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Hemoglobin Binding and Catalytic Heme Extraction by IsdB Near Iron Transporter Domains

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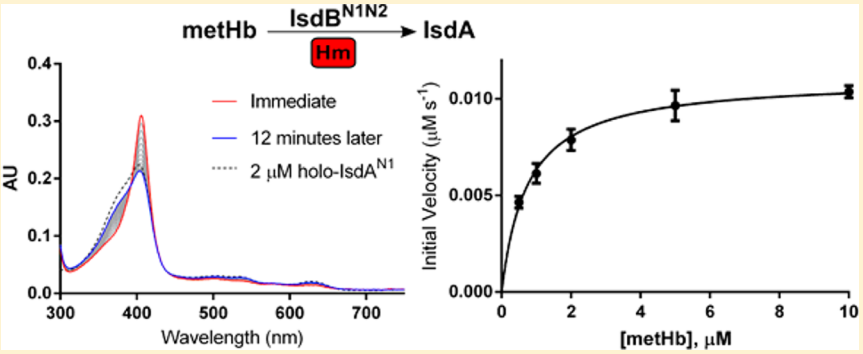
Supporting Information



ABSTRACT: The Isd (iron-regulated surface determinant) system is a multiprotein transporter that allows bacterium *Staphylococcus aureus* to take up iron from hemoglobin (Hb) during human infection. In this system, IsdB is a cell wall-anchored surface protein that contains two near iron transporter (NEAT) domains, one of which binds heme. IsdB rapidly extracts heme from Hb and transfers it to IsdA for relay into the bacterial cell. Using a series of recombinant IsdB constructs that included at least one NEAT domain, we demonstrated that both domains are required to bind Hb with high affinity (K_D = 0.42 ± 0.05 μM) and to extract heme from Hb. Moreover, IsdB extracted heme only from oxidized metHb, although it also bound oxyHb and the Hb−CO complex. In a reconstituted model of the biological heme relay pathway, IsdB catalyzed the transfer of heme from metHb to IsdA with a K_m for metHb of 0.75 ± 0.07 μN and a k_{cat} of 0.22 ± 0.01 s^{-1}. The latter is consistent with the transfer of heme from metHb to IsdB being the rate-limiting step. With both NEAT domains and the linker region present in a single contiguous polypeptide, high-affinity Hb binding was achieved, rapid heme uptake was observed, and multiple turnovers of heme extraction from metHb and transfer to IsdA were conducted, representing all known Hb−heme uptake functions of the full-length IsdB protein.

Staphylococcus aureus is a Gram-positive coccus and a common member of the normal human flora, colonizing approximately one-third of the population, primarily on the hands and nostrils.[1] It can also cause serious infections because of its large array of virulence factors: both the genuinely pathogenic (such as leukocidin and the hemolysins)[2] and those geared more toward survival in the host (such as adhesins and immune evasion systems).[3] One important aspect of survival for bacteria is the acquisition of iron from the host because the level of free iron present in the human body is many orders of magnitude lower than that required for bacterial growth. The vast majority of iron in the human body occurs as part of the heme molecule, most of which is found in the oxygen carrier protein hemoglobin (Hb). To take advantage of this abundant iron source *S. aureus* possesses a system of proteins designed to use heme and hemoglobin as an iron source, the iron-regulated surface determinant (Isd) system.

The Isd system consists of four peptidoglycan-anchored surface proteins (IsdA, IsdB, IsdC, and IsdH) that reversibly bind heme, an ABC transporter (IsdF) with an associated lipoprotein (IsdE), and two intracellular heme-degrading enzymes (IsdG and IsdI).[4] The function of a ninth component, IsdD, remains unknown. Each surface protein contains one to three copies of a conserved NEAT (for near iron transporter) domain.[5] NEAT domains adopt an eight-stranded β-sandwich fold[6−9] and mediate heme and hemoprotein binding by the surface proteins. In particular, IsdB contains two NEAT domains (N1 and N2) and acts as the primary Hb receptor for the cell.[10] IsdB is critical for the use of Hb as an iron source by *S. aureus*, both *in vitro* and during infection.[10,11] In fact, IsdB is the most highly upregulated member of the *isd* gene cluster under all tested iron restriction conditions[12,13] and is the second most highly upregulated transcript in a comprehensive microarray of *S. aureus* cultured in purified human blood or serum.[13] A low Hb concentration (~2 μM) is found in healthy human plasma because of the normal intravascular hemolysis of aging red blood cells;[14] furthermore, *S. aureus* encodes a

Received: November 28, 2013
Published: March 20, 2014

number of hemolysins, one of which (γ-hemolysin) is significantly upregulated during growth in human blood.[13] The role of IsdB is to extract the heme from Hb at the cell surface for transfer to IsdA or IsdC, which then relays it to the membrane transporter (IsdEF) for internalization.[15] IsdH is composed of three NEAT domains (N1−N3) and is also able to bind Hb but does not appear to be dominant for the use of Hb as an iron source during infection.[10]

The amino acid sequence of the IsdB N1 domain is 46 and 65% identical with those of the IsdH N1 and IsdH N2 domains, respectively, and the amino acid sequence of the IsdB N2 domain is 56% identical with that of the IsdH N3 domain (Figure 1). Furthermore, both IsdB N2 and IsdH N3 domains

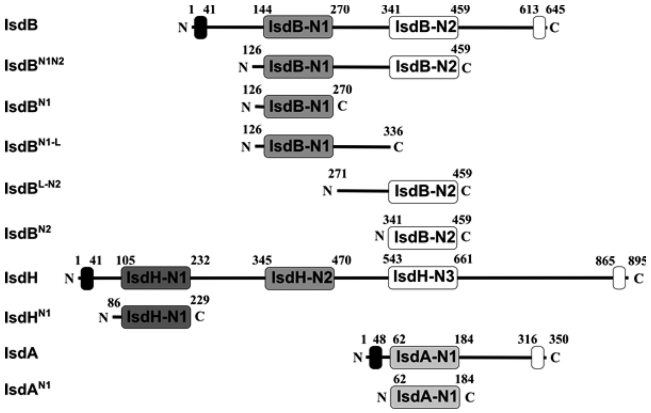


Figure 1. Sequence features of Isd surface proteins discussed in this study and recombinant constructs used. The full-length sequences of IsdB, IsdH, and IsdA are included for context but were not used as cloned constructs. The black N-terminal boxes indicate the signal sequences for secretion, and the white C-terminal boxes indicate the sortase sequences for cell wall peptidoglycan anchoring of the full-length proteins. A high level of sequence identity between NEAT domains is indicated by similar NEAT domain coloring.

have been shown to bind heme,[9,16] and IsdH N1 and IsdH N2 domains were shown to bind Hb as isolated domains.[16−19] Specifically, the IsdH N1 domain was shown to bind only to the α-subunit of Hb (αHb), whereas the IsdH N2 domain binds to both α- and β-subunits.[18] A crystal structure of the IsdH N1 domain in complex with metHb has delineated the binding interface of the complex.[18] A motif rich in aromatic residues that is conserved among the IsdB N1, IsdH N1, and IsdH N2 domains was shown to be required for Hb binding by the IsdH N1 domain[16] and is now known to be part of the binding interface between αHb and the IsdH N1 domain.[18] Moreover, the IsdH N2 and IsdH N3 domains must be present in a contiguous unit with the linker region intact to perform the rapid extraction of heme from metHb.[19]

In contrast, little is understood about IsdB−Hb interactions beyond the observation that full-length IsdB does bind Hb[15,17,20] and remove heme from metHb.[15] Because of the high level of sequence identity between IsdB and IsdH domains, the presence of the conserved aromatic motif, and their apparent similarity in overall function, the function of the IsdB N1 domain has been suggested to be binding Hb in a fashion analogous to that of the IsdH N1 domain. We had previously shown that the IsdB N2 domain binds heme and transfers heme to the IsdA N1 domain,[9] and herein, we have determined which domain(s) of IsdB is necessary and/or sufficient for Hb binding and uptake of heme from Hb. To this end, soluble recombinant constructs that contained various portions of the IsdB coding region were produced (Figure 1). Hb purified from human blood was used for all studies in its reduced form with oxygen bound (oxyHb), in its reduced form with carbon monoxide bound (HbCO), or in its oxidized form (metHb), as indicated.

EXPERIMENTAL PROCEDURES

Cloning. Plasmids encoding recombinant constructs with an N-terminal His_6 tag and thrombin cleavage site were generated in pET28a(+) vectors containing various portions of the IsdB coding region: $IsdB^{N1N2}$ (residues 126−459), $IsdB^{N1}$ (residues 126−270), $IsdB^{N1-L}$ (residues 126−336), $IsdB^{L-N2}$ (residues 271−459), and $IsdB^{N2}$ (residues 341−459), as well as one construct of an IsdH NEAT domain, $IsdH^{N1}$ (residues 86−229) and the IsdA NEAT domain, $IsdA^{N1}$ (residues 62−184). Briefly, IsdB constructs were subcloned from a GST-tagged construct cloned from *S. aureus* N315 chromosomal DNA,[9] using a modified whole plasmid polymerase chain reaction method.[21] The IsdH construct was generated by total gene synthesis (GenScript). Cloning of the IsdA construct was conducted as previously described.[6] All clones were confirmed by DNA sequencing (Agencourt).

Protein Expression and Purification. Recombinant IsdB and IsdH constructs were overexpressed in *Escherichia coli* BL21(DE3) cells. A 2 L bacterial culture was grown from 2 mL of overnight culture in Luria-Bertani (LB) broth supplemented with 25 μg/mL kanamycin at 30 °C to an OD_{600} of 0.7−0.9, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside, and grown for an additional 18 h at 25 °C. Cells were pelleted by centrifugation, resuspended in 20 mL of 50 mM HEPES (pH 7.4), 150 mM NaCl, and 10 mM imidazole, and then lysed at 4 °C using an EmulsiFlex-C5 homogenizer (Avestin). Insoluble material was removed by centrifugation; the soluble lysate contained a mixture of apo- and holo-His_6 ($IsdB^{N1N2}$, $IsdB^{L-N2}$, and $IsdB^{N2}$) or only apoprotein ($IsdB^{N1}$, $IsdB^{N1-L}$, and $IsdH^{N1}$). The apoprotein could be separated from the cellular protein and holoprotein (where applicable) using a HisTrap nickel affinity column (GE Healthcare) by elution with an imidazole gradient. The apoprotein was dialyzed against 50 mM HEPES (pH 7.4) and 50 mM NaCl and then cleaved with thrombin at a 1:500 ratio by weight of His_6 protein to remove the His_6 tag, leaving behind a two-amino acid (Gly-Ser) N-terminal artifact. The recombinant protein was then dialyzed against 50 mM HEPES (pH 7.4) for cation exchange chromatography using a Source 15S column (GE Healthcare) in the case of IsdB constructs and anion exchange chromatography using a Source 15Q column (GE Healthcare) in the case of $IsdH^{N1}$. The purified protein was obtained by elution with a NaCl gradient. The resulting pure [>95% by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)] apoprotein was dialyzed against either 20 mM HEPES (pH 7.4) and 80 mM NaCl (μ = 0.1) for pull downs or steady-state kinetics or 20 mM NaH_2PO_4 (pH 7.4) and 50 mM NaCl (μ = 0.1) for isothermal titration calorimetry studies. The NEAT domain of IsdA ($IsdA^{N1}$) was produced as described previously.[22]

Hemoglobin Preparation. Human blood (~9 mL) from a healthy volunteer was collected in 3.2% sodium citrate tubes by a health practitioner following appropriate institutional protocols. The blood was spun at 1500*g* and 4 °C for 10 min, and the supernatant (plasma) was removed. The red cell pellet was washed three times with a 3-fold excess of ice-cold 0.9% NaCl and collected by centrifugation at 2000*g* and 4 °C

dx.doi.org/10.1021/bi500230f | *Biochemistry* 2014, 53, 2286−2294

for 10 min each. The red cells were then lysed osmotically by addition of two cell pellet volumes of 50 mM Tris (pH 8.6) and 2 mM EDTA (4 C), followed by incubation on ice for 30 min. The resulting solution was centrifuged at 11000*g* and 4 °C for 30 min, and the supernatant was removed. To precipitate out the stroma, solid NaCl was added to a final concentration of 50 mg/mL of Hb solution, while the mixture was being stirred. Stroma were removed by centrifugation at 11000*g* and 4 °C for 30 min. The supernatant containing Hb was dialyzed against 50 mM Tris (pH 8.6) and 1 mM EDTA at 4 °C overnight. The next day, a small aliquot of the dialyzed red cell lysate was applied to a Source 15Q anion exchange column (GE Healthcare) equilibrated with 50 mM Tris (pH 8.6), and HbA (~95% of the human hemoglobin complement) was separated from other minor types of hemoglobin and other red cell proteins by elution with a NaCl gradient. Purified HbA was dialyzed against 50 mM Tris (pH 8.0) at 4 °C. The remaining dialyzed red cell lysate was flash-frozen in liquid nitrogen in small aliquots for use as needed.

Carbonmonoxyhemoglobin (HbCO) was produced as described by Safo and Abraham.[23] Briefly, a few grains of sodium dithionite were added to a vial of purified HbA to produce deoxyHb; the resulting solution was purged for 20 min with nitrogen and then for 5 min with carbon monoxide. MetHb was produced by oxidizing oxyHb with a 1.2-fold molar excess of potassium ferricyanide, followed by incubation for 10 min at room temperature and desalting on a Sephadex G-50 column. Hemoglobin concentrations were determined by the pyridine hemochrome method as previously described.[24]

Oligomerization states of oxyHb and metHb preparations were confirmed by size exclusion chromatography coupled with multiangle light scattering; briefly, samples were separated on a WTC-030S5 size exclusion column equilibrated in 50 mM Tris (pH 8.0), with an in-line MALS detector (MiniDAWN TREOS) and a refractive index detector (Optilab T-rEX; all from Wyatt Technology Corp.).

IsdB Pull-Down Assay. Aliquots (16 × 25 μL) of nickel bead slurry (Chelating Sepharose Fast Flow, GE Healthcare; stored in 20% ethanol) were washed twice with 1 mL of dH_2O, followed by a 1 mL wash with binding buffer, consisting of 20 mM HEPES (pH 7.4), 80 mM NaCl, and 10 mM imidazole. The small amount of imidazole was included to abrogate nonspecific binding of IsdB constructs to the beads but did not affect the binding of Hb to the nickel beads. For controls (Hb or IsdB construct alone), 50 μL of 20 μM IsdB or 20 μN (in heme) Hb was added to the beads and kept on ice for the duration of the pull-down experiment. For pull-down experiments, Hb was added to a level of 20 μN to the beads and kept on ice for 15 min, after which an equimolar amount of IsdB was added for a total final volume of 50 μL and incubated on ice for a further 30 min. All samples were gently agitated occasionally. After incubation, the 50 μL eluate was removed, and beads with bound protein were washed twice with 500 μL of binding buffer and then eluted with 25 μL of 50 mM HEPES (pH 7.4), 150 mM NaCl, and 500 mM imidazole. Each eluate (12 μL) was run on a 15% SDS−PAGE gel at 150 V for 75 min and stained using Coomassie Blue.

Alternatively, the reverse pull-down experiment was conducted, with His_6-tagged IsdB constructs bound to beads pulling down Hb. The samples were processed in a manner identical to that described above, except that the binding buffer contained 75 mM imidazole, a concentration at which His_6-IsdB could bind the nickel beads but Hb could not.

Electronic Spectra of Heme Transfer. Spectra (250−750 nm) of hemoglobins, IsdB constructs, or mixtures thereof were recorded in a conventional spectrophotometer (Cary50) with an optical path length of 1 cm in a quartz cell at room temperature (22 °C). OxyHb or metHb (2 μN each) was mixed with various IsdB constructs at 20 μM, and spectra were immediately recorded; further recordings were taken as indicated.

Isothermal Titration Calorimetry (ITC). ITC was conducted on a MicroCal ITC-200 instrument. All samples were dialyzed overnight against 20 mM NaH_2PO_4 (pH 7.4) and 50 mM NaCl; dialysis buffer was sterile-filtered and then used for sample dilution and washing the ITC instrument. IsdB or IsdH (250 μM each) was used as the titrant, with 25 μN HbCO in the cell; in the case of $IsdB^{N1-L}$ and $IsdB^{L-N2}$, additional runs with 700 μM IsdB titrated into 70 μN HbCO were also performed, under the same conditions. Twenty injections of 2 μL each at 180 s intervals were performed at 25 °C. Binding isotherms were analyzed using a single-site binding model with the MicroCal-modified version of Origin 7.0.

Steady-State Heme Transfer Kinetics. The relay of heme from metHb to $IsdA^{N1}$ by catalytic amounts of $IsdB^{N1N2}$ was characterized by exploiting spectral differences between metHb and holo-$IsdA^{N1}$. In a conventional spectrophotometer (Cary60) maintained at 25 °C, a catalytic amount of $IsdB^{N1N2}$ (50 nM; 10-fold lower than the lowest metHb concentration) was used to perform a relay of heme from metHb (concentration ranging from 0.5 to 10 μN) to $IsdA^{N1}$ (50 μM) under steady-state conditions. IsdB and IsdA were added to the reaction cuvette along with buffer, and the cuvette was equilibrated to 25 °C for 2 min prior to the addition of metHb to initiate the relay reaction. The absorbance at 408 nm was monitored for the first 60 s of the reaction. Each reaction was conducted in triplicate; the slopes from 0 to 30 s were averaged to give an initial velocity at each metHb concentration.

Transfer of Heme from MetHb to $IsdB^{N2}$ through $IsdB^{N1-L}$. The uptake of heme from metHb by a combination of $IsdB^{N1-L}$ and $IsdB^{N2}$ was monitored by electronic spectroscopy. MetHb (2 μN) was mixed with 20 μM $IsdB^{N2}$ and 0.2−20 μM $IsdB^{N1-L}$, and spectra from 250 to 750 nm were recorded every 12 s for 1 min and then every 1 min for 5 min. Complete transfer results in a characteristic holo-$IsdB^{N2}$ spectrum.

RESULTS

Purification of Hb. Hb was purified from blood graciously provided by a donor. Spectra of prepared 5 μN Hb in varying states are shown in Figure S1 of the Supporting Information and show the typical spectral features associated with these forms of Hb. OxyHb was exclusively found in its tetrameric form (~63 kDa), and metHb was a mixture of tetrameric and dimeric forms, consistent with its higher tetramer−dimer dissociation constant (Figure S2 of the Supporting Information). Because of the multiple potential oligomerization states, for the sake of clarity hemoglobin concentrations are reported on a heme basis and thus by normality rather than molarity.

Regions of IsdB Required To Bind Hb. Recombinant IsdB contructs (Figure 1) containing a NEAT domain or a NEAT domain and the interdomain linker (L) were tested for Hb binding. We used the well-known property of Hb to bind to Ni-NTA beads[25] to test which IsdB constructs could be bound and pulled down out of solution by immobilized Hb. $IsdB^{N1}$, $IsdB^{N1-L}$, $IsdB^{L-N2}$, and $IsdB^{N2}$ were not pulled down by either oxyHb or metHb; only $IsdB^{N1N2}$ was pulled down and could be

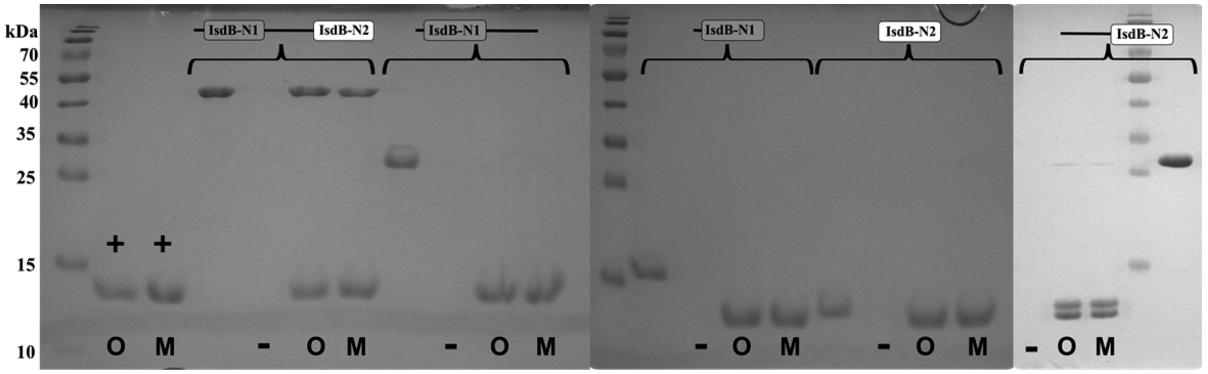


Figure 2. Hemoglobin pull-down assay of IsdB constructs. OxyHb (O) or metHb (M) (20 μN each) was immobilized on nickel beads, followed by incubation with 20 μM IsdB. The bound protein was eluted with buffer and 500 mM imidazole. The positive controls (+) show Hb binding to the beads in the absence of IsdB. The negative controls (−) show the lack of IsdB binding in the absence of Hb. To the left of each negative control lane is a lane containing 1 μg of the respective IsdB construct tested for reference (except for $IsdB^{L\text{-}N2}$, in which the reference is to the right of the ladder).

pulled down by either oxyHb or metHb (Figure 2). Interestingly, these data suggested that only a complete unit comprising both NEAT domains and the intervening linker could bind any form of Hb tested with better than micromolar affinity. Furthermore, $IsdB^{N1N2}$ reconstituted with heme prior to addition to immobilized Hb resulted in much smaller amounts of $IsdB^{N1N2}$ pulled down, suggesting that the affinity of the interaction is decreased possibly because of a conformational change that occurs in IsdB upon heme binding (Figure S3 of the Supporting Information).

The thermodynamics of Hb binding by IsdB constructs was characterized by ITC. Titration of $IsdB^{N1N2}$ into HbCO resulted in an exothermic reaction (Figure 3A). Analysis of the data with a single-site binding model indicated high-affinity binding (K_D = 422 ± 47 nM; ΔH = −6.47 ± 0.19 kcal mol^{-1}; ΔS = 7.45 ± 0.83 cal mol^{-1} K^{-1}; average of three runs). The stoichiometry of binding was 0.76 ± 0.02, which is equivalent to approximately three $IsdB^{N1N2}$ molecules per HbCO tetramer. Similar results were obtained for the reverse reaction, including the stoichiometry. Note that Hb possesses two unequal subunits and $IsdB^{N1N2}$ may interact with each differently; therefore, the K_D and stoichiometry measurements may be considered the average for a mixture of interactions. Previously, a K_D of 50 nM was reported for full-length IsdB with Hb from an unidentified source using surface plasmon resonance.[17]

Under the same conditions and at the same concentrations of $IsdB^{N1}$ (Figure 3C), $IsdB^{N1\text{-}L}$ (Figure S4A of the Supporting Information), or $IsdB^{N2}$ (Figure S4B of the Supporting Information), no interaction could be observed with HbCO by ITC, confirming the lack of a high-affinity interaction observed in the pull-down reactions. When the concentrations of reactants were significantly increased, no interaction between $IsdB^{L\text{-}N2}$ and HbCO was observed (Figure 3D); however, a lower-affinity interaction between $IsdB^{N1\text{-}L}$ and HbCO was observed (Figure 3B). The heats of titration did not approach zero over the course of the experiment, indicating that saturation of HbCO by $IsdB^{N1\text{-}L}$ did not occur. Thus, the fit to a single-site binding model was poor but gave an estimated K_D of ∼130 μM (average of two runs), approximately 300-fold weaker than the interaction between $IsdB^{N1N2}$ and HbCO.

$IsdB^{N1N2}$ Cannot Remove Heme from OxyHb but Removes All Heme Groups from MetHb. Using the same panel of IsdB constructs, the overall competency in the extraction of heme from Hb was investigated by electronic spectroscopy, exploiting the large visible spectral differences between Hb and heme-bound IsdB.[15] Initially, the uptake of heme from oxyHb was assayed; however, when $IsdB^{N1N2}$ (or any IsdB construct) was mixed with oxyHb, minimal spectral changes were observed after 60 min, indicating no significant heme transfer (Figure S5 of the Supporting Information). Previous reports of heme extraction by IsdB employed Hb that is oxidized to the metHb state. In serum, the abundant protein haptoglobin is able to bind to Hb dimers, which autoxidize more quickly than tetramers.[26,27] Given that IsdH can bind haptoglobin and both IsdH and IsdB can bind the haptoglobin−Hb complex, the surface of the bacterial cell is likely to be enriched for metHb. Hb is also known to scavenge nitric oxide produced by endothelial cells, which oxidizes the heme iron.[28] Altogether, metHb is the most likely physiologically relevant Hb heme source for *S. aureus* during human colonization or infection.

When an excess of $IsdB^{N1N2}$ was mixed with metHb, the visible spectrum resembled that of holo-IsdB, which binds heme through the IsdB N2 domain with a 1:1 stoichiometry; the IsdB N1 domain does not bind heme.[9] Indeed, the spectrum overlaid well with that of $IsdB^{N1N2}$ reconstituted with the same amount of heme, indicating that quantitative removal of heme from Hb occurred (Figure 4A). The transfer of heme to $IsdB^{N1N2}$ occurred within the sample mixing and spectrum collection time (<20 s). When $IsdB^{N1\text{-}L}$ and $IsdB^{N2}$ were mixed with metHb, an immediate spectral shift to the holo-IsdB spectrum was also observed (Figure 4B). When $IsdB^{N1}$ and $IsdB^{N2}$ or $IsdB^{N1}$ and $IsdB^{L\text{-}N2}$ were mixed with metHb, slow spectral changes were observed, estimated to be approximately equivalent to the heme off rate for metHb (Figure 4C,D). Thus, both IsdB NEAT domains and the linker region were required for the rapid uptake of heme from metHb. Also, connectivity between $IsdB^{N1}$ and the linker region was essential for the rapid transfer of heme to $IsdB^{N2}$, as the same domains are present in $IsdB^{N1}$ and $IsdB^{L\text{-}N2}$ but rapid heme uptake did not occur.

High-affinity binding of $IsdB^{N1\text{-}L}$ or $IsdB^{N2}$ to Hb was not essential for rapid heme uptake under these conditions. To determine if $IsdB^{N1\text{-}L}$ and $IsdB^{N2}$ could form a complex in the presence of Hb, reconstituting the $IsdB^{N1N2}$ protein, further pull-down assays were employed. Hb did not pull down either $IsdB^{N1}$ or $IsdB^{N2}$ when presented with both (Figure S3D of the Supporting Information, lanes 1 and 2), but Hb did pull down some $IsdB^{N1\text{-}L}$ in the presence of $IsdB^{N2}$ (Figure S3D of the

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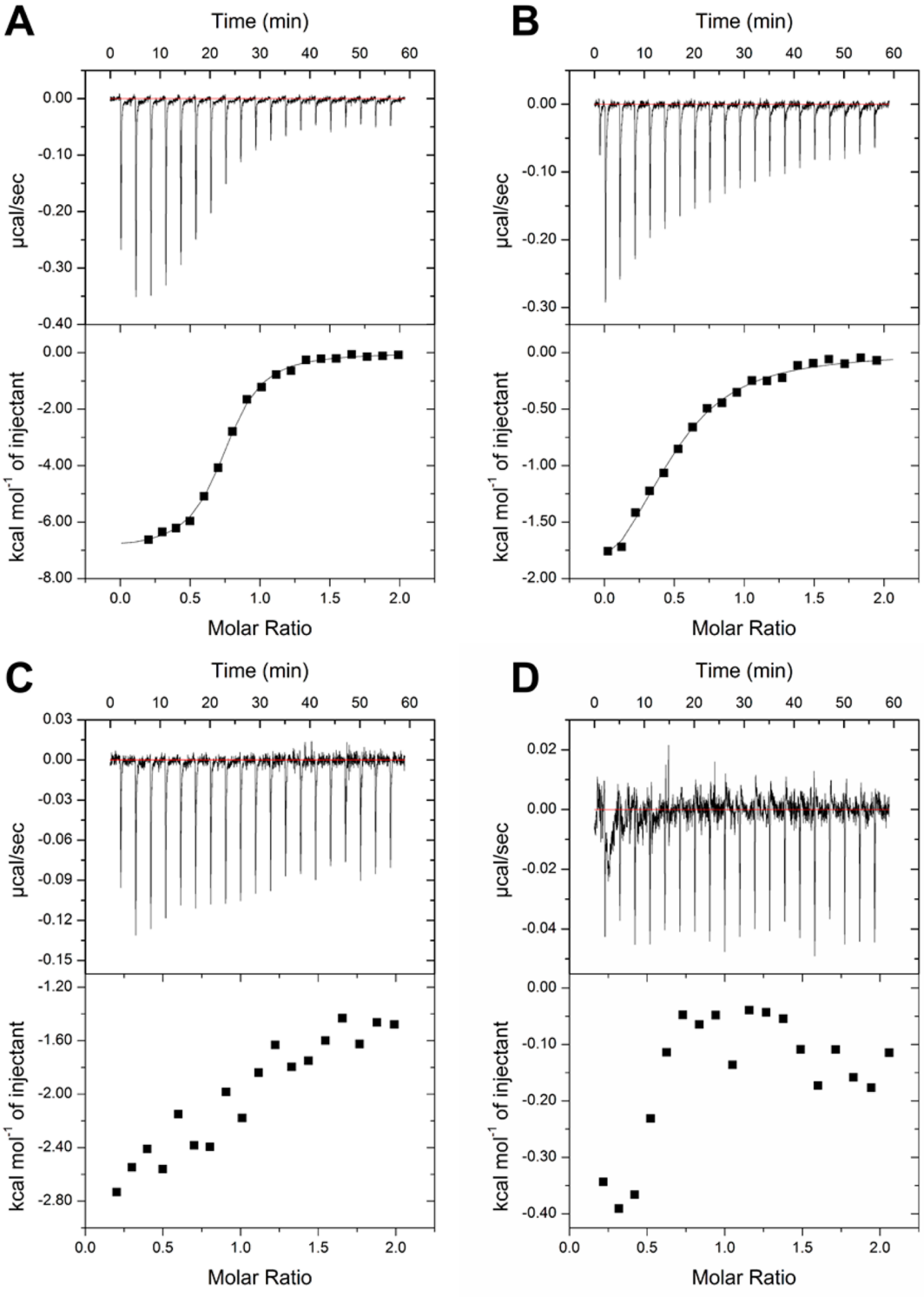

Figure 3. Representative ITC data for titration of (A) 250 μM IsdB^{N1N2} into 25 μN HbCO, (B) 700 μM IsdB$^{N1\text{-}L}$ into 70 μN HbCO, (C) 250 μM IsdB^{N1} into 25 μN HbCO, and (D) 700 μM IsdB$^{L\text{-}N2}$ into 70 μN HbCO. Injections (20 × 2 μL) were made at 180 s intervals in 20 mM NaH_2PO_4 (pH 7.4) and 50 mM NaCl at 25 °C.

Supporting Information, lanes 3 and 4). In the reverse pull-down experiment, immobilized His$_6$-IsdB$^{N1\text{-}L}$ did not pull down IsdB^{N2} (Figure S3B of the Supporting Information, lane 8). However, in the presence of Hb, His$_6$-IsdB$^{N1\text{-}L}$ did pull down Hb and IsdB^{N2} when they were presented together (Figure S3B of the Supporting Information, lanes 11 and 12). Conversely, His$_6$-IsdB$^{L\text{-}N2}$ did not pull down IsdB^{N1} in the presence of Hb (Figure S3C of the Supporting Information, lanes 5 and 6).

IsdB^{N1N2} Is a Catalyst for the Transfer of Heme between MetHb and IsdA^{N1}. The extraction of heme from Hb at the cell surface was modeled by incubating a catalytic amount of the IsdB construct in the presence of excess Hb and IsdA^{N1}, the sole NEAT domain of IsdA. The steady-state rate of

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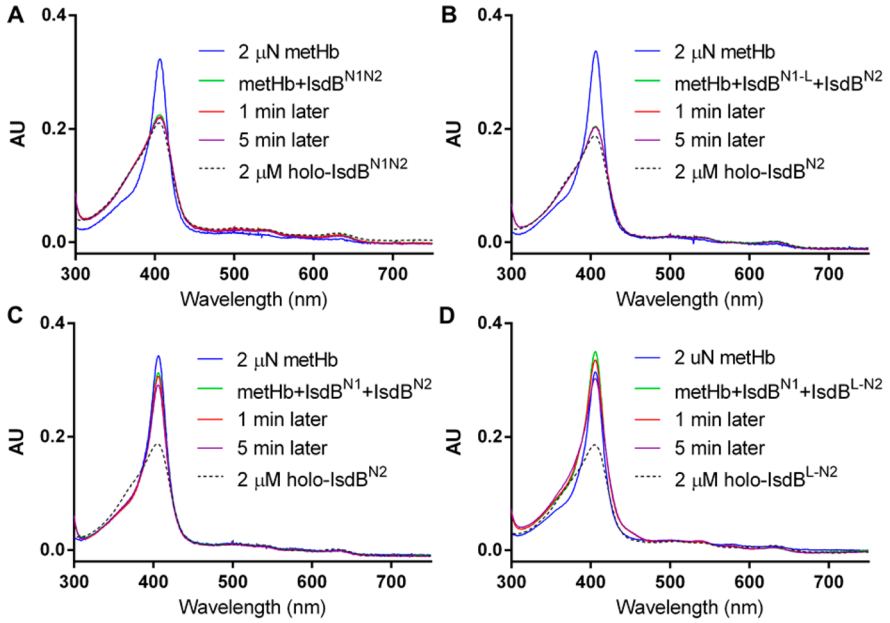

Figure 4. Electronic absorption spectra of 2 μN metHb mixed with 20 μM IsdB constructs (as indicated). A decrease in the magnitude of the Soret band is associated with heme transfer. The heme-reconstituted IsdB apoprotein, diluted to 2 μM by heme concentration, is shown for comparison.

transfer of heme by IsdB^{N1N2} from metHb to IsdA^{N1} was monitored using visible electronic spectroscopy (Figure 5A). IsdB^{N1N2} catalyzed complete heme transfer from metHb, as judged by comparison of the final spectrum to that of IsdA^{N1} loaded with a similar amount of heme. As a control, the same experiment but without IsdA^{N1} resulted in no change to the spectrum of metHb (Figure S6A of the Supporting Information). Also, the absence of IsdB^{N1N2} resulted in the slow transfer of heme to IsdA^{N1}, consistent with solution-based heme transfer occurring at the metHb heme off rate (Figure S6B of the Supporting Information). Furthermore, the spectra of IsdB and IsdA when bound to heme are sufficiently different (Figure S6C of the Supporting Information) that heme transfer kinetics between these proteins was defined previously.[9,29] The kinetics of the IsdB^{N1N2}-mediated heme transfer reaction was followed at a single wavelength (408 nm) for 1 min under steady-state conditions at 25 °C. The initial velocities obtained were linearly dependent on IsdB^{N1N2} concentration and independent of IsdA^{N1} concentration. Reactions were performed with varying metHb concentrations, and the data were fit to Michaelis−Menten kinetics, yielding a K_m for metHb of 0.75 ± 0.07 μN and a k_{cat} of 0.22 ± 0.01 s^{-1} (Figure 5B).

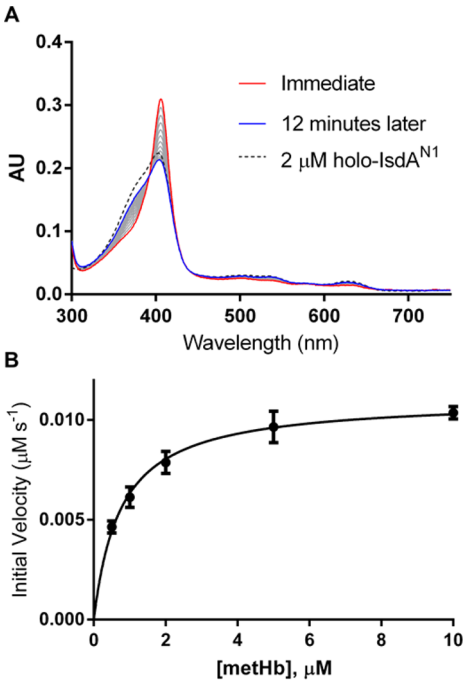

Figure 5. Steady-state transfer of heme from metHb to IsdA^{N1} by IsdB^{N1N2}. (A) Large-scale spectral changes consistent with heme transfer occurred when a catalytic amount of IsdB^{N1N2} (50 nM) was mixed with 2 μN metHb and an excess of acceptor IsdA^{N1} (50 μM). Data for heme-reconstituted IsdA^{N1}, diluted to 2 μM by heme concentration, are shown for comparison. (B) Plot of the initial rates of transfer of heme from metHb to IsdA^{N1} via a catalytic amount of IsdB^{N1N2} (50 nM) as a function of metHb concentration. The curve is a fit of Michaelis−Menten kinetics.

The combination of catalytic amounts of IsdB^{N1-L} and IsdB^{N2} did not catalyze the rapid transfer of heme from metHb to IsdA^{N1}. This result is in contrast to the observed extraction of heme from metHb by the same combination of IsdB fragments at excess concentrations (Figure 4B). Possibly, a ternary complex is formed when heme is relayed from metHb to IsdA^{N1} through IsdB^{N1N2}, which is not formed for the "cleaved" polypeptide. Alternatively, the reduced affinity of IsdB^{N1-L} and IsdB^{N2} for metHb may have impaired the required interaction when present in catalytic amounts rather than in excess. A third possible explanation is that unlike IsdB^{N1N2}, IsdB^{N1-L} and IsdB^{N2} are unable to perform multiple turnovers of extraction of heme from metHb.

This latter hypothesis was investigated by testing the potential of varying concentrations of IsdB^{N1-L} and IsdB^{N2} in conducting the uptake of heme from metHb. The transfer of heme from metHb (2 μN) to IsdB^{N2} (20 μM) was assayed by electronic spectroscopy in the presence of 0.2−20 μM IsdB^{N1-L} (Figure S7 of the Supporting Information). Only in the presence of equimolar (2 μM) or higher concentrations

dx.doi.org/10.1021/bi500230f | Biochemistry 2014, 53, 2286−2294

IsdB^{N1-L} was near-complete heme transfer from metHb observed. With a 10-fold smaller amount of IsdB^{N1-L} (0.2 μM), less than half of the metHb heme was transferred to IsdB^{N2} after 5 min. Under these conditions, IsdB^{N1-L} alone is unable to act as a partner for the uptake of heme from metHb to IsdB^{N2}. Therefore, under the conditions of the steady-state experiment, the combination of IsdB^{N1-L} and IsdB^{N2} was not fully catalytic. An explanation for the limited turnover in the IsdB^{N1-L} + IsdB^{N2} reaction may be that once extraction of heme from metHb has occurred, IsdB^{N1-L} and IsdB^{N2} are bound together in a nonproductive complex. This hypothesis is supported by the observation that IsdB^{N1-L} did not pull down IsdB^{N2} alone but did pull down IsdB^{N2} in the presence of metHb (Figure S3 of the Supporting Information, lanes 11 and 12).

DISCUSSION

Both IsdB NEAT domains and the intervening linker region must be present and contiguous for high-affinity Hb binding (Figures 2 and 3). No single NEAT domain of IsdB tested was competent for Hb binding on its own, yet linked together, they bind Hb with nanomolar affinity. Possibly, the IsdB^{N1} construct produced misfolded protein, explaining the lack of Hb binding activity; however, a recent paper has detailed the NMR structure of IsdB^{N1}, using a recombinant construct that differs from ours by the addition of only three amino acids (we omitted the N-terminal Leu125 and C-terminal Glu271 and Asp272).[30] Given that a nearly identical construct resulted in an NMR structure in which the backbone resonances produced the canonical β-strand rich topography of NEAT domains, our IsdB^{N1} construct is likely properly folded. IsdB^{N1} was also found to be required for binding of Hb to *S. aureus* cells *in vivo*, as cells expressing a version of IsdB lacking the NEAT1 domain could not bind Hb.[31] This is consistent with our data, which conclude that IsdB^{N1} is essential for high-affinity Hb binding because IsdB^{N1}, the linker region, and IsdB^{N2} must all be present and contiguous for Hb binding to occur. Although no interaction between IsdB^{N1} or IsdB^{N1-L} and Hb was demonstrated with Hb immobilized on nickel beads, immobilized His$_6$-tagged IsdB^{N1-L} did pull down significant amounts of Hb (Figure S3 of the Supporting Information) and weak binding of non-His$_6$-tagged IsdB^{N1-L} for HbCO could be shown by ITC (Figure 3B). The preferred interaction interface of Hb with nickel beads may have limited binding of IsdB^{N1-L}.

In contrast, a single NEAT domain of IsdH, the IsdH N1 domain, binds Hb with high affinity.[18] On the basis of a level of amino acid sequence identity between IsdB^{N1} and IsdH^{N1} of 46%, these domains were proposed to share the same function. This difference in binding affinity may be related to the pI for IsdB^{N1N2} (8.5), which is markedly higher than that of the analogous construct in IsdH, IsdH^{N2N3} (5.0), as calculated using the Compute pI/M_w tool on ExPASy.org.[32] Thus, at physiological pH (7.4), IsdB and IsdH would be oppositely charged, which is likely to impact interaction with Hb (pI of 6.9). Despite the obvious disparities in binding between IsdB and IsdH, it is noteworthy that IsdB^{N1}, IsdH^{N1}, and IsdH^{N2} share a conserved five-amino acid aromatic motif (FYHYA in the IsdB N1 and IsdH N2 domains and YYHFF in the IsdH N1 domain) in a sequence alignment.[16,33] This aromatic motif was shown to be important for high-affinity Hb binding by IsdH^{N1} and IsdH^{N2}; mutation of any one of the residues resulted in a 41−153-fold decrease in Hb binding affinity, and complete abrogation of Hp binding.[16] The motif was also found to be intimately associated with the Hb α chain in the crystal structure of an IsdH^{N1}−metHb complex.[18] In the IsdB^{N1} NMR structure, the backbone resonances of only 10 residues could not be assigned; four of these were Phe164−Tyr167, within the aromatic motif.[30] In the determination of the NMR structure of IsdH^{N1}, the equivalent residues of the aromatic motif were also not assigned to resonances.[17,33] Recently, a study demonstrated that mutation of residues in the IsdB aromatic motif abrogated binding of Hb to *S. aureus* cells and slowed the rate of transfer of heme from metHb to IsdB *in vitro*.[31] Also, swapping out the IsdB^{N1} aromatic motif for the IsdH^{N1} aromatic motif resulted in cells that were unable to bind Hb, and IsdB with the chimeric aromatic motif was significantly impaired in removing heme from metHb.[31] The conservation of the aromatic motif suggests that it is an important Hb-binding determinant of IsdB, though not the sole determinant.

Interestingly, a study probing for binding of Hb to the N-terminal (IsdB$_N$) and C-terminal (IsdB$_C$) halves of IsdB demonstrated Hb binding by the N-terminal half, and it was suggested that this was due to the presence of IsdB^{N1} within this construct.[34] Because the IsdB$_N$ construct appears to contain the signal sequence (which would not appear in the mature protein) as well as the ∼80 amino acids N-terminal to our IsdB^{N1} construct, the extra ∼125 N-terminal residues may contribute to Hb binding. Thus, the region N-terminal to the IsdB N1 domain may be involved in binding to Hb but appears to be dispensable for high-affinity Hb binding and heme extraction by IsdB^{N1N2}.

Though ITC was unable to provide an unambiguous stoichiometry of the interaction between IsdB^{N1N2} and Hb, on the basis of the ability to remove all four heme groups from tetrameric Hb, IsdB^{N1N2} could interact with both α and β chains. IsdH^{N1} was shown to bind to only α chains by ITC, and only interaction with the α chain is observed in the crystal structure of the complex with metHb.[18] However, IsdH^{N1} added to tetrameric HbCO results in an observed stoichiometry of 0.7:1, similar to that observed for the interaction with IsdB^{N1N2} (Figure S4C of the Supporting Information). Thus, the interaction with α and β chains decreases the apparent stoichiometry for IsdH, and potentially, the observed stoichiometry of IsdB^{N1N2} may differ when it is bound to isolated Hb monomers. Furthermore, an IsdH construct comprising IsdH^{N2} (Hb binding), the linker region, and IsdH^{N3} (heme binding), called IsdH^{N2N3} (64% identical to IsdB^{N1N2}), was recently shown to perform quantitative removal of heme from metHb, as well.[35] A crystal structure of IsdH^{N2N3} in complex with tetrameric metHb clearly demonstrates that an IsdH^{N2} domain interacts with each of the α and β Hb chains.[35]

Although the entire IsdB^{N1N2} polypeptide was required to be contiguous for high-affinity binding to metHb, rapid extraction of heme from metHb did not display the same requirement. Both IsdB^{N1N2} and the mixture of IsdB^{N1-L} and IsdB^{N2} when present in excess could quantitatively extract heme from metHb within 20 s. This observation corresponds well to data for nearly identical concentrations of metHb and full-length IsdB, in which the reaction was completed in ∼10 s.[15] Rapid heme uptake by the mixture of IsdB^{N1-L} and IsdB^{N2} contrasts with findings for the analogous construct in IsdH. IsdH^{N2N3} could rapidly extract heme from metHb, but IsdH^{N2-L} and IsdH^{N3} were unable to perform rapid heme extraction.[19] The ability of IsdB^{N1-L} and IsdB^{N2} to perform rapid extraction of heme from metHb suggests that formation of a stable, high-affinity complex is unnecessary for heme extraction and that a

"cleaved" IsdB polypeptide is capable of reconstituting the function of the intact polypeptide. However, catalytic amounts of $IsdB^{N1-L}$ and $IsdB^{N2}$ did not perform the relay of heme from metHb to $IsdA^{N1}$. Furthermore, $IsdB^{N1-L}$ did not perform multiple turnovers of transfer of heme from metHb to $IsdB^{N2}$. Therefore, these IsdB fragments clearly do not achieve the full functionality present in $IsdB^{N1N2}$.

Studies with $IsdH^{N2N3}$ have revealed that the linker region between the NEAT domains forms an inflexible three-helix bundle that acts as a rigid spacer holding the NEAT domains ~40 Å apart, resulting in a rigid, elongated dumbbell shape.[19] As the IsdB and IsdH linker regions have 70% identical amino acid sequences and give similar circular dichroism spectra,[19] IsdB likely also shares this overall shape. The crystal structure of the $IsdH^{N2N3}$–metHb complex revealed that $IsdH^{N2}$ interacted closely with the Hb chains as previously shown[18] and that the linker region and $IsdH^{N3}$ did not interact with Hb, although $IsdH^{N3}$ was poised directly over the Hb heme pockets.[35] The domains of IsdH (N2, linker, and N3) fold independently and do not make extensive interdomain interactions.[19,36] Similar NMR data are not available for IsdB, and interdomain interactions may differ, which may explain the requirement for all three IsdB domains (N1, linker, and N2) for hemoglobin binding and uptake of heme from metHb.

Heme was successfully relayed to $IsdA^{N1}$ when $IsdB^{N1N2}$ was present in catalytic amounts, demonstrating that the $IsdB^{N1N2}$ fragment possesses all the features of IsdB required for extraction of heme from metHb and transfer to IsdA. The k_{cat} of 0.22 s^{-1} (at 25 °C) for the overall transfer reaction is similar to an observed rate of transfer of heme from metHb to full-length IsdB (0.3 s^{-1}),[15] and it is conceivable that increasing the temperature of the reaction mixture to the human body temperature (37 °C) would increase the transfer rates further. The rate of the overall transfer reaction of heme from metHb to $IsdA^{N1}$ through $IsdB^{N1N2}$ is >400-fold slower than the rate of transfer of heme from $IsdB^{N2}$ to $IsdA^{N1}$ (82 s^{-1}),[9] indicating that the extraction of heme from metHb is rate-limiting for the overall heme transfer reaction. $IsdB^{N1N2}$ has a specificity constant (k_{cat}/K_m) of 2.93×10^5 M^{-1} s^{-1}, comparable to those of some classical enzymes such as ribonuclease and tRNA synthetase. The overall rate is sufficiently rapid that it is conceivable that a ternary complex is formed among metHb, $IsdB^{N1N2}$, and $IsdA^{N1}$. IsdB and IsdA are colocalized on the cell wall and can be co-immunoprecipitated *in vivo*,[37] but efforts to show an interaction with the recombinant protein *in vitro* have not been successful. Noteworthy is the fact that the normal concentration of plasma Hb due to red blood cell turnover by intravascular hemolysis in healthy adults is approximately 150 mg/dL, or 2.3 μM,[14] 3 times the measured K_m for the metHb in the heme transfer reaction, suggesting that IsdB on the *S. aureus* cell surface could be more than half saturated even without the action of hemolysins. However, free serum Hb is rapidly bound by haptoglobin, which may alter the heme uptake kinetics of IsdB. The involvement of haptoglobin in the heme transfer reaction is a subject for further study.

In summary, we have described the minimal functional unit of IsdB for Hb binding, heme extraction, and transfer to IsdA. None of the domains investigated could bind Hb with high affinity alone; however, when all domains were present in one polypeptide ($IsdB^{N1N2}$), nanomolar-affinity Hb binding was observed. Therefore, in addition to $IsdB^{N1}$, either $IsdB^{N2}$, the linker, or both bind Hb when they are present in the $IsdB^{N1N2}$ polypeptide. The linker region in IsdB proved to be essential for the Hb binding and heme extraction functions of IsdB. Without the linker region present, the NEAT domains of IsdB could neither bind Hb (even in combination) nor remove heme from Hb through activated transfer, as opposed to solution-based transfer. Adding the linker to $IsdB^{N1}$ ($IsdB^{N1-L}$) facilitated the rapid transfer of heme to $IsdB^{N2}$, although with one or few turnovers. Interestingly, addition of the linker region to $IsdB^{N2}$ resulted in a nonfunctional construct. The linker may participate in Hb binding or be required to optimize NEAT domain Hb binding orientation, or its connectivity to $IsdB^{N1}$ may produce steric strain in the Hb molecule promoting the loss of heme to $IsdB^{N2}$. Furthermore, although IsdB can bind oxyHb, metHb, and HbCO, only metHb serves as a substrate for heme extraction. Lastly, $IsdB^{N1N2}$ can serve as a catalyst for a heme transfer reaction that reconstitutes metHb, $IsdB^{N1N2}$, and $IsdA^{N1}$ under physiologically relevant infection conditions. The observed K_m of $IsdB^{N1N2}$ for metHb provides a mechanism for *S. aureus* colonization in an iron-restricted environment such as the human body.

■ ASSOCIATED CONTENT

Supporting Information

Additional characterization data as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by a grant from CIHR (MOP-49597) to M.E.P.M. C.F.M.B. and M.M.V. hold NSERC CGSM and CGSD scholarships, respectively.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the anonymous blood donor and the Centre for Blood Research Phlebotomy Suite at The University of British Columbia. C.F.M.B. thanks Dr. van Petegem for use of the ITC and Kelvin Lau for help with ITC experiments and interpretation.

■ ABBREVIATIONS

NEAT, near iron transporter; Isd, iron-regulated surface determinant; Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; ITC, isothermal titration calorimetry.

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