-GS-N3}}\) mutant in which the linker is replaced with a glycine-serine nonapeptide acquires heme slowly from Hb.

IsdH\(^{\text{N2N3}}\) adopts an ordered elongated dumbbell-shaped structure in which its NEAT domains are separated by a helical linker domain. The NMR structure of the linker domain (called IsdHlinker) reveals that it adopts a three-helix bundle. First observed in the IgG-binding domain of \(S. \text{aureus}\), three-helix bundles serve as robust scaffolds for molecular recognition and are ubiquitously found in structural proteins, enzymes, and DNA-binding proteins (61, 62). Because the N and C termini in IsdHlinker are positioned at opposite ends of the bundle, in the context of the IsdHN2N3 receptor, the linker domain presumably acts as a spacer that holds the N2 and N3 domains apart by \(\sim 40 \text{ Å}\). This is compatible with the assigned NMR spectra of the intact 39-kDa IsdH\(^{\text{N2N3}}\) receptor, because a comparison with the NMR spectra of IsdHlinker reveals that only residues located at the ends of the helical bundle near the connection points to the N2 and N3 domains exhibit large chemical shift differences. Moreover, the NMR chemical shifts of residues in the isolated IsdH\(^{\text{N2}}\) domain and IsdH\(^{\text{N2N3}}\) are similar, suggesting that N2 is not involved in extensive interdomain interactions in the structure of IsdH\(^{\text{N2N3}}\). Interestingly, although IsdH\(^{\text{N2N3}}\) adopts an elongated structure, the domains do not appear to be connected by flexible loops. Inspection of the heteronuclear NOE data of IsdH\(^{\text{N2N3}}\) reveals nearly uniform values over the length of the polypeptide, including amino acids that connect the domains. Notably, several residues at the N and C termini of the linker domain that are unstructured in the isolated IsdHlinker become ordered when they are located in IsdHN2N3 (in IsdHN2N3, 2 and 11 residues preceding and following the linker domain, respectively, exhibit elevated NOE values in IsdHN2N3 as compared with IsdHlinker). Thus, the three domains within IsdH\(^{\text{N2N3}}\) adopt an extended conformation in which their positioning is fixed with respect to one another. IsdB can be assumed to adopt a similar structure because it shares significant sequence homology with IsdHN2N3, and we have shown that its linker region also adopts a helical conformation.

From the ESI-MS data, IsdH\(^{\text{N2N3}}\) extracts heme from Hb via the ordered process shown in Fig. 10A. On the cell surface, IsdB and IsdH can be expected to encounter Hb in its \((\alpha\beta)_2\) tetrameric and \(\alpha\beta\) dimeric forms, whose relative abundance depends on protein concentration. When IsdH\(^{\text{N2N3}}\) binds to the \((\alpha\beta)_2\) tetramer, it promotes its dissociation into \(\alpha\beta\) dimers, which is presumably caused by receptor-induced steric strain that ruptures the weaker \(\alpha_1\beta_1\) interface of the tetrameric Hb (63). Dimer formation is expected to facilitate heme transfer to IsdH\(^{\text{N2N3}}\) because dimeric Hb releases heme more readily than
the \((\alpha\beta)_2\) tetramer; compared with the tetramer, the rate of heme loss from the \(\alpha\) and \(\beta\) chains in the isolated \((\alpha\beta)\) dimer is 2 and 10 times faster, respectively (34). In the second step, heme is transferred from the \((\alpha\beta)\) dimer to the N3 domain within the IsdHN2N3 receptor. Our data do not reveal which globin chain, if any, serves as the preferred heme donor for IsdHN2N3. It is possible that heme is first removed from the \(\beta\) subunit because it has intrinsically weaker affinity for heme as compared with the \(\alpha\) subunit (34). Alternatively, structural distortions induced in the dimer by the receptor may trigger heme transfer from the \(\alpha\) chain, creating semi-\(\beta\) Hb from which heme is known to be rapidly released (34). In the final step, after the loss of one of its heme molecules, the \((\alpha\beta)\) dimer dissociates completely. Formation of monomeric species is probably driven by the greater tendency of Hb dimers to dissociate (59). As the monomeric \(\alpha\) and \(\beta\) chains quickly lose their heme to the environment, both globins could be expected to readily release their ligand to IsdH (34). A similar transfer reaction is expected to occur when IsdH encounters an \((\alpha\beta)\) Hb dimer, but it would bypass the need for tetramer dissociation. An alternative heme transfer pathway is also possible. In it, the receptor would remove heme directly from the tetramer or concurrently with tetramer dissociation. Heme removal from the tetramer could be advantageous because it would produce semi-Hb tetramers that are prone to dissociate (64). However, as described immediately below, heme capture from the Hb tetramer is not an obligate step in the transfer reaction.

FIGURE 9. **Comparison of experimental and back-calculated RDC values of backbone amide protons in IsdHN2N3**. A, observed \(^{1}D_{NH}\) RDCs for the N2 domain were plotted versus back-calculated RDCs from a homology model of IsdHN2. B, experimentally measured \(^{1}D_{NH}\) RDCs for the linker domain were plotted versus back-calculated RDCs from the solution structure of the isolated linker. C, observed \(^{1}D_{NH}\) RDCs for the N3 domain were plotted versus back-calculated RDCs from the crystal structure of IsdHN3 (Protein Data Bank entry 2Z6F). The correlation factors \(R^2\) are indicated in the plots.

FIGURE 10. **Model of heme extraction by IsdH**. A, a model for the mechanism of heme acquisition by the surface receptor IsdHN2N3. A schematic diagram shows the binding equilibria involved in the extraction process. Wild-type IsdHN2N3 binds to the \(\alpha\) chain of Hb promoting its dissociation into \((\alpha\beta)\) dimers. Heme acquisition by the receptor protein results in further dissociation of Hb into its monomeric subunits. See "Discussion" for details. B, a model of IsdHN2N3 in complex with Hb. IsdHN2N3 (red) was modeled based on the solution structure of IsdHN1 (Protein Data Bank entry 2H3K). The complex model with Hb was generated by superposition over the crystal structure of the IsdHN1-Hb complex (Protein Data Bank entry 3SZK). A possible orientation of the linker (blue) and IsdHN3 (green, Protein Data Bank entry 2Z6F) allowing productive heme transfer from a Hb \((\alpha\beta)\) dimer (yellow-orange) to IsdH is indicated. The orientation of the subdomains (N1, linker, and N2) within IsdHN2N3 has not been experimentally determined, and only one possible orientation is shown. The protein backbones are shown as schematics. The heme groups in Hb are shown in stick representation.

The (\(\alpha\beta\)) tetramer; compared with the tetramer, the rate of heme loss from the \(\alpha\) and \(\beta\) chains in the isolated (\(\alpha\beta\)) dimer is 2 and 10 times faster, respectively (34). In the second step, heme is transferred from the (\(\alpha\beta\)) dimer to the N3 domain within the IsdHN2N3 receptor. Our data do not reveal which globin chain, if any, serves as the preferred heme donor for IsdHN2N3. It is possible that heme is first removed from the \(\beta\) subunit because it has intrinsically weaker affinity for heme as compared with
Several lines of evidence indicate that binding of the IsdH<sub>N2N3</sub> receptor to tetrameric Hb induces steric strain in Hb that causes it to dissociate into dimers and that this process does not require heme transfer to IsdH<sub>N2N3</sub> (Fig. 10A). The most compelling evidence comes from the ESI-MS data of IsdH<sub>N2N3</sub> and IsdH<sub>N2N3(Y642A)</sub>, which indicate that both proteins readily disrupt the tetramer. Because IsdH<sub>N2N3</sub> binds heme with lower affinity, this indicates that structural perturbations in Hb induced by receptor binding are sufficient to cause it to dissociate. This process requires two NEAT domains that are connected by a structured linker because the Hb tetramer does not dissociate when it is bound to an IsdH<sub>N2</sub>-G5-N3 mutant in which the linker domain is replaced with a flexible glycine-serine peptide. The idea that an intact bi-NEAT domain receptor is required to dissociate the tetramer is also consistent with a recent crystal structure of the IsdH<sub>N1</sub>-Hb complex, which revealed that binding of the isolated N1 NEAT domain to Hb induced only modest structural changes in Hb (50). As we have shown, IsdH<sub>N2N3</sub> adopts a rigid structure in its apo state; this suggests that binding of IsdH<sub>N2N3</sub> to Hb results in atomic overlap between the proteins that causes the tetramer to dissociate. A model of the structure of the IsdH<sub>N2N3</sub>-Hb complex illustrates a possible orientation of the receptor protein on Hb (Fig. 10B). The orientation of the subdomains (N1, linker, and N2) within IsdH<sub>N2N3</sub> has not been experimentally determined, and only one possible orientation is shown. The model was constructed using the NMR structure of IsdB<sub> linker</sub>, the crystal structure of the isolated N3 domain, and a homology model of the N2 domain based on the structure of IsdH<sub>N1</sub>. Based on the recently reported crystal structure of the isolated N1 domain bound to Hb, the N2 domain in IsdH<sub>N2N3</sub> can be expected to engage the a subunit of Hb via its A-helix (50). Contacts from N2 presumably originate from residues located within surface loops positioned at one end of its b-barrel structure because these residues are conserved in N1 and N2. The relative positioning of the remainder of the IsdH<sub>N2N3</sub> protein and its contacts to Hb cannot be predicted from our NMR data. However, assuming that IsdH<sub>N2N3</sub> adopts an extended structure, the N3 domain could, in principle, be positioned adjacent to the heme pockets of either the a or b subunits. Unlike IsdB, the IsdH protein contains an N-terminal NEAT domain (N1) that binds to the a subunit of Hb (Fig. 1A) (50). It is possible that the N1 and N2 domains in IsdB simultaneously engage the Hb tetramer via its two a subunits. Alternatively, N1 and N2 may not simultaneously engage the same tetramer. In this scenario, Hb binding by N1 may function to increase the efficiency of heme capture by increasing the local concentration of Hb that is proximal to IsdH<sub>N2N3</sub>. A more detailed understanding of the mechanism of extraction and the origin of molecular strain induced by the receptor on Hb will require studies of the full-length IsdB protein and the structure determination of IsdH<sub>N2N3</sub> in both its free and Hb-bound states.

We have demonstrated that the NEAT domains within IsdB function synergistically to capture heme from Hb. Interestingly, several other pathogenic species of Gram-positive bacteria display surface proteins implicated in heme capture that contain more than one NEAT domain (26). At present, only a few of these proteins have been characterized biochemically. S. pyogenes encodes the membrane-anchored Shr protein, which has two NEAT domains, and, similar to IsdB and IsdH, it has been proposed to acquire heme via a receptor-Hb complex (65, 66). B. anthracis produces a Hb hemoporph called IsdX2 that contains five NEAT domains (67, 68). All of its domains bind Hb, and some are multifunctional because they can also bind heme. It will be interesting to see if subsets of these domains are also connected by structured linker segments that enable their NEAT domains to function synergistically. Despite the prevalence and importance of multi-NEAT domain proteins in Gram-positive bacteria, this present study is the first to address in detail the possible interactions between NEAT domains, the role of the linker segments, and functional synergy between these regions. Further research will be required to reveal if the mechanism of extraction described here can be generalized to other NEAT-containing Hb receptors. This work could lead to small molecule antibiotics that work by limiting microbial access to heme-iron.

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