Structure of the Hemoglobin-IsdH Complex Reveals the Molecular Basis of Iron Capture by Staphylococcus aureus**

Claire F. Dickson1, Kaavya Krishna Kumar1, David A. Jacques2, G. Reza Malmirchegini3, Thomas Spirig4, Joel P. Mackay5, Robert T. Clubb6, J. Mitchell Guss6, and David A. Gell1

From the 1Menzies Research Institute Tasmania, University of Tasmania, Hobart, Tasmania 7000, Australia, the 2School of Molecular Bioscience, University of Sydney, Sydney, New South Wales 2006, Australia, and the 3Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095

Background: IsdB and IsdH proteins from Staphylococcus aureus strip heme iron from human hemoglobin.

Results: The IsdH-hemoglobin complex shows how globin-binding and heme-binding NEAT domains of IsdH cooperate to remove heme from both chains of hemoglobin.

Conclusion: The supradomain architecture of IsdH confers activity by precisely positioning the heme acceptor domain.

Significance: Multiple IsdH-hemoglobin interfaces may be targets for new antibiotics.

Staphylococcus aureus causes life-threatening disease in humans. The S. aureus surface protein iron-regulated surface determinant H (IsdH) binds to mammalian hemoglobin (Hb) and extracts heme as a source of iron, which is an essential nutrient for the bacteria. However, the process of heme transfer from Hb is poorly understood. We have determined the structure of IsdH bound to human Hb by x-ray crystallography at 4.2 Å resolution, revealing the structural basis for heme transfer. One IsdH molecule is bound to each α and β Hb subunit, suggesting that the receptor acquires iron from both chains by a similar mechanism. Remarkably, two near iron transporter (NEAT) domains in IsdH perform very different functions. An N-terminal NEAT domain binds α/β globin through a site distant from the globin heme pocket and, via an intervening structural domain, positions the C-terminal heme-binding NEAT domain perfectly for heme transfer. These data, together with a 2.3 Å resolution crystal structure of the isolated N-terminal domain bound to Hb and small-angle x-ray scattering of free IsdH, reveal how multiple domains of IsdH cooperate to strip heme from Hb. Many bacterial pathogens obtain iron from human hemoglobin using proteins that contain multiple NEAT domains and other domains whose functions are poorly understood. Our results suggest that, rather than acting as isolated units, NEAT domains may be integrated into higher order architectures that employ multiple interaction interfaces to efficiently extract heme from host proteins.

Staphylococcus aureus is a Gram-positive bacterial pathogen that causes infections of the skin and invasive disease in many tissues and organs. S. aureus is the leading cause of surgical site infections, skin and soft tissue infections, and infective endocarditis (1). Methicillin-resistant S. aureus strains that are resistant to all β-lactam antibiotics cause hospital- and community-acquired infections with high mortality rates (2, 3). New antibacterial treatments are urgently needed, but their development requires a better understanding of the mechanisms that underlie S. aureus pathogenesis.

Proteins displayed on the surface of pathogenic bacteria are at the frontline of the host-pathogen interface and interact with tissues of the host to carry out functions in adhesion, cell invasion, and acquisition of nutrients. Prominent among these functions is the capture of iron from the host (4). As well as being required at the active sites of many essential proteins, iron performs an important signaling role in pathogenesis and regulates the activation of nearly 400 S. aureus genes, including many required for colonization of the host (5). Under normal physiological conditions, all iron in the human body exists in complex with proteins. Successful pathogens have therefore evolved effective mechanisms to capture iron directly from these proteins. For example, Gram-negative pathogenic Neisseria species express the outer membrane transferrin-binding protein A (TbpA) and TbpB, which extract iron from serum transferrin (6). Hb is the most abundant iron source in humans, and many bacteria express proteins to capture this iron, such as iron-regulated surface determinant H (IsdH)5 expressed on the surface of S. aureus.

In mammals, ~70% of the total body iron is iron protoporphyrin IX (heme) that is bound to Hb. S. aureus can obtain all of its iron requirements from Hb (7–9), which it gains access to by secreting hemolytic toxins that lyse erythrocytes. Hb is cap-

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5 The abbreviations used are: IsdH, iron-regulated surface determinant H; NEAT, near iron transporter; SAXS, small angle x-ray scattering; r.m.s.d., root mean square deviation; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; Hal, heme-acquisition leucine-rich repeat protein; IlaA, iron-regulated leucine rich surface protein A; Shr, streptococcal hemoprotein receptor.
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The domain architectures of S. aureus IsdH and IsdB (Fig. 1) are similar, with an extended region of homology that includes all three recognized domains of IsdB, including two near iron binding or heme binding activity are represented by white and black boxes, respectively, and sequence identity is indicated. Known structures of the isolated C-terminal NEAT domain (33), the helical linker (27), and the N-terminal NEAT domain (11) of IsdB are shown. IsdB shares a region of extended homology with IsdB, comprising IsdH\textsuperscript{N1}, the helical linker domain, and IsdH\textsuperscript{N3} (IsdH\textsuperscript{N1-N3}).

The domain architectures of S. aureus IsdH and IsdB (Fig. 1) are similar, with an extended region of homology that includes all three recognized domains of IsdB, including two near iron transporters (NEAT) domains and a recently identified three-helix linker domain (27). A homologue of IsdB/H occurs in the bacterium Bacillus cereus (30–33), and NEAT domains in IsdA and IsdC (not shown) are similar, with an extended region of homology that includes the helical linker (27), and the N-terminal NEAT domain (43), the helical linker (27), and the N-terminal NEAT domain (11) of IsdB/H are shown. IsdB shares a region of extended homology with IsdB, comprising IsdH\textsuperscript{N1}, the helical linker domain, and IsdH\textsuperscript{N3} (IsdH\textsuperscript{N1-N3}).

Although the structures and ligand binding activities of the IsdH\textsuperscript{N1}, IsdH\textsuperscript{N3}, and IsdH\textsuperscript{N2} NEAT domains are known (11, 12, 27, 39, 43), the isolated domains do not release or capture heme from Hb, and the heme capture process is still poorly understood.

Because individual domains do not recapitulate Hb–receptor function, we have analyzed a functional three-domain fragment of IsdH (IsdH\textsuperscript{N2-N3}, Fig. 1). Here we report the structures of IsdH\textsuperscript{N2} and IsdH\textsuperscript{N2-N3} in complex with Hb using x-ray crystallography, providing the first insights into how the domains of the Hb receptors of S. aureus cooperate to extract heme from Hb.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—IsdH\textsuperscript{N2} (residues 321–467), human Hb, and individual Hb chains were obtained as described previously (11). For x-ray crystallographic and SAXS studies, IsdH (residues 326–660) carrying a Tyr-642 to Ala mutation (IsdH\textsuperscript{N2-N3(Y642A)}) was expressed and purified as described previously (27). For heme transfer experiments, IsdH\textsuperscript{N2-N3} (residues 321–655) and IsdB\textsuperscript{N3} (residues 542–655) from S. aureus strain TCH1516 were cloned into pET15b (Novagen) for expression with an N-terminal hexahistidine tag. The proteins were expressed and purified to yield final products with the additional N-terminal sequence MGSSHHHHHHSSGLVPRGSHMLE. IsdH\textsuperscript{N2-N3} was purified over immobilized metal affinity chromatography resin (His-Select Nickel Affinity Gel, Sigma). The load condition was 50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole. The bound protein was washed in equilibration buffer containing 25 mM imidazole and eluted in 100 mM imidazole. Additional purification was performed by anion-exchange (Q-Sepharose, GE Healthcare); proteins were loaded in 10 mM sodium phosphate, pH 7.0, and eluted over a gradient of 100–250 mM NaCl. A final gel filtration step over a Superose 12 column (GE Healthcare Life Sciences) equilibrated in 150 mM sodium phosphate, pH 7.0, was performed.

**X-ray Crystallography**—Hb and IsdH\textsuperscript{N2} were mixed together in 1:2 molar ratio (5 mg/ml Hb) and crystallized by hanging drop vapor diffusion. Crystallization conditions included 0.2 M sodium formate, 0.1 M Bis-Tris propane, pH 7.5, 20% (w/v) PEG3350, and produced crystals of 50–100 \(\mu\)m. The crystals were cryoprotected with 30% glycerol and flash-cooled in a cold nitrogen stream (100 K). Diffraction data to 2.3 Å resolution were collected at 100 K with an x-ray beam wavelength of 0.95370 Å at the Micro Crystallography MX2 beamline at the Australian Synchrotron (Clayton, Australia). Data were indexed and scaled using HKL2000 and SCALPACK (44), respectively. The structure was solved by molecular replacement using PHASER (45), which gave a unique solution when using \(c_\beta\)Hb dimer (Protein Data Bank (PDB) code 2DN1) and an alanine model of IsdH\textsuperscript{N1} (PDB code 3SZK) generated by CHAINSAW (46) as independent search models. The structure was refined using REFMAC5 (47), with manual map inspection and model building being performed in COOT (48). The quality of the model was regularly checked for steric clashes, incorrect stereochemistry, and rotamer outliers using MolProbity (49). The final structure had 98.57% of residues in the Ramachandran preferred region, with no outliers according to MolProbity. Coordinates and structure factors can be found at...
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Research Collaboratory for Structural Bioinformatics (RCSB) PDB entry 4FC3.

The IsdH^{N2-N3}(Y642A)-Hb complex was buffer-exchanged into 20 mM HEPES, pH 7.5, and crystallized by hanging drop vapor diffusion. Crystals of 200–500 μm grew in 0.2 M diammonium citrate, 13% (w/v) PEG3350, 0.7% l-butanol, pH 4.6, at 294 K. Crystals were cryoprotected in stabilizing solution containing 25% ethylene glycol before flash freezing in liquid nitrogen. Diffraction data to 4.2 Å resolution were collected at 100 K with an x-ray beam wavelength of 0.95369 Å on the Australian Synchrotron MX2 beamline. Integration and scaling were performed with HKL-2000 and SCALPACK (44). Molecular replacement was performed with Phaser (45) using the crystal structures of Hb (PDB 3P5Q), IsdH^{N2} (PDB 4FC3), and IsdH^{N3} (PDB 2E7D and 2Z6F) as search models. Clear regions of helical density corresponding to the helical linker domain, which was left out of molecular replacement, provided confidence in the experimental model. The NMR structure of the linker (PDB 2LHR) (27) was placed manually into the model. Anomalous signal was observed at the predicted positions of the iron atoms in the heme groups coordinated by the four globin chains. Although the IsdH^{N2} and IsdH^{N3} domains are structurally similar, Phaser unambiguously placed them in unique locations within the complex. The solution was refined with Buster version 2.10.0 (50). Refinement was weighted heavily toward the geometry of the high-resolution structures used for molecular replacement, and included noncrystallographic symmetry restraints (51) and translation/liberation/screw refinement (groups were defined as single domains). Difference electron density joining the domains of IsdH indicated the positions of linking residues, but these could not be built with confidence and so were not included in the model. MolProbity (49) was used to verify the geometry, and the Ramachandran statistics are as follows; 96.2% of residues were found in favored regions; 99.9% were found in allowed regions; 0.06% were found in disallowed regions. Coordinates and structure factors can be found at RCSB PDB entry 4IJ2.

SAXS—Samples of IsdH^{N2-N3}(Y642A) were buffer-exchanged by gel filtration to obtain matched buffer controls. SAXS data were collected on an Anton Paar SAXSess instrument with a sealed tube source. I(0) values and P(r) curves were calculated using GIFT (Anton Paar, Graz, Austria) and PRIMUS (72), and experimental molecular weights were calculated from the calibrated I(0) values (52). No significant aggregation or interparticle interference was found by Guinier or P(r) analysis. Ab initio shape reconstruction from the experimental scattering data was carried out using DAMMIF (53), and 10 independent models were averaged using DAMAVER (54). Although there was some variation between the models (normalized spatial discrepancy = 0.983), no outliers were identified, and filtering of the average envelope to retain the most highly occupied area gave a dumbbell-shaped envelope, which was superposed with the IsdH^{N2-N3} crystal structure using SUPCOMB (55).

Heme Transfer—Heme transfer was monitored by UV-visible spectroscopy. Apo-IsdH^{N2-N3} was produced using the acetic acid heme extraction method of Ascoli et al. (56). Apo-IsdH^{N2-N3}(Y642A) was obtained heme-free upon purification from Escherichia coli. Hb samples were converted to ferric Hb (Hb containing ferric heme), a form of Hb that is produced following lysis of red blood cells, by the addition of a 5-fold molar excess of potassium ferricyanide and monitored using UV-visible spectrophotometry. Excess oxidant was removed by buffer-exchange over G-25 Sepharose (GE Healthcare). Hb was mixed with apo-IsdH^{N2-N3} at the ratios indicated under “Results.” Reactions were performed in 150 mM sodium phosphate, pH 7.0, at 4 °C. Absorbance spectra (350–700 nm) were recorded at 40-s intervals on a JASCO UV-630 spectrophotometer equipped with a temperature-controlled sample chamber. To determine the percentage of heme transferred from Hb to the Isd protein at each time point, the acquired UV-visible spectrum was fit to a linear combination of ferric Hb and fully heme-loaded holo-IsdH^{N2-N3} spectra.

RESULTS

IsdH^{N2} Binds to a Site Comprising Portions of the A and E Helices of α Hb—To begin to understand the function of IsdH^{N2-N3}, we crystallized an IsdH^{N2}-Hb complex from a mixture containing a 2:1 molar ratio of IsdH^{N2} to Hb tetramer and determined the x-ray crystal structure to 2.3 Å resolution (Table 1 and Fig. 1). Phases were obtained by molecular replacement using the structures of Hb (PDB 2DN1) (57) and IsdH^{N1} (PDB 3SZK) (11). The asymmetric unit of the IsdH^{N2}-Hb crystals comprises one Hb dimer with one IsdH^{N2} molecule bound to the α Hb subunit (Fig. 2A). IsdH^{N2} (Fig. 2B) binds in the same position on α Hb as IsdH^{N1} (Fig. 2C), and a comparable number of residues at both interfaces participate in hydrogen bonds and salt bridges, including interactions with Asp-74, Lys-11, and Thr-8 of α Hb (Fig. 2, B and C). A short α helix in IsdH^{N2} (primary sequence FYHYAS), containing a series of aromatic side chains, forms part of the IsdH^{N2-α} Hb-binding interface (Fig. 2D). A helix with similar hydrophobic character, but different primary sequence (YYHFFS), occurs in IsdH^{N1} (11, 12). A Phe side chain from a different position in the IsdH^{N2} or

### Table 1

| Data collection | IsdH^{N2}-Hb | IsdH^{N2-N3}(Y642A)-Hb |
|----------------|--------------|------------------------|
| Space group    | C222         | P2,2,2                 |
| α, β, γ (°)    | 67.04, 149.85, 86.26 | 132.90, 185.30, 103.21 |
| Resolution (Å) | 50.0-2.26 (2.26-2.32) | 49.7-4.23 (4.24-4.32) |
| R_meas. (%)    | 12.2 (57.3)  | 9.2 (72.7)             |
| R_work (%)     | 12.5 (2.3)   | 10.17 (1.83)           |
| Completeness (%) | 93.2 (93.2) | 99.9 (100)             |
| Redundancy     | 3.8 (3.5)    | 3.8 (3.8)              |
| No. of reflections | 19,451   | 18,511                 |
| R_work/R_meas. | 0.219/0.256 | 0.299/0.310            |
| Protein        | 3282        | 13,375                 |
| Water          | 96          | 172                    |
| B-factors      | 24.63       | 86.14                  |
| Ligand/ion     | 17.94       | 65.65                  |
| Bond lengths (Å) | 0.005    | 0.008                  |
| Bond angles (°) | 0.760     | 0.84                   |

**RESULTS**

IsdH^{N2} binds to a site comprising portions of the A and E helices of α Hb—To begin to understand the function of IsdH^{N2-N3}, we crystallized an IsdH^{N2}-Hb complex from a mixture containing a 2:1 molar ratio of IsdH^{N2} to Hb tetramer and determined the x-ray crystal structure to 2.3 Å resolution (Table 1 and Fig. 2). Phases were obtained by molecular replacement using the structures of Hb (PDB 2DN1) (57) and IsdH^{N1} (PDB 3SZK) (11). The asymmetric unit of the IsdH^{N2}-Hb crystals comprises one Hb dimer with one IsdH^{N2} molecule bound to the α Hb subunit (Fig. 2A). IsdH^{N2} (Fig. 2B) binds in the same position on α Hb as IsdH^{N1} (Fig. 2C), and a comparable number of residues at both interfaces participate in hydrogen bonds and salt bridges, including interactions with Asp-74, Lys-11, and Thr-8 of α Hb (Fig. 2, B and C). A short α helix in IsdH^{N2} (primary sequence FYHYAS), containing a series of aromatic side chains, forms part of the IsdH^{N2-α} Hb-binding interface (Fig. 2D). A helix with similar hydrophobic character, but different primary sequence (YYHFFS), occurs in IsdH^{N1} (11, 12). A Phe side chain from a different position in the IsdH^{N2} or
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IsdH\textsuperscript{N1} aromatic \(\alpha\) helix is buried in the groove between helices A and E of \(\alpha\) Hb (Fig. 2, B and C), suggesting that the aromatic motif is functionally conserved. The \(\alpha\) Hb subunit retains its native structure in complex with either IsdH\textsuperscript{N2} (r.m.s.d. of 0.8 Å over 136 Ca atoms as compared with PDB 2DN1) or IsdH\textsuperscript{N3} (r.m.s.d. of 0.8 Å over 136 Ca atoms as compared with PDB 2DN1), which suggests that the IsdH\textsuperscript{N1} and IsdH\textsuperscript{N2} domains alone do not destabilize the \(\alpha\) globin fold or promote heme release, in agreement with previous functional studies (11, 27). In addition, the IsdH\textsuperscript{N2}, binding site is distant from the entrance to the globin heme pocket, making it unlikely to be directly involved in heme extraction. We conclude that IsdH\textsuperscript{N2} performs an Hb recognition/targeting role within the native IsdH receptor.

The Three Domains of IsdH\textsuperscript{N2,N3} Are Assembled into a Higher Order Structure That Binds to \(\alpha\) and \(\beta\) Hb Chains and Positions the Globin Heme Pocket to Achieve Heme Transfer—To trap a stable IsdH\textsuperscript{N2,N3}-Hb complex for x-ray crystallographic studies, we expressed IsdH\textsuperscript{N2,N3} with a Tyr-642 to Ala mutation in the helical linker domain (Fig. 3A). This arrangement is consistent with interdomain interactions detected in an NMR spectroscopic analysis of the free receptor (27). Despite differences in the \(\alpha\) and \(\beta\) Hb chain sequences, IsdH is clearly bound to equivalent sites on both globin subunits (Fig. 3C). On the \(\alpha\) subunit, the IsdH\textsuperscript{N2} domain docks in the same position in the IsdH\textsuperscript{N2,N3(Y642A)}-Hb and IsdH\textsuperscript{N2}-Hb structures, suggesting that the Hb-targeting function of IsdH\textsuperscript{N2} is preserved in the context of the full IsdH receptor and providing independent validation of both structures. At the IsdH\textsuperscript{N2,N3(Y642A)}-\(\beta\) interface, the IsdH\textsuperscript{N2} domain binds at a site on the A and E helices of \(\beta\) Hb, analogous to the binding site on \(\alpha\) Hb. Of the 15 residues that comprise the IsdH\textsuperscript{N2}-interacting face of \(\alpha\) Hb, there are three nonconservative substitutions in \(\beta\) Hb: \(\alpha\) Ala-5 is substituted by \(\beta\) Glu-6; \(\alpha\) Asn-9 is substituted by \(\beta\) Ala-10; and \(\alpha\) Lys-11 is substituted by \(\beta\) Thr-12. The latter two changes are expected to disrupt hydrogen-bonding interactions with IsdH\textsuperscript{N2} (Fig. 2B). Nevertheless, interactions between isolated IsdH\textsuperscript{N2} and \(\beta\) Hb can be detected in gel filtration (11), suggesting that the IsdH\textsuperscript{N2,N3(Y642A)}-Hb crystal captures a weak mode of interaction that is mechanistically important (as described below).

In three of the IsdH receptor molecules, the helical linker domain positions the IsdH\textsuperscript{N3} domain directly over the heme...
The pocket of the bound globin subunit. The electron density observed for the fourth IsdHN3 domain, which was expected to lie over the heme pocket of the other Hb subunit, was not sufficiently strong to place this IsdHN3 domain with confidence (Fig. 3A, dashed circle), and it was therefore not included in the model. The three complete IsdHN2-N3(Y642A) molecules in the complex have essentially identical structures (backbone r.m.s.d. 1.2 Å in pairwise comparisons), indicating that, in addition to binding the same site on Hb and Hb, IsdHN2-N3 binds both globin chains in the same conformation (Fig. 3C). In this conformation, the IsdHN3 domain is positioned directly over the heme pocket on the globin, such that the heme would need to move only ~12 Å, through the entrance to the globin heme pocket, to effect transfer to IsdH (Fig. 3D, dashed arrow). Interestingly, electron density at the heme-binding site of the IsdHN3 domains (Fig. 3D, green), together with weak anomalous signal, suggests that the receptors have partial heme occupancy despite the inactivating Tyr-642 to Ala mutation. At the resolution of our x-ray data, however, it is not possible to say whether the globin heme pocket structure is altered by interaction with IsdHN3. In addition, the heme-binding strands (residues Val-637-Gln-645) of IsdHN3 are not well defined in the electron density. As a result, we are not able to discern whether localized structural changes occur in Hb to promote heme transfer. Nevertheless, the close proximity of IsdH and Hb heme-binding sites in the IsdHN2-N3-Hb complex is consistent with a direct protein-to-protein heme relay.

The Three Domains of IsdHN2-N3 Are Pre-organized to Position the Heme Acceptor Site over the α/β Hb Heme Pocket—To investigate whether changes in IsdHN2-N3 conformation occur upon binding to Hb, we performed SAXS on the free IsdHN2-N3(Y642A) receptor. Model-free analysis of the SAXS

**FIGURE 3. Crystal structure of IsdHN2-N3 bound to Hb.** A, four IsdHN2-N3 receptors bind to one Hb tetramer in the asymmetric unit of the crystal (PDB 4IJ2). Three of the IsdH N2-N3 receptors are complete, comprising IsdH, linker, and IsdHN3 domains. The position of the IsdHN3 domain in the fourth receptor molecule is not well defined (the expected location is marked by a dashed ellipse). B, stereo diagram showing the 2Fo − Fc electron density map for one full IsdHN2-N3 receptor contoured at 1. C, detail of the IsdHN2-N3-α (blue/orange) and IsdHN2-N3-β (teal/yellow) interaction complexes. Residues from the loop regions, which are not modeled in the structure, are shown with dashed lines. D, the IsdHN3-α interface with Fc − Fc difference map shown at 3 σ (green), indicating electron density that is not accounted for by atoms of the IsdHN2-N3-Hb model. The structure of heme-bound IsdH (PDB 2Z6F, red) is superimposed to indicate the expected binding position of the heme group (lines), which coincides with a peak of Fc − Fc difference electron density in the IsdHN2-N3-Hb complex (green). Heme transfer from globin to the IsdH receptor requires a relatively small translation of only 12 Å between the E and F helices of the globin (dashed arrow).
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data yielded the expected molecular weights for all samples, which were within 10% of the theoretical molecular weights calculated from the sequences (Table 2). We generated theoretical scattering curves for the three full receptors present in the complex with Hb using the program CRYSOLO (59). These theoretical curves fit extremely well to the solution scattering of free IsdHN2-N3, with χ values of 0.924, 0.904, and 0.866 (Fig. 4A), indicating that no major structural change occurs upon receptor binding and providing independent validation of the 4.2 Å resolution crystal structure. Ab initio shape reconstruction of the free IsdH receptor reveals close agreement with the crystal structure of the Hb-bound receptor (Fig. 4B). These results argue strongly that the receptor does not undergo a major conformational change upon binding to Hb. Instead, the domains of the receptor are pre-organized to position the heme acceptor site over the globin heme pocket.

IsdHN2-N3 Acquires Heme from α and β Hb—The structure of the IsdHN2-N3-Hb complex shows how IsdH could access heme in both the α and the β subunits of Hb. Physical interaction between IsdHN2 and isolated α and β Hb subunits has been demonstrated in solution, with the binding to β Hb being considerably weaker (11). To investigate whether IsdHN2-N3 can remove heme from α and β chains of Hb, and hence probe the functional significance of β chain interactions, we studied the transfer of the heme from ferric Hb to apo-IsdHN2-N3 using UV-visible spectroscopy. In the absence of heme, apo-IsdHN2-N3 shows negligible absorption from 350–750 nm (Fig. 5A, dashed line). Holo-IsdHN2-N3, when bound to ferric Heme (Fig. 5A, dotted and dashed line), has a characteristic spectrum that distinguishes it from ferric Hb (Fig. 5A, solid line). After mixing ferric Hb with apo-IsdHN2-N3, UV-visible spectra were acquired as a function of time, and the fraction of ferric Hb and holo-IsdHN2-N3 at each time point was determined by least squares fitting to a linear combination of the spectra shown in Fig. 5A. At a mixing ratio of 5 apo-IsdHN2-N3 to 1 Hb tetramer, there was rapid and quantitative transfer of heme from Hb to IsdHN2-N3, with only ~5% ferric Hb remaining after 10 min at 4 °C (Fig. 5B, bottom, dotted and dashed curve). As an alternative approach to assess the number of Hb heme groups accessed by IsdHN2-N3, we measured the final UV-visible spectrum after a 10-min incubation of ferric Hb with different amounts of apo-IsdHN2-N3. We observed a linear relationship between apo-IsdHN2-N3-Hb molar mixing ratio and the total spectral change at 406 nm, up to a molar ratio of ~3.5:1 (Fig. 5C), which was consistent with a 4:1 IsdHN2-N3-Hb interaction model. Together, these experiments show that heme is effectively removed from both α and β chains under these conditions.

Although IsdH can quantitatively deplete heme from Hb, it is possible that heme is released into solution from destabilized Hb and scavenged by IsdHN2-N3 receptors that are not physically bound to Hb chains. To investigate whether a physical interaction between the IsdH receptor and Hb is required to extract heme from all sites (α and β chains), we utilized the isolated IsdHN3 heme-binding domain. The isolated IsdHN3 domain is fully functional to bind heme from solution but is unable to capture heme from ferric Hb over the course of 10 min (Fig. 5B, gray circles), as shown previously by Spirig et al. (27). When IsdHN2-N3 was mixed with Hb in a 2:1 ratio, in the presence or absence of additional IsdHN3, the heme transfer curves were identical, with ~50% of heme groups removed from Hb in each case (Fig. 5B, compare dashed and solid curves), indicating that IsdHN3 only captures heme from Hb in the context of the intact receptor. The absence of any detectible competition for heme binding between IsdHN3 and IsdHN2-N3 confirms that interaction between free IsdHN3 and the globin heme pocket is extremely weak in the absence of the IsdHN2 domain. Together, the above data indicate that heme extraction from Hb (i) takes place directly within an IsdH-Hb complex, (ii) requires contacts mediated through the IsdHN2 domain, and

### Table 2: Molecular parameters from SAXS

|                  | IsdH<sub>N2-N3(Y642A)</sub> | IsdH<sub>N2-N3/Y642A</sub> | IsdH<sub>N3(Y642A)</sub> |
|------------------|-----------------------------|-----------------------------|-----------------------------|
| Structural parameters |                             |                             |                             |
| Sample concentration (mg/ml) | 7                           | 4.5                          | 3                           |
| I(0) (cm<sup>-1</sup>) (P(r) analysis)<sup>a</sup> | 0.1770                      | 0.1186                      | 0.0807                      |
| I(0) (cm<sup>-1</sup>) (P(r) analysis)<sup>b</sup> | 0.183 ± 0.001               | 0.122 ± 0.001               | 0.0821 ± 0.0006             |
| R<sub>i</sub> (Å) (P(r) analysis)<sup>a</sup> | 27.75                       | 28.96                       | 28.99                       |
| R<sub>i</sub> (Å) (P(r) analysis)<sup>b</sup> | 28.1 ± 0.2                  | 30.4 ± 0.2                  | 30.2 ± 0.02                 |
| I(0) (cm<sup>-1</sup>) (Guinier analysis)<sup>a</sup> | 0.183 ± 0.001               | 0.121 ± 0.001               | 0.082 ± 0.001               |
| I(0) (cm<sup>-1</sup>) (Guinier analysis)<sup>b</sup> | 27.7 ± 0.4                  | 29.5 ± 0.5                  | 29.6 ± 0.8                  |
| D<sub>0</sub> (Å) | 85                          | 90                          | 85                          |
| Molecular mass determination |                             |                             |                             |
| Partial specific volume (cm<sup>3</sup> g<sup>-1</sup>) | 0.732                       | 0.732                       | 0.732                       |
| Contrast (Δρ × 10<sup>5</sup> cm<sup>-1</sup>) | 2.808                       | 2.808                       | 2.808                       |
| Molecular mass M<sub>i</sub> (from I(0))<sup>a</sup> | 36,405                      | 36,830                      | 38,470                      |
| Molecular mass M<sub>i</sub> (from I(0))<sup>b</sup> | 37,639                      | 37,575                      | 39,090                      |
| M<sub>i</sub> calculated from primary sequence (monomer) | 38,788                      | 38,788                      | 38,788                      |

<sup>a</sup> Values generated with GIFT.
<sup>b</sup> Values generated with PRIMUS.
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We demonstrate here how multiple domains in IsdH cooperate to effect heme extraction from Hb. Excellent agreement between the IsdH N2-N3-Hb crystal structure and the solution SAXS scattering of the free receptor indicates that the three domains of IsdH N2-N3 are pre-organized to position IsdH N3 at the entrance to the globin heme pocket when the IsdH N2 domain docks with its cognate site between the A and E helices of α and β Hb. The isolated heme-binding IsdH N3 domain interacts with Hb too weakly to function in heme uptake, indicating that physical positioning of this domain in the context of the intact receptor is an essential feature of the heme transfer mechanism. A high level of sequence homology between IsdH N2-N3, IsdB, and an IsdB homologue from the related pathogen S. lugdunensis (28) indicates that our results are relevant to all three proteins.

Heme is extracted from Hb and moved across the bacterial cell wall to the bacterial membrane in a series of direct protein-to-protein transfer steps (Fig. 6). Transport by protein-to-protein relay is frequently employed in nature to transfer reactive species or signaling molecules in both prokaryote and eukaryote cells. Examples include copper transport (60), electron transport (61), and relay of phosphoryl groups in signal transduction in plants (62), animals (63), and bacteria (64). In these examples, precise positioning of the donor and acceptor proteins is critical to cargo transfer, and both donor and acceptor proteins have co-evolved to perform efficient ligand transfer. In contrast, Hb has evolved to minimize heme dissociation, and partial unfolding of the globin is required in order for heme to enter/exit the globin heme pocket (65). We speculate that the challenge of extracting heme from the deep binding cleft in Hb cannot be met by interaction through a single interface. Hence, the IsdB/H receptors are anchored through a site distant from the heme pocket (Fig. 6, step 1), which holds the IsdH N2 domain in contact with Hb while at the same time allowing conformational changes in the heme pocket to take place (Fig. 6, step 2). Consistent with this idea, mutagenesis studies of the Hb receptor A (HgbA) from Gram-negative Haemophilus ducreyi indicate that there is likewise a physical separation of the Hb-binding and heme uptake interfaces (66, 67). This similarity suggests that an architecture involving multiple interaction interfaces might have a functional advantage for heme extraction from Hb in a range of systems.

Protein-protein interactions that mediate transport or signaling are typically extremely transient in nature (60, 61, 64). In the Isd pathway, weak interactions (Kd > 5 mM) are responsible for rapid heme transfer between S. aureus IsdA and IsdC (41). The structure of IsdH N2-N3 predicts that the IsdB/H receptors would need to dissociate from Hb to relay heme to IsdA/C (Fig. 6, step 3), and hence there is necessarily a balance between the IsdB/H-Hb complex persisting long enough for heme extraction, but not so long that transfer to IsdA/C becomes impractically slow. In this light, it is possible that weaker binding of IsdH to β Hb, as compared with α Hb, is an adaptation to the intrinsically more rapid heme dissociation from the β subunits of Hb dimers/tetramers (42). Notably, IsdH contains an additional N-terminal Hb-binding domain, IsdH N1, which is not present in IsdB. IsdH N1 and IsdH N2 bind to the same site on α Hb and so may compete for binding under some circumstances. IsdH N2 displays no detectible interaction with β Hb (11) and so is not expected to interfere with IsdH N2-N3 binding to β Hb. The precise role that IsdH N2 plays, in the context of full-length IsdH, will now need to be established. Notwithstanding these factors, our data indicate that heme capture by IsdH and IsdB occurs by a similar mechanism for α and β chains. Kinetic data also support a single mechanism of heme uptake from α and β chains; Zhu et al. (18) showed that heme is almost completely transferred from Hb to IsdB in under 2 min at 22 °C with a single rate constant.

In IsdH and IsdB, the same NEAT domain fold has evolved to specifically and exclusively bind either the surface of Hb or a heme molecule. The Pfam protein fold database identifies over 2000 NEAT domain sequences in Gram-positive species across the phylum Firmicutes, including many pathogens that cause severe human disease such as S. pyogenes, B. anthracis, C. perfringens, and L. monocytogenes, as well as S. aureus. Most NEAT
domains are identified as heme-binding modules, based on conservation of key residues involved in heme interactions. The most conserved NEAT-containing protein is IsdC. IsdC contains a single NEAT domain that delivers heme to the heme permease complex in the cytoplasmic membrane (IsdE/F), which is also highly conserved in Firmicutes, suggesting that IsdC/E/F are components of an ancestral heme-scavenging pathway. In contrast, upstream of IsdC, there is variation in the number of NEAT proteins and the domain architectures of these proteins in different bacterial species, reflecting diversification in the mechanisms for heme capture and heme relay to IsdC. NEAT domain proteins that target Hb as an iron source include IsdX1, IsdX2, and Hal from B. anthracis (30–33), IlsA from B. cereus (34), and Sh from S. pyogenes (35, 68). Each of these proteins contains one or more heme-binding NEAT domains and additional domains with poorly characterized function (Fig. 7). Interestingly, the diverse domain architecture suggests that heme capture from Hb may have evolved multiple times. Although the IsdH/N2 and linker domain sequences appear to be unique to S. aureus and closely related species, different domains could play conceptually similar roles to target and support heme-binding NEAT domains. For example, in Sh, sequences adjacent to the N-terminal NEAT domain have been implicated in Hb binding (68). The structures and properties of these domains have not been determined. In addition, Sh, IsdX2, and Hal contain leucine-rich repeat domains; these domains are well established as versatile protein interaction motifs (69). The secreted hemophore IsdX2 takes heme from Hb via four heme-binding NEAT domains (32, 33). A fifth NEAT domain does not bind heme, but does interact with Hb (32), and so may play structural or Hb-targeting function in the context of the full-length IsdX2. IsdH/N2–N3 is the first example from Gram-positive bacteria where a structure comprising multiple domains of an Hb receptor has been determined, revealing how these domains cooperate to achieve function. The IsdH/N2–N3/Hb structure predicts that IsdH can access iron from all forms of Hb that are likely to be encountered in serum following erythrocyte lysis. Dissociation of the Hb tetramer occurs in vivo following erythrocyte lysis, due to dilution effects and autooxidation processes. In addition, removal of heme from one or more globin chains is expected to destabilize Hb, leading to increased formation of monomers (27, 42). Importantly, our results suggest that a similar mechanism of heme extraction will operate for tetramer, dimer, or monomer
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globin species. Hb released by hemolysis is also bound by the serum scavenger protein haptoglobin, which inhibits globin denaturation and targets Hb to the scavenger receptor CD163 on macrophages. IsdBH binds Hb-haptoglobin complexes and the IsdH N1 domain is reported to bind haptoglobin and hemoglobin independently (7, 10, 12, 22), but heme uptake from Hb-haptoglobin complexes has not been measured. The structure of an Hb-haptoglobin complex was solved recently (70), and comparison of that structure with the IsdH N2–N3-Hb complex shows that haptoglobin does not block IsdH N2 or IsdH N3 domain interactions with the globin, suggesting that Hb-haptoglobin is available as an iron source for S. aureus.

In summary, our data reveal the mechanism by which S. aureus, an important human pathogen, obtains the iron that is essential for infection. The pre-organized, multidomain architecture of the IsdH/Hb-encapping unit is a solution that couples highly specific recognition of Hb to a functional heme extraction module and might represent a general strategy exploited by a wide range of pathogenic bacteria.

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REFERENCES

1. Kleven, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Fridkin, S. K. (2007) Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA 298, 1763–1771

2. Klein, E., Smith, D. L., and Laxminarayan, R. (2007) Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999–2005. Emerg. Infect. Dis. 13, 1840–1846

3. Dukic, V. M., Lauderdale, D. S., Wilder, J., Daum, R. S., and David, M. Z. (2013) Epidemics of community-associated methicillin-resistant Staphylococcus aureus in the United States: a meta-analysis. PLoS One 8, e52722

4. Nairz, M., Schroll, A., Sonnweber, T., and Weiss, G. (2010) The struggle for iron - a metal at the host-pathogen interface. Cell. Microbiol. 12, 1691–1702

5. Torres, V. J., Attia, A. S., Mason, W. J., Hood, M. I., Corbin, B. D., Beasley, F. C., Anderson, K. L., Staff, D. L., McDonald, W. H., Zimmerman, L. J., Friedman, D. B., Heinrichs, D. E., Dunnan, P. M., and Skaar, E. P. (2010) Staphylococcus aureus fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. Infect. Immun. 78, 1618–1628

6. Noinaj, N., Easley, N. C., Oke, M., Mizuno, N., Gumbar, J., Boura, E., Steere, A. N., Zak, O., Aisen, P., Tajkhorshid, E., Evans, R. W., Gorringe, A. R., Mason, A. B., Steven, A. C., and Buchanan, S. K. (2012) Structural basis for iron piracy by pathogenic Neisseria. Nature 483, 53–58

7. Dryla, A., Gelbmann, D., von Gabain, A., and Nagy, E. (2003) Identification of a novel iron regulated staphylococcal surface protein with haptoglobin-hemoglobin binding activity. Mol. Microbiol. 49, 37–53

8. Skaar, E. P., Humayun, M., Bae, T., DeBord, K. L., and Schneewind, O. (2004) Iron-source preference of Staphylococcus aureus for heme. Infect. Immun. 72, 544–550

9. Pilpa, R. M., Robson, S. A., Villareal, V. A., Wong, M. L., Phillips, M., and Clubb, R. T. (2009) Functionally distinct NEAT (NeAr Transporter) domains within the Staphylococcus aureus IsdH/HarA protein extract heme from hemoglobin. J. Biol. Chem. 284, 1166–1176

10. Skaar, E. P., Humayun, M., Bae, T., DeBord, K. L., and Schneewind, O. (2004) Iron-source preference of Staphylococcus aureus for heme. J. Biol. Chem. 279, 16477–16487

11. Mack, J., Vermeiren, C., Heinrichs, D. E., and Stillman, M. J. (2004) In vivo heme scavenging by Staphylococcus aureus IsdC and IsdD proteins. Biochim. Biophys. Res. Commun. 320, 781–788

12. Abe, R., Caeveiro, J. M., Kozuka-Hata, H., Oyama, M., and Tsumoto, K. (2012) Mapping ultra-weak protein-protein interactions between heme transporters of Staphylococcus aureus. J. Biol. Chem. 287, 16477–16487

13. Moyer, N., Tiedemann, M. T., Pluym, M., Cheung, J., Heinrichs, D. E., and Stillman, M. J. (2008) Demonstration of the iron-regulated surface determinant (Isd) heme transfer pathway in Staphylococcus aureus. J. Biol. Chem. 283, 28125–28136

14. Torres, V. J., Attia, A. S., Mason, W. J., Hood, M. I., Corbin, B. D., Beasley, F. C., Anderson, K. L., Staff, D. L., McDonald, W. H., Zimmerman, L. J., Friedman, D. B., Heinrichs, D. E., Dunnan, P. M., and Skaar, E. P. (2010) Staphylococcus aureus fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. Infect. Immun. 78, 1618–1628

15. Mack, J., Vermeiren, C., Heinrichs, D. E., and Stillman, M. J. (2012) The multi-protein heme shuttle pathway in Staphylococcus aureus: Isd cog-wheel kinetics. J. Am. Chem. Soc. 134, 16578–16585

16. Zhu, H., Xie, G., Liu, M., Olson, J. S., Fabian, M., Dooley, D. M., and Lei, B. (2008) Pathway for heme uptake from human hemoglobin by the iron-regulated surface determinants of Staphylococcus aureus. J. Biol. Chem. 283, 18450–18460

17. Mazmanian, S. K., Saar, E. P., Gaspar, A. H., Humayun, M., Gornicki, P., Jelenka, J., Joachmiak, A., Missiakas, D. M., and Schneewind, O. (2003) Passage of heme iron across the envelope of Staphylococcus aureus. Science 299, 906–909

18. Morrissey, J. A., Cockayne, A., Hammacott, J., Bishop, K., Denman-Johnson, A., Hill, P. J., and Williams, P. (2002) Conservation, surface exposure, and in vivo expression of the Frp family of iron-regulated cell wall proteins in Staphylococcus aureus. Infect. Immun. 70, 2399–2407

19. Kempel, K., Herbst, F. A., Moche, M., Hecker, M., and Becker, D. (2011) A quantitative proteomic view on secreted, cell-surface-associated and cytoplasmic proteins of the methicillin-resistant human pathogen Staphylococcus aureus under iron-limited conditions. J. Proteome Res. 10, 1657–1666

20. Visai, L., Yanagisawa, N.,Josefsson, E., Tarkowski, A., Pazzali, I., Roojakkers, S. H., Foster, T. J., and Speziale, P. (2009) Immune evasion by Staphylococcus aureus conferred by iron-regulated surface determinant protein IsdH. Microbiology 155, 667–679

21. Skaar, E. D., Dickey, S. E., and Saar, E. P. (2009) Subcellular localization of the Staphylococcus aureus heme iron transport components IsdA and IsdB. Infect. Immun. 77, 2624–2634

22. Cheng, A. G., Kim, H. K., Burts, M. L., Krausz, T., Schneewind, O., and Missiakas, D. M. (2009) Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. FASEB J. 23, 3393–3404

23. Miro, J. M., Anguera, I., Cabell, C. H., Chen, A. Y., Stafford, J. A., Corey, G. R., Olaison, L., Eykyn, S., Hosen, B., Abrutyn, E., Raoult, D., Bayer, A., and Bowler, G. (2005) Staphylococcus aureus native valve infective endocarditis: report of 566 episodes from the International Collaboration on Endocarditis Merged Database. Clin. Infect. Dis. 41, 507–514

24. Spirig, T., Malmirchegini, G. R., Zhang, J., Robson, S. A., Sjodt, M., Liu, M., Krishna Kumar, K., Dickson, C. F., Gell, D. A., Lei, B., Luo, J. A., and Clubb, R. T. (2013) Staphylococcus aureus uses a novel multi-domain receptor to break apart human hemoglobin and steal its heme. J. Biol. Chem. 288, 1065–1078

25. Zapotoczna, M., Heilbrunner, S., Speziale, P., and Foster, T. J. (2012) Iron regulated surface determinant (Isd) proteins of Staphylococcus lugdunensis. J. Bacteriol. 194, 6453–6467

26. Anguera, I., Del Río, A., Miró, J. M., Matínez-Lacasa, X., Marco, F., Gumà,
The text is not suitable for natural reading as it seems to be a scrambled or corrupted version of a scientific document. The text contains numerous errors and does not form coherent sentences. It appears to be a mix of scientific terms, proper names, and other elements that do not fit together logically. Without further context, it is challenging to understand the intended content.
69. Kobe, B., and Kajava, A. V. (2001) The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struct. Biol.* **11**, 725–732

70. Andersen, C. B., Torvund-Jensen, M., Nielsen, M. J., de Oliveira, C. L., Hersleth, H. P., Andersen, N. H., Pedersen, J. S., Andersen, G. R., and Moestrup, S. K. (2012) Structure of the haptoglobin-haemoglobin complex. *Nature* **489**, 456–459

71. Silva, M. M., Rogers, P. H., and Arnone, A. (1992) A third quaternary structure of human hemoglobin A at 1.7-A resolution. *J. Biol. Chem.* **267**, 17248–17256

72. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1277–1282