Abstract

Gram-positive Staphylococcus aureus is a common member of the normal human flora, but can also cause serious infections. Survival and growth of S. aureus is dependent on the acquisition of iron from the host, wherein the majority of iron occurs as part of the heme molecule in the oxygen-carrier protein hemoglobin (Hb). S. aureus possesses a system of proteins designed to use heme and hemoglobin as an iron source: the iron-regulated surface determinant (Isd) system. IsdB is the primary Hb receptor and extracts heme from Hb at the cell surface for transfer to IsdA or IsdC, which then transfer it to the membrane transporter for internalization. IsdB contains two NEAT domains (IsdB-N1 and IsdB-N2) which were hypothesized to carry out the Hb-binding, heme binding and heme transfer functions of the protein.

Heme binding by IsdB-N2 was characterized biochemically and the crystal structure of heme-reconstituted IsdB-N2 was solved. IsdB-N2 bore the canonical eight-stranded β-sandwich NEAT domain fold and used a conserved Tyr residue to coordinate heme-iron, as well as a non-conserved Met residue, resulting in a novel Tyr-Met hexacoordinate heme-iron. Biochemical differences between equivalent mutations produced in IsdB\textsuperscript{N2} and IsdB\textsuperscript{N1N2} introduced the possibility of intraprotein domain interactions.

The molecular mechanism for heme transfer from IsdB-N2 to IsdA-N1 was investigated using stopped-flow spectroscopy and the kinetics of heme transfer from IsdB-N2 to IsdA-N1 were modeled. The rate of heme transfer between the isolated NEAT domains was similar to that measured for the full-length proteins.

Only a recombinant construct with both domains in a contiguous unit (IsdB\textsuperscript{N1N2}) could bind Hb with high affinity. Spectroscopic analysis demonstrated that both domains were also required
to extract heme from Hb. In a reconstituted model of the biological heme relay pathway, IsdB catalyzed heme transfer from Hb to IsdA at a rate 370-fold slower than heme transfer from IsdB$^{N2}$ to IsdA$^{N1}$, revealing that heme transfer from Hb to IsdB is the rate-limiting step in this pathway. Finally, the serum Hb-binding protein haptoglobin blocked heme uptake from Hb by IsdB, revealing new areas for exploration of function. These studies provide insight into mechanisms of host-pathogen interactions during infection.
Preface

Several parts of this work were published in refereed journals. Below is a description of contributions made by fellow scientists.

Chapter 1


Several members of the Murphy lab collaborated to write a structural review of the Isd system. I wrote the sections on related Isd systems and the summary; I partially based the Introduction section on NEAT domains in this thesis on a section written by J. Grigg.

Chapter 3


J. Grigg cloned the IsdA<sup>N1</sup> expression plasmid and identified the initial crystallization conditions. A. Arrieta cloned the IsdB<sup>N2</sup> expression plasmid. I performed crystal optimization and solved the crystal structures, and performed all spectroscopic and kinetic analyses, with the help of J. Grigg.

Chapter 4


M. Verstraete cloned the IsdB<sup>N1N2</sup> and IsdB<sup>N1</sup> expression plasmids. I cloned the IsdB<sup>N1-L</sup> expression plasmid and performed all experiments. L. Eltis provided expertise and advice to guide experimental design and interpretation.
Chapter 5


I performed all experiments described with help from L. Eltis and M. Murphy on experimental design. Portions of this chapter, as well as section 4.2.5, are part of a manuscript in preparation.

Ethics Approval

This project required Ethics Approval for purifying hemoglobin from fresh human blood. Approval was provided by the UBC Clinical Research Ethics Board, Certificate Number H11-03395, under Project Title “Hemoglobin binding by the IsdB receptor of Staphylococcus aureus.”
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List of Abbreviations

apoMb  Apomyoglobin
EDTA  Ethylenediaminetetraacetic acid
Fe$^{2+}$  Ferrous iron
Fe$^{3+}$  Ferric iron
Fur  Ferric uptake regulator transcription factor
Hb  Hemoglobin
HbA  Human hemoglobin A (the major adult form)
HbCO  Carbonmonoxyhemoglobin
His$_6$  Hexahistidine affinity purification tag
Hp  Haptoglobin
Hp-Hb  Haptoglobin-hemoglobin complex
Isd  Iron--regulated surface determinant
IsdB-N2  C-terminal NEAT2 domain of IsdB in *S. aureus*
IsdB$^{N2}$  Recombinant protein construct comprising IsdB-N2
ITC  Isothermal titration calorimetry
$K_{4,2}$  Tetramer-dimer dissociation constant of Hb
$k_{\text{cat}}$  Catalytic constant
$K_D$  Dissociation constant
$K_m$  Michaelis constant
$k_{\text{obs}}$  Observed rate constant
metHb  Methemoglobin
NEAT  Near iron Transporter domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>oxyHb</td>
<td>Oxyhemoglobin</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
</tr>
<tr>
<td>r.m.s.d.</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC-MALS</td>
<td>Size exclusion chromatography coupled with multi-angle light scattering</td>
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Chapter 1: Introduction

1.1 *Staphylococcus aureus*

*S. aureus* is a member of the phylum Firmicutes, Gram positive bacteria characterized by genomes with a low percentage of G+C, and the order Bacillales, which also includes the genera Bacillus and Listeria. The genus *Staphylococcus* comprises approximately 40 known species of catalase positive bacteria which are facultative anaerobes and are also halotolerant; most *S. aureus* strains are also coagulase positive (1). *S. aureus* is most commonly found as a member of the normal human flora, colonizing approximately one-third of the population, primarily on the hands and nostrils (2). Numerous *S. aureus* strains also colonize other mammalian species, in particular cattle, leading to an agriculturally significant condition called bovine mastitis; interestingly, although there is host specificity among animal- or human-associated lineages, the strains themselves are closely related, with the genetic basis for host specificity not entirely clear (3). In humans, *S. aureus* is one of the main agents of nosocomial infections (4) and can cause a range of disease, from mild (skin infections such as boils and folliculitis) to severe, life-threatening bloodstream infections (5). Colonization is an important risk factor for subsequent *S. aureus* infection; in one study, more than 82% of isolates in bacteremia were identical to the strain carried in the patient’s anterior nares (6). *S. aureus* possesses a large number of virulence factors that contribute to its ability to cause infection, ranging from secreted toxins and adhesins to strategies for avoiding or subverting the innate and humoral immune responses (7-9).

The history of antibiotic-resistant strains of *S. aureus* is as long as the history of antibiotic use in the 20th century. Shortly after the introduction of penicillin, a β-lactam antibiotic, resistant strains of *S. aureus* emerged which possessed enzymes to cleave the β-lactam ring (β-lactamases) (10). Methicillin, another β-lactam antibiotic which could not be cleaved by β-
lactamases, was introduced in 1959, with the first methicillin-resistant strains emerging only two years later (10). The gene allowing for methicillin resistance, mecA, encodes an alternate form of the transpeptidase protein which is inhibited by β-lactam antibiotics; thus, methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant not only to methicillin, but to all β-lactam antibiotics, including the penicillins and cephalosporins (10). Until the 1990s MRSA infections were generally healthcare-associated (HA-MRSA), but at that time MRSA infections in patients without previous healthcare exposure began to quickly emerge and spread, and are now known to be part of a separate lineage, community-acquired MRSA (CA-MRSA) (10). An ongoing debate is whether infection with MRSA (as opposed to methicillin-sensitive *S. aureus*, MSSA) causes greater rates of morbidity and mortality, but MRSA infections certainly incur greater costs and involve longer hospital stays (11).

Until recently, MRSA strains have remained susceptible to antibiotics other than the β-lactam class and the glycopeptide antibiotic vancomycin has been the preferred treatment for MRSA infections; unfortunately, resistance to vancomycin is emerging as well (10). Linezolid, which targets the 23S rRNA of the ribosome, was licensed globally in 2000-2001; while widespread resistance to linezolid has not been seen, linezolid-resistant MRSA strains have been found (12). Strict reporting and monitoring of antibiotic resistant strains of *S. aureus*, as well as rigorous infection control procedures in hospitals, may help to bring the antibiotic-resistance crisis under control. In the absence of a vaccine against *S. aureus*, studies targeting its virulence mechanisms and nutritional requirements have shed light on how this pathogen causes such significant disease in humans.
1.2 Iron as a nutritional requirement

Iron is an essential nutrient to every known life-form, with the notable exception of *Borrelia burgdorferi*, the causative agent of Lyme Disease, which can use manganese in place of iron (13). Iron is a transition metal which can occupy numerous oxidation states; under physiological conditions, it exists mainly in ferric (Fe$^{3+}$) or ferrous (Fe$^{2+}$) form (14). Iron is used extensively within biological systems to carry out essential processes which are energetically unfavourable, using only organic molecules, such as oxygen transport (as in hemoglobin and myoglobin) and some oxidation-reduction reactions (such as those involved in electron transport or enzymatic catalysis) (15,16). The Fe$^{2+}$/Fe$^{3+}$ redox couple is particularly useful in biology because the reduction potential can be finely tuned within the range of biologically significant potentials (from -0.5V to 0.6V) by changing iron ligands (15,16). Due to its unoccupied $d$ orbitals, iron can act as a ligand to many biological or inorganic molecules, and can be found within proteins as part of iron-sulfur clusters, in complex with protoporphyrin IX as a heme molecule, or bound directly to protein ligands as mononuclear or dinuclear iron (14).

However, iron is difficult to obtain for most organisms. Iron is the second most abundant metal in the earth’s crust, but under normal physiological conditions (aerobic environment, pH ~7), the soluble ferrous form of iron quickly oxidizes to the insoluble ferric form, which precipitates in ferric hydroxide aggregates, unavailable to many organisms (17). The total soluble ferric iron concentration under physiological conditions is estimated to be $1.4 \times 10^{-9}$ M, far too low to support optimal growth of an organism (18); for instance, the human body requires $\sim 10^{-3}$ M iron (by body volume) and most microbes require $\sim 10^{-6}$ M iron (19), although *S. aureus* in particular seems to have much lower iron requirements and can grow readily in media with iron levels of $5 \times 10^{-8}$ M (20). Moreover, redox cycling of iron under aerobic conditions can produce
hydroxyl radicals, which are highly damaging to biological macromolecules (21). Thus, biological organisms have evolved mechanisms to obtain and store iron which must overcome iron’s intrinsic low solubility and high toxicity.

1.3 Human iron homeostasis

The average human body contains ~ 4 g of iron, the majority of which (> 2 g) exists as heme in hemoglobin (Hb) (Figure 1-1) (21). Approximately 300 mg is also present as heme in myoglobin, with ~ 600 mg found in reticuloendothelial macrophages, which have phagocytosed dying erythrocytes to recycle their iron, ~ 30 mg per day (21). Approximately 1 g of elemental iron is stored in the liver, within ferritin molecules, and only a very small amount (~ 8 mg) is present in other cellular or serum proteins, such as transferrin (Tf), a circulating iron delivery protein (21).

Humans absorb iron through their diet at a rate of 1-2 mg of iron per day, crucial to replace excreted iron and maintain iron homeostasis (21). Interestingly, humans do not have a dedicated excretion system for iron; iron loss occurs through sloughing of mucosa and desquamation of epithelial cells (21).

1.3.1 Biological states of hemoglobin

Hemoglobin (Hb) is a tetrameric molecule composed of 2 α chains and 2 β chains whose major function in the human body is to deliver oxygen to the tissues and remove carbon dioxide. Hb is normally found within highly specialized anuclear erythrocytes that have a limited life span, ~ 120 days in circulation. Hemoglobin makes up ~ 90% of the dry weight of the erythrocyte and is present at concentrations of approximately 150 g/L blood in the average human; with ~ 5 L of blood in the average human, there is ~ 750 g of hemoglobin circulating, and 1% (or ~ 7 g) is turned over every day.
Figure 1-1. Human iron homeostasis. Approximately 70% of the human body’s iron is stored within erythrocytes as part of hemoglobin (Hb). Erythrocytes do not have a nucleus and thus cannot repair themselves; the average life of an erythrocyte is 120 days, after which they are engulfed by macrophages and the iron is recycled. The average human body loses 1-2 mg of iron a day; iron is absorbed from the diet to counter these losses, which account for only ~0.03% of the body’s total iron. 99.97% of the iron in the body remains stored or cycled within body compartments. Adapted from (14) and (21).

Within the confines of the erythrocyte, the Hb-heme molecules remain in the reduced ferrous form, but sometimes spontaneously oxidize to the ferric form known as methemoglobin (metHb). A cytoplasmic system of proteins within the erythrocyte including cytochrome b5 and its reductase reduces ferric Hb back to ferrous Hb and maintains the level of metHb at less than 1% of total hemoglobin content (22). This system is crucial for oxygen transport within the body because metHb is unable to carry oxygen. Heme-iron coordination in Hb is octahedral, with five
or six ligands: four ligands are provided by nitrogen atoms from the porphyrin ring, the fifth (proximal) ligand is a His from the Hb protein and the sixth (distal) ligand varies. Ferrous heme-iron can coordinate gaseous ligands such as oxygen (oxyhemoglobin, oxyHb) or carbon monoxide (carboxyhemoglobin, HbCO) in the sixth position, or have that position open (deoxyhemoglobin, deoxyHb). Oxygen coordination to ferric heme is not favorable under physiological conditions (neutral pH) and water is normally in the sixth position. At alkaline pH, water is ionized to the hydroxyl radical; at acidic pH, a His on the distal side can become a ligand resulting in bis-His, or hemichrome, coordination (23,24).

The vast majority of Hb turnover occurs extravascularly. Macrophages of the reticuloendothelial system recognize signs of erythrocyte aging or damage and phagocytose the senescent erythrocyte. However, ~10-20% of hemolysis occurs intravascularly, releasing free Hb into the bloodstream (25); normal plasma Hb levels are ~6 μM (by heme) (26). Hb oxidation state is highly dependent upon its concentration; dilution from the interior of the cell into the bloodstream increases the rate at which Hb dissociates from tetramers into dimers (the tetramer-dimer dissociation constant, known as $K_{4,2} = 0.7 \mu M$), which in turn increases the rate at which Hb autoxidizes to metHb (27,28). Moreover, reduced Hb scavenges nitric oxide from the blood vessel endothelium which results in metHb production as well (29). Nitric oxide is a regulator of vascular tone and excessive plasma Hb can produce endothelial dysfunction through scavenging of nitric oxide (30). MetHb forms dimers in dilute solution ($K_{4,2} = 30 \mu M$) that lose heme much more readily than oxyHb, which can further damage biological molecules and tissues (31-33). Cell-free Hb is removed from the plasma by haptoglobin (Hp), an abundant serum protein dedicated to Hb binding and present at levels of at least 10 mg/mL in the plasma (34). Hp binds Hb essentially irreversibly, but can only bind dimeric Hb, which is typically in the metHb state.
As part of the Hp-Hb complex, Hb is no longer reactive with tissues and is thus detoxified; Hp-Hb complexes are then recognized by the receptor CD163 on the surface of macrophages and hepatocytes, and removed from circulation. The first structure of the Hp-Hb complex (porcine Hp-Hb; 82% amino acid sequence identity to human Hp-Hb) has only recently been elucidated, revealing that Hp binds precisely at the Hb dimer-tetramer interface and explaining its specificity for Hb dimers. The structure shows that Hp binds close to, but not in contact with, the heme group, possibly stabilizing heme binding by metHb.

1.3.2 Nutritional immunity

Iron trafficking is controlled within the body and little free iron is available. Tf (found in the serum and lymph) and lactoferrin (found in mucosal secretions such as tears) bind iron with high affinity. Excess Tf is present relative to free iron, such that under normal conditions Tf is only one-third saturated. In fact, the estimated concentration of extracellular free iron in human tissues is on the order of $10^{-24}$ M, numerous orders of magnitude lower than the typical bacterial iron requirements for growth, which are on the order of $10^{-6}$ M. The naturally low levels of free iron in the human body, aside from mitigating the toxicity of free iron, also have the beneficial effect of limiting microbial growth within the human host; this has been referred to as a form of innate immunity called “nutritional immunity.”
Figure 1-2. Structure of the porcine Hp-Hb complex. The structure of the porcine Hp-Hb complex (PDB ID: 4F4O) is shown with protein chains in cartoon and heme groups modeled as sticks. (A) The biological unit. Hp is a dimer with an unusual strand-swap in one domain (the \( \alpha \) domain in human Hp), and the other domain (the \( \beta \) domain in human Hp) binds to a Hb dimer. (B) A closer examination of the binding interface between the Hp \( \beta \) domain and the Hb dimer shows Hp loops protruding into the cleft between \( \alpha \) and \( \beta \) Hb. (C) Hp does not interact with the Hb heme moieties or overlap the heme-binding pocket.

1.4 Iron and heme uptake by *Staphylococcus aureus*

Bacteria that grow in humans, either pathogenic or commensal, must contend with the significant challenge of iron acquisition in the face of nutritional immunity. *S. aureus* first recognizes iron restriction through the ferric uptake regulator, Fur, a dimeric protein common to both Gram positive and Gram negative bacteria (40). Fur is a transcriptional regulator that senses low intracellular iron. Under iron-replete conditions, Fur is bound to iron and thus binds to 19 bp
DNA sequences known as “Fur boxes” upstream of target genes and typically represses transcription; under iron-restricted conditions, Fur is not bound to iron and repression of gene expression is lifted (40). In *S. aureus*, genes upregulated when Fur repression is lifted are largely those involved in iron acquisition (e.g., the *sir, sbn, hts* and *isd* operons), as well as virulence factors (e.g., the cytotoxins *hla, hlg*, and *lukED*) and adhesins (e.g., the fibrinogen adhesin *clfB*) (41-43). As noted above, in the human body, iron stores are found in two main forms: elemental iron, and heme-iron. *S. aureus* encodes multiple mechanisms to take advantage of both iron pools.

**1.4.1 *S. aureus* iron acquisition systems**

*S. aureus* acquires elemental iron from its environment through the actions of the Feo (ferrous iron transport) system, a ubiquitous Fe$^{2+}$-iron transporter that scavenges free ferrous iron (44). However, as the amount of bioavailable ferrous iron in the human body is small, *S. aureus* also employs other methods of iron acquisition through the use of siderophores. Siderophores are low-molecular weight molecules which are synthesized within a cell and exported, where they chelate ferric iron owing to their extremely high iron affinity; iron-filled siderophores are then taken back up by the cell through specific transporters. *S. aureus* produces two siderophores, staphyloferrin A and staphyloferrin B, as well as being able to take up exogenous hydroxamate siderophores through expression of the Fhu (ferric hydroxamate uptake) transporter (45). Both staphyloferrin A and staphyloferrin B are able to scavenge iron from Tf and thus permit *S. aureus* to grow with Tf as the sole iron source (46).
1.4.2  *S. aureus* heme acquisition

When presented with both heme and iron-bound Tf as the only potential iron sources, *S.
aureus* preferentially takes up heme-iron (47). *S. aureus* is only known to encode a single heme
acquisition system, termed the **Iron-regulated surface determinant (Isd)** system (Figure 1-3).

![Diagram of S. aureus Isd heme uptake system and genetic organization.](image)

**Figure 1-3.** Schematic of the *S. aureus* Isd heme uptake system and genetic organization.
(A) The peptidoglycan-anchored components are anchored at different depths. All characterized
components of the Isd system bind heme; of the four surface proteins, only IsdB and IsdH can also bind
hemoglobin and remove the heme moiety. The predominant heme transfer pathway is indicated with
arrows. (B) Genetic organization of Isd system components. *isdA-G* are clustered together in one region,
with most genes encoded in a single operon; *isdA* and *isdB* are transcribed individually and divergently
from the Isd operon. *isdH* and *isdI* are found elsewhere on the chromosome. Arrows indicate the position
of promoters and direction of transcription; Fur boxes are found upstream of each promoter.
Identified in 2002 and 2003, the system consists of four surface proteins covalently anchored to the peptidoglycan (IsdA, IsdB, IsdC and IsdH) that reversibly bind heme, an ABC transporter (IsdF) with associated lipoprotein (IsdE), and two intracellular heme-degrading enzymes (IsdG and IsdI) (48-50). The function of a ninth component, IsdD (a predicted membrane protein), remains unknown. Lastly, a sortase (SrtB) is encoded in a gene cluster with IsdCDEFG; its function is to anchor IsdC to the peptidoglycan, whereas the remaining Isd surface proteins (IsdABH) are anchored by sortase A, the housekeeping sortase of the cell (49). Experimental and genetic evidence points to all genes/operons of this system possessing a Fur box upstream of the promoter and thus being classically Fur-regulated (expression repressed in the presence of iron) (50). The Isd system is not essential for the growth of S. aureus. The gene encoding IsdF may be deleted without affecting growth of S. aureus on heme as a sole iron source, suggesting possible alternate heme transporters that the Isd surface proteins may pass heme to (50).

Early protease-protection experiments showed that the surface components of the system are arranged at different depths within the thick Gram-positive cell wall, leading to the hypothesis that this arrangement would enhance heme transfer from the extracellular milieu to the cellular membrane (50). The surface components of the Isd system indeed transfer heme unidirectionally, funneling heme to IsdC which is the only cell-wall anchored protein capable of transferring heme efficiently to IsdE (51,52). IsdB and IsdH stand apart in the Isd system in that they are the only components also able to bind hemoproteins; IsdH can bind Hp, Hb and the Hp-Hb complex, whereas IsdB can bind Hb and the Hp-Hb complex but not Hp alone (53,54). Thus, heme is stripped from Hb at the surface by IsdB and IsdH, transferred in a relay fashion to IsdA then IsdC, and then to the lipoprotein IsdE (55) where it is transferred to the permease IsdF and
transported across the membrane to the cytoplasm and the porphyrin ring is degraded by the homologous enzymes IsdG and IsdI to liberate the central iron ion for use by the cell (56,57).

1.5 NEAT domains of the Isd system

The four surface proteins of the Isd system (IsdABCH) share a common feature: they carry from 1 to 3 copies of a conserved protein fold known as a NEAT (for NEAr Transporter) domain (Figure 1-4). This domain of ~125 residues was identified bioinformatically as being in the genomic neighbourhood of putative iron uptake systems (58). Each cell-wall anchored Isd protein encodes an N-terminal secretion signal, a C-terminal sortase signal (for cell wall anchoring) and at least one NEAT domain. Amino acid sequence identity between NEAT domains varies widely with a range from 11-65% (59). NEAT domains are numbered from the N-terminus to the C-terminus; I will be using a dash to indicate the domain within the protein (as in “IsdB-N2”) and superscript to indicate a recombinant protein construct containing that domain (as in “IsdB$^{N2}$”).

Figure 1-4. NEAT domains of the *S. aureus* Isd system. Secretion signals are represented by a blue box at the N-terminus of each protein, and sortase (cell-wall anchoring) signals are represented by a yellow box at the C-terminus. Sortase signals are the sequences LPXTG for IsdABH, and NPQTN for IsdC. Heme binding NEAT domains are indicated with a red hexagon. Amino acid sequence identity > 45% is indicated by similar NEAT domain colouring.
The first crystal structure of a NEAT domain to be reported was for IsdA-N1, and revealed an eight-stranded immunoglobulin-like β-sandwich fold (60) (Figure 1-5A). Heme-iron is pentacoordinate with Tyr166 acting as the sole protein ligand; Tyr170 forms a hydrogen bond with the coordinating Tyr, and Ser82 forms a hydrogen bond with a heme propionate group (Figure 1-5B). A His residue is located on the distal side of the heme-iron; although His can coordinate heme-iron, it does not do so in this structure. Sequence alignments of the S. aureus Isd NEAT domains revealed that a Tyr in the same position as Tyr166 was conserved in the IsdC NEAT domain and the C-terminal NEAT domains of IsdB and IsdH; therefore, it was hypothesized that these NEAT domains would also be heme binding (60). Studies using isolated NEAT domains showed that indeed, only C-terminal NEAT domains bound heme, by spectroscopic and magnetic circular dichroism techniques (61). Most individual NEAT domains of the surface Isd proteins have now been characterized for heme and hemoprotein binding and demonstrated to mediate either heme binding (52,59-64) or hemoprotein binding (53,59,65); no domain has been shown to perform both functions. Interestingly, the Tyr (e.g. Tyr170 in the case of IsdA) four residues downstream of the coordinating Tyr (IsdA: Tyr166) is invariant in all S. aureus NEAT domains, regardless of heme binding ability. Crystal structures of holo-IsdC\textsuperscript{N1} (62) and holo-IsdH\textsuperscript{N3} (64) revealed that the overall NEAT domain fold is highly conserved despite low (15%) sequence identity. The crystal structure of holo-IsdA\textsuperscript{N1} overlays with the holo crystal structures of IsdC\textsuperscript{N1} and IsdH\textsuperscript{N3} with r.m.s.d. values of 1.76 and 1.47 Å over all Cα atoms, respectively (66). The heme-iron ions of IsdH\textsuperscript{N3} and IsdC\textsuperscript{N1} are also pentacoordinate, with the protein ligand provided by the predicted conserved Tyr residue. However, neither IsdH\textsuperscript{N3} nor IsdC\textsuperscript{N1} have a potential heme-iron ligand on the distal side of the heme (60,62,64).
1.5.1 Hemoprotein binding by IsdH NEAT domains

IsdH was first identified independently from the Isd system, but at approximately the same time (65); its Hp-binding activity was discovered at that time and led to its designation as HarA (for haptoglobin receptor A). The authors noted two highly homologous domains (which they termed HarA-D1 and HarA-D2) and found that HarA-D1 and HarA-D2 bound Hp, Hb and Hp-Hb complexes (65). These domains are now called IsdH-N1 and IsdH-N2, and are homologous to IsdB-N1. In fact, IsdH and IsdB are the most homologous proteins of the Isd surface proteins. IsdB-N1 shares 46% and 65% amino acid sequence identity with IsdH-N1 and IsdH-N2, respectively; the C-terminal (heme binding) NEAT domains IsdB-N2 and IsdH-N3 share 56% identity but are very distantly related to the N-terminal NEAT domains of IsdB and IsdH (< 20% sequence identity). Moreover, a large portion of IsdB, consisting of IsdB-N1, IsdB-N2, and the intervening linker region (IsdB-N1N2) shares 64% amino acid sequence identity with the homologous region in IsdH, IsdH-N2N3. These data indicate that IsdB and IsdH likely arose
through gene duplication, with IsdH-N1 further arising from a subsequent duplication of IsdH-N2.

Consistent with lacking the conserved Tyr noted in C-terminal (heme-binding) NEAT domains (60), IsdH\textsuperscript{N1} does not bind heme (59). Two solution structures of IsdH\textsuperscript{N1} were reported almost simultaneously (53,59) and the topologies were nearly identical to that of IsdA\textsuperscript{N1}. This demonstrated that differences in ligand specificity between heme-binding NEAT domains such as IsdA-N1 and hemoprotein-binding NEAT domains were not due to large differences in the secondary or tertiary structure of the folded NEAT domains. However, it was noted that the Hb-binding Isd proteins (IsdB and IsdH) shared a conserved feature not found in the heme-binding domains: a motif of four to five consecutive aromatic residues (FYHYA in IsdB-N1 and IsdH-N2; YYHFF in IsdH-N1) (53,59). Later, this motif was shown to be important for high-affinity Hb binding by IsdH\textsuperscript{N1} and IsdH\textsuperscript{N2}; mutation of any one of the residues resulted in a 41 – 153-fold decrease in Hb binding affinity as determined by surface plasmon resonance, and complete abrogation of Hp binding, indicating that these aromatic residues were a crucial component of the interaction surface between IsdH, Hb and especially Hp (67). Gel filtration studies initially suggested that two IsdH\textsuperscript{N1} domains interacted with each Hb $\alpha/\beta$ dimer (59). This stoichiometry was recently confirmed for IsdH\textsuperscript{N2} by gel filtration coupled with multi-angle light scattering using chemically separated Hb chains; however, IsdH\textsuperscript{N1} interacted only with $\alpha$Hb (68). The same report confirmed the specificity of the interaction between IsdH\textsuperscript{N1} and $\alpha$Hb by producing a crystal structure of the IsdH\textsuperscript{N1}-metHb complex, in which IsdH\textsuperscript{N1} was found bound exclusively to $\alpha$Hb (Figure 1-6A); the same structure also revealed an intimate association between the aromatic motif of IsdH\textsuperscript{N1} and $\alpha$Hb (Figure 1-6B) (68).
Figure 1-6. Structure of the IsdH\textsuperscript{N1}-metHb complex. (A) IsdH\textsuperscript{N1} interacts exclusively with the \(\alpha\) chain of metHb. IsdH\textsuperscript{N1} is coloured in magenta, with \(\alpha\)Hb in green and \(\beta\)Hb in cyan. The heme moieties of each Hb chain are also shown and are distant from the IsdH\textsuperscript{N1} binding site. (B) A closer view of the binding interface with the IsdH\textsuperscript{N1} aromatic motif residues shown as sticks. Four of the five residues in this motif closely abut an \(\alpha\)Hb helix.

Recently, it was shown that a construct of IsdH comprising IsdH\textsuperscript{N2} (Hb-binding), the linker region, and IsdH\textsuperscript{N3} (heme binding), called IsdH\textsuperscript{N2N3}, could remove heme from metHb (69). Electrospray ionization mass spectrometry and heme transfer data showed that IsdH\textsuperscript{N2N3} accomplished this in part by inducing steric strain on the Hb tetramer, resulting in dissociation into dimers and monomers and promoting heme release (69). It was also shown by NMR that the linker region was a highly structured three-helix bundle, and the overall structure of IsdH\textsuperscript{N2N3} was predicted to be that of an elongated dumbbell (69). Replacement of the linker region with a flexible Gly-Ser linker dramatically reduced the heme uptake activity of the IsdH\textsuperscript{N2N3} construct, revealing for the first time that the linker region was functionally important to the heme uptake process (69).

Even more recently, a crystal structure of IsdH\textsuperscript{N2N3} in complex with tetrameric metHb was reported and confirms the predicted elongated dumbbell shape; an IsdH\textsuperscript{N2} domain interacts with each of the \(\alpha\) and \(\beta\) Hb chains, with the heme binding domain IsdH\textsuperscript{N3} not interacting with Hb but being positioned directly over the heme pockets (Figure 1-7A) (70). In this structure, the heme-iron coordinating Tyr, Tyr642, has been mutated to an Ala residue; interestingly, the loop
containing that residue was not modeled in 3 out of the 4 chains in the asymmetric unit. The hydroxyl group of Tyr646 is 12.6 Å from the heme-iron in the fourth chain (Figure 1-7B).

![Figure 1-7](image)

**Figure 1-7. Structure of IsdH^{N2N3 Y642A} in complex with metHb.**
(A) One IsdH^{N2N3 Y642A} molecule (yellow and magenta) surrounds each Hb chain (green and cyan). The IsdH^{N2} domains directly interact with the Hb molecule; the linker region and IsdH^{N3} domains do not interact with Hb. The Hb heme molecules are depicted in orange. (B) The sole chain of IsdH^{N2N3 Y642A} in which the heme-binding loop was modeled is shown in grey; Tyr646 and Y642A are depicted as sticks. The heme pocket of an αHb molecule (in salmon) is close by, with the heme again depicted in orange as sticks.

### 1.6 IsdB, the hemoglobin receptor

IsdB contains two NEAT domains (IsdB-N1 and IsdB-N2) and acts as the primary Hb receptor for the cell (54). IsdB is critical for the use of Hb as an iron source by *S. aureus*, both in vitro and during infection (54,71). In fact, *isdB* is the most highly upregulated member of the *isd* gene cluster under all tested iron restriction conditions (41,72) and is the second most highly upregulated transcript in a comprehensive microarray study comparing *S. aureus* cultured in purified human blood (100-fold upregulated) or serum (200-fold upregulated) to iron replete media (72). In a mouse tissue cage model of *S. aureus* infection, *isdB* transcription is upregulated over 1000-fold (compared to culturing in iron-rich conditions) (41). A low concentration of Hb (approximately 6 µM) is found in healthy human plasma due to normal intravascular hemolysis of aging red blood cells (73); furthermore, *S. aureus* encodes a number of hemolysins, one of
which (γ-hemolysin) is significantly upregulated during growth in human blood (72). IsdH is also able to bind Hb, but does not appear to be dominant for the use of Hb as an iron source during infection (54). In fact, IsdH expression has not been demonstrated to be upregulated under conditions of iron restriction, infection or in blood or serum (41,43,72); although all isd genes and operons are under the control of the Fur repressor, it is clear that further levels of control over gene expression exist as well.

1.6.1 IsdB as a vaccine component

IsdB is highly expressed on the surface of cells during colonization or infection, bears no homology to any human protein, and is highly conserved across all S. aureus strains (74), making it an excellent target for antimicrobial intervention. Naturally occurring antibodies against IsdB were first described in 2002 in a screen of patient serum against a staphylococcal protein library (75). While the most potent S. aureus antigens are peptidoglycan and lipoteichoic acid, comprising most of the naturally-occurring anti-staphylococcal antibodies, high levels of IgG antibodies against IsdB were also found in both healthy (carriers and non-carriers) and infected subjects (76). Subsequently, several groups attempted to make subunit vaccines using IsdB due to its potent antigenicity. Merck Ltd based a vaccine solely on recombinant IsdB (V710); frustratingly, while the vaccine induced a strong antibody response in mice and afforded some protection from lethal challenge with S. aureus, the antibody response in rhesus macaques was much lower (77) and the project was halted in Phase II/III clinical trials as immunization with V710 failed to prevent bacteremia or wound infection in post-surgical patients (78). In numerous attempts to produce a vaccine against S. aureus, vaccine efficacy in mouse infection models and high antibody titers in humans do not correlate well with human protective immunity (79). However, an S. aureus vaccine including multiple bacterial components did elicit a stronger
immune response: a 2006 paper outlining a multivalent vaccine comprised of IsdA, IsdB, SdrD and SdrE showed greater protection in a mouse model than any of the components alone (80). It is unclear whether this vaccine is still in development. Moreover, another group has created a subunit fusion vaccine using a chimera of IsdB (the region around IsdB-N2) and the alpha-toxin protein Hla, which has also demonstrated greater protection in a mouse model than either component alone (81). IsdB is still an active target for vaccine research and a vaccine against S. aureus may yet be found to include IsdB.

1.7 Hypothesis and aims

In spite of the clear importance of IsdB as a Hb receptor for heme uptake by the S. aureus cell, little is understood about IsdB-Hb interactions beyond the observation that full-length IsdB does bind Hb (50,51,53) and remove heme from metHb (51). Owing to the high sequence identity between IsdB and IsdH domains, the presence of the conserved aromatic motif and their apparent similarity in overall function, the function of IsdB-N1 has been speculated (59,67) or stated (52,61,68-71,82) to bind Hb in a fashion analogous to IsdH-N1; however, this has never been shown experimentally. IsdB-N2 had been shown to bind heme (61), but the crystal structure had not been reported. The overall goal of this project has been to understand the basis for Hb recognition and binding by IsdB as well as heme extraction from Hb and subsequent transfer to IsdA, hypothesizing that the IsdB NEAT domains would have specific roles in these processes.

Using a combination of x-ray crystallography and biochemical techniques, I studied the structure and function of the heme binding NEAT domain of IsdB, IsdB-N2. I found that IsdB$^{N2}$ was structurally similar to other heme binding NEAT domains but employed unprecedented Tyr-Met heme-iron coordination. I characterized IsdB$^{N2}$ heme binding parameters and heme transfer
to IsdA$^{N1}$, and examined the effects of point mutations of heme-interacting residues in the heme pocket on these parameters as well.

In order to determine which regions of IsdB were necessary and sufficient for Hb binding and heme extraction, I used biochemical techniques including isothermal titration calorimetry (ITC) and stopped-flow spectroscopy to examine numerous constructs comprising different regions of IsdB. I found that only with both NEAT domains and the linker region present in a single contiguous polypeptide were all known Hb-heme uptake functions of the full-length IsdB protein performed: high affinity Hb binding was achieved, rapid heme uptake was observed, and multiple turnovers of heme extraction from metHb and transfer to IsdA were carried out.

In order to more closely replicate biologically relevant conditions of infection, I also investigated the effects of the Hb-binding serum protein haptoglobin (Hp) and another Hb-binding S. aureus surface protein (IsdH$^{N1}$) on the heme extraction process. I found that while IsdB$^{N1N2}$ could bind the Hp-Hb complex, Hp blocked heme uptake from metHb by IsdB$^{N1N2}$. IsdB$^{N1N2}$ and IsdH$^{N1}$ did not interact in the absence of Hb and IsdH$^{N1}$ did not appear to have an effect on heme uptake from metHb by IsdB$^{N1N2}$.

Lastly, I produced point mutations of heme-interacting residues in the heme pocket in IsdB$^{N1N2}$ with the hypothesis that they could trap a Hb-IsdB$^{N1N2}$ complex for crystallization. I found notable differences between the same mutations in IsdB$^{N1N2}$ and IsdB$^{N2}$, raising the new hypothesis that there are interdomain interactions that play a role in how heme is bound by IsdB.
Chapter 2: Methods

2.1 Cloning of recombinant protein expression systems

Plasmids were generated encoding recombinant constructs with an N-terminal His\textsubscript{6} tag and thrombin cleavage site in pET28a(+) vectors containing various portions of the IsdB coding region: IsdB\textsuperscript{N1N2} (residues 126-459), IsdB\textsuperscript{N1} (residues 126-270), IsdB\textsuperscript{N1-L} (residues 126-336), IsdB\textsuperscript{L-N2} (residues 271-459) and IsdB\textsuperscript{N2} (residues 341-459), as well as one construct of an IsdH NEAT domain, IsdH\textsuperscript{N1} (residues 86-229) and the IsdA NEAT domain, IsdA\textsuperscript{N1} (residues 62-184). Constructs described in this study are listed in Table 2-1.

Briefly, IsdB constructs were subcloned from a GST-tagged construct cloned from \textit{S. aureus} N315 chromosomal DNA (83) using a modified whole plasmid polymerase chain reaction method (84). Site-directed variants of IsdB\textsuperscript{N2} were created by subcloning from the IsdB\textsuperscript{N2} clone, using a modified whole plasmid polymerase chain reaction method (84). Site-directed variants of IsdB\textsuperscript{N1N2} were created by subcloning from IsdB\textsuperscript{N1N2} using the Quikchange site-directed mutagenesis kit (Agilent Technologies). All clones were verified by sequencing (Agencourt, Beverly, USA).

The NEAT-domain coding region of IsdA (IsdA\textsuperscript{N1}; Ser62-Ala184) was subcloned from the previous construct in pET28a, complete with the N-terminal His\textsubscript{6} tag and ribosome binding site (60), into pBAD-18 (85) using the restriction digestion enzymes XbaI and HindIII as previously described (63). The IsdH construct was generated by total gene synthesis (GenScript).
Table 2-1. Plasmids for recombinant protein expression systems.

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<th>Product (S. aureus Newman residue #)</th>
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2.2 Protein expression and purification

The following general procedure was used; differences are described below.

Recombinant protein was 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<sub>600 nm</sub> of 0.7 - 0.9, then induced with 0.5 mM of isopropyl β-D-thiogalactopyranoside and grown for another 18 hours at 25°C. Cells were pelleted by centrifugation and resuspended in 20 mL of 20 mM sodium phosphate (pH 7.4), 500 mM NaCl then lysed at 4°C using an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada). Insoluble material was removed by centrifugation; the soluble lysate contained a mixture of apo and holo His<sub>6</sub>-protein, and apo protein could be separated at 4°C using a HisTrap nickel affinity column (GE Healthcare, New Jersey, USA) by elution with an imidazole gradient. The apo protein was dialyzed against 50 mM Tris (pH 8.0), 100 mM NaCl then cleaved with thrombin at a 1:500 ratio by weight of His<sub>6</sub>-protein to remove the His<sub>6</sub> tag leaving behind a two
amino acid (Gly-Ser) N-terminal artifact. Recombinant protein was then dialyzed against 50 mM MOPS (pH 7.0) for cation exchange chromatography using a Source 15S column (GE Healthcare, New Jersey, USA) and purified protein was obtained by elution with a NaCl gradient. The resulting pure (>95% by SDS-PAGE) apo protein was dialyzed against either 20 mM Tris (pH 8.0) for crystallization, or 50 mM Tris (pH 8.0) and 100 mM NaCl for spectroscopic and kinetic studies.

IsdB<sup>N2</sup> and IsdB<sup>N2</sup> variants for studies of heme binding and transfer to IsdA<sup>N1</sup>: prepared using the above method.

IsdB<sup>N1</sup>, IsdB<sup>N1-L</sup>, IsdB<sup>N1N2</sup>, IsdB<sup>L-N2</sup>, IsdB<sup>N2</sup> and IsdH<sup>N1</sup> for studies of Hb binding and heme extraction from Hb: induced cells were resuspended in 20 mL of 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM imidazole instead. Purified apoprotein was dialyzed against 50 mM HEPES (pH 7.4) and 50 mM NaCl for thrombin cleavage and 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<sup>N1</sup>. Apoprotein was dialyzed against either 20 mM HEPES (pH 7.4), 80 mM NaCl (μ = 0.1) for pulldowns or steady-state kinetics, or 20 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 50 mM NaCl (μ = 0.1) for isothermal titration calorimetry studies.

IsdB<sup>N1N2</sup> variants for Hb binding and heme extraction studies: prepared as IsdB<sup>N1N2</sup>, except the nickel column binding buffer was 50 mM Tris (pH 8.0), 100 mM NaCl, 10 mM imidazole. Protein was pure enough after the nickel column to forego a second column, and dialyzed into 20 mM HEPES (pH 7.4), 80 mM NaCl without cleaving His<sub>6</sub> tag.

IsdA<sup>N1</sup>: The NEAT domain of IsdA (IsdA<sup>N1</sup>) was produced as described elsewhere (52).
Selenomethionine-labeled (Se-Met) IsdB\textsuperscript{N2}, prepared by the method previously described by Van Duyne \textit{et al.} (86) and purified as described for native protein by the general method.

2.3 Heme reconstitution

Purified apo-protein was incubated for 1 h at 4°C with 1.5 molar equivalents of hemin dissolved in 0.1 M NaOH and diluted in 0.1 M phosphate buffer (pH 7.4). Excess hemin was removed by centrifugation and non-specifically bound hemin was removed by gel filtration chromatography on a Sephadex G-50 column (1 x 6 cm). Concentration of the holo protein was then determined by the pyridine hemochrome assay using the extinction coefficient $\varepsilon_{418} = 191.5$ mM\textsuperscript{-1} cm\textsuperscript{-1} as previously described (87).

2.4 Holo-IsdB\textsuperscript{N2} crystal structure determination

Holo native and Se-Met IsdB\textsuperscript{N2} crystals were grown by hanging drop vapour diffusion at room temperature at a 1:1 ratio of protein:well solution composed of 0.1 M Tris (pH 8.0), 0.1 M MgCl\textsubscript{2} and 25% polyethylene glycol 3350. Crystals were briefly washed in mother liquor and flash frozen in liquid nitrogen. Data were collected at the Stanford Synchrotron Radiation Lightsource on beamline 9-2. Data were processed and scaled using HKL2000 (88). Crystals grew in the space group $P2_12_12_1$ with four molecules in the asymmetric unit.

Se-Met crystals were generated initially for anomalous phasing, but in the interim a suitable model was deposited in the PDB (IsdH\textsuperscript{N3}, 60% identity; PDB ID 2E7D) and molecular replacement with a single chain (with no heme) from that structure using the program MolRep (89) from the CCP4 program suite (90) yielded interpretable phases. Se-Met IsdB\textsuperscript{N2} was built using ARPWarp (91) and manual building was completed using Coot (92). A Ramachandran plot revealed that 92.5% of residues were in the most favoured regions with the remaining 7.5% in additional allowed regions. Subsequently native holo-IsdB\textsuperscript{N2} crystals were generated and the
program MolRep was again used for phasing by molecular replacement with the Se-Met structure as the search model. The structure was modified using Coot (92) and refined with Refmac5 (93). Multiple conformations of side chains were modelled by visual examination of $F_o - F_c$ maps. Waters were added using the ARP/Waters (91) function in Refmac5 and model quality parameters were assessed using Procheck (94). A Ramachandran plot revealed that 94.3% of residues were in the most favoured regions with the remaining 5.7% in additional allowed regions. Figures are of the native structure and were generated using PyMOL (95). Data collection and refinement statistics for both the native and Se-Met IsdB$_{N2}$ structures are shown in Table 3-1.

2.5 Determination of IsdB$_{N2}$ heme binding stoichiometry

Heme binding was tracked by difference absorption spectroscopy in the Soret region (around 400 nm) at room temperature. Aliquots of hemin (1.3 μL) solubilized in 0.1 M NaOH and diluted in 50 mM Tris pH 8.0, 100 mM NaCl were added to 1 mL of a 5 μM solution of apo-IsdB$_{N2}$ or a reference cuvette containing buffer alone. Spectra were recorded 10 minutes after addition of heme and titrations covered a concentration range from 0.5 to 12 μM heme. Saturation was defined as a plateau in the absorption difference between the reference cuvette and the experimental cuvette. For reference, absorption spectra (250-650 nm) of purified reconstituted proteins were measured. All spectra were recorded using a Cary 50 Bio UV-visible spectrophotometer (Agilent Technologies, Mississauga, Canada) with an optical path length of 1 cm in a quartz cell at room temperature.
2.6  Trp fluorescence quenching by heme

Heme binding was monitored by fluorescence quenching of the tryptophan residue (Trp392) at the base of the heme pocket, based on the method described by Eakanunkul et al. (96). Fluorescence emission spectra from 300-450 nm were recorded at 20°C with excitation at 295 nm in 50 mM Tris (pH 8), 100 mM NaCl with apo-IsdB\(^{N2}\) concentrations of 1 μM in 1 mL volume using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Mississauga, Canada). Heme (prepared as described above, in various dilutions) was added to the buffered protein solution in 0.5-1.5 μL aliquots and allowed to reach equilibration (which ranged from 6-12 minutes depending on the variant) before readings were taken. The titrations covered a heme concentration of 0.1-15 μM. The dissociation constant (\(K_D\)) was calculated from the decrease in the fluorescence intensity between 300-450 nm as a function of increasing heme concentration, using an equation for equilibrium binding which accounts for ligand depletion as described by Stein et al. (97). All reactions were done in triplicate.

2.7 Determination of rates of heme transfer to apomyoglobin

The rates of heme dissociation from IsdB\(^{N2}\) were measured by single-wavelength stopped-flow spectroscopy with apomyoglobin (apoMb) as a heme scavenger (98). ApoMb was prepared from myoglobin (Sigma-Aldrich, St. Louis, USA) as previously described (99). Heme dissociation reactions were carried out with 10 μM holo-IsdB\(^{N2}\) (reconstituted as described above) in one syringe (final concentration 5 μM) and 100 μM apomyoglobin (final concentration 50 μM) in the second syringe, both in 50 mM Tris (pH 8), 100 mM NaCl at room temperature in a SX.18MV stopped-flow reaction analyzer (Applied Photophysics, Leatherhead, UK). Reactions were monitored by recording the absorbance at 408 nm for 1000 seconds (wild-type; ~7 half-lives) or 600 seconds (M362L variant; ~11 half-lives); 1000 readings at logarithmic intervals.
were acquired using Pro-Data SX software, regardless of time frame. The change in absorbance at 408 nm (maximal absorbance for holomyoglobin) was plotted against time and fit by a double exponential equation to obtain the first-order rate constants for heme dissociation using GraphPad Prism version 5.02 for Windows (GraphPad Software, La Jolla, USA). All reactions were done in triplicate.

2.8 Determination of rates of IsdB$^{N2}$ heme transfer to IsdA$^{N1}$

Differences in spectral characteristics between holo-IsdB$^{N2}$ and holo-IsdA$^{N1}$ were exploited to monitor the transfer of heme from holo-IsdB$^{N2}$ to apo-IsdA$^{N1}$ by stopped-flow spectroscopy, conducted using an SX.18MV stopped-flow reaction analyzer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a photodiode array detector. The reactions were carried out with 4 μM holo-IsdB$^{N2}$ in one syringe (final concentration 2 μM) and concentrations of apo-IsdA$^{N1}$ ranging from 20-100 μM (final concentrations 10-50 μM) in the second syringe; a minimum five-fold excess of the IsdA acceptor was used to attain pseudo-first order conditions. One hundred spectra were recorded at logarithmic intervals from 0-10 seconds from approximately 300-650 nm, acquired using the Xscan software (Applied Photophysics, Leatherhead, UK). The drive syringe chamber and optical cell were maintained at 25 °C by a circulating water bath. The change in absorbance over time at the wavelength of maximal change (418.9 nm for wild-type and 425.3 nm for M362L variant) for a given concentration was fit by a single exponential equation to determine the observed transfer rate ($k_{obs}$). All reactions were done in quadruplicate.
2.9 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 1500g, 4 °C for 10 min., and the supernatant (plasma) was removed. The red cell pellet was washed three times with a three-fold excess of ice-cold 0.9% NaCl and collected by centrifugation at 2000g, 4 °C 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), 2 mM EDTA (4 °C), followed by incubation on ice for 30 minutes. The resulting solution was centrifuged at 11,000g, 4 °C for 30 min. and the supernatant was removed. In order to precipitate out the stroma, solid NaCl was added to a final concentration of 50 mg per mL of Hb solution, with stirring. Stroma were removed by centrifugation at 11,000 g, 4 °C for 30 min. The supernatant containing Hb was dialyzed against 50 mM Tris (pH 8.6), 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; this constituted the oxyHb sample mentioned in the study. Remaining dialyzed red cell lysate was flash frozen in liquid nitrogen in small aliquots for use as needed.

2.9.1 Carbonmonoxyhemoglobin preparation

Carbonmonoxyhemoglobin (HbCO) was produced as described by Safo and Abraham (100). 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, then 5 min. with carbon monoxide.
2.9.2 Methemoglobin preparation

Methemoglobin (metHb) was produced by oxidizing oxyHb with a 1.2-fold molar excess of potassium ferricyanide, followed by incubation for 10 minutes at room temperature and desalting on a Sephadex G-50 column. Hemoglobin concentrations were determined by the pyridine hemochrome method as previously described (87).

2.9.3 Determination of Hb oligomerization states

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

2.10 IsdB pulldown assay

16 x 25 μL aliquots of nickel bead slurry (Chelating Sepharose Fast Flow, GE Healthcare; stored in 20% ethanol) were washed twice with 1 mL of dH₂O, followed by a 1 mL wash of binding buffer, consisting of 20 mM HEPES (pH 7.4), 80 mM NaCl, 10 mM imidazole. The small amount of imidazole was included to abrogate non-specific binding by IsdB constructs to the beads, but did not affect binding by Hb to the nickel beads. For controls (Hb or IsdB constructs alone), 50 μL of 20 μM IsdB or 20 μN (in heme) Hb were added to the beads and kept on ice for the duration of the pulldown experiment. For pulldowns, Hb was added to 20 μN to the beads and kept on ice for 15 minutes, following which an equimolar amount of IsdB was added for a total final volume of 50 μL and incubated on ice for a further 30 minutes. 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, then eluted with 25 µL of 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 mM imidazole. 12 µL of each eluate was run on a 15% SDS-PAGE gel at 150 V for 1 hour, 15 minutes and stained using Coomassie Blue.

Alternatively, the reverse pulldown was carried out, with His6-tagged IsdB constructs bound to beads pulling down Hb. The samples were processed in an identical manner to the above, except that the binding buffer contained 75 mM imidazole, a concentration at which His6-IsdB could bind the nickel beads but Hb could not.

2.11 Electronic spectra of heme transfer endpoint from hemoglobins

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

2.12 Isothermal titration calorimetry (ITC)

ITC was carried out on a MicroCal ITC-200 instrument. All samples were dialyzed overnight against 20 mM NaH₂PO₄ (pH 7.4), 50 mM NaCl; dialysis buffer was sterile-filtered and then used for sample dilution and washing the ITC instrument. 250 µM IsdB/IsdH was used as the titrant, with 25 µN HbCO in the cell; in the case of IsdB¹¹⁻L and IsdB¹⁻N², additional runs with 700 µM IsdB titrated into 70 µN HbCO were also performed, under the same conditions. 20 injections of 2 µL each at 180 second 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.
2.13 Steady-state heme transfer kinetics

Relay of heme from metHb to IsdA\textsuperscript{N1} by catalytic amounts of IsdB\textsuperscript{N1N2} was characterized by exploiting spectral differences between metHb and holo-IsdA\textsuperscript{N1}. In a conventional spectrophotometer (Cary60) maintained at 25 °C, a catalytic amount of IsdB\textsuperscript{N1N2} (50 nM; 10-fold less than lowest metHb concentration) was used to perform a relay of heme from metHb (concentration ranging from 0.5-10 μM) to IsdA\textsuperscript{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 minutes prior to addition of metHb to initiate the relay reaction. The absorbance at 408 nm was monitored for the first 60 seconds of the reaction. Each reaction was carried out in triplicate; the slopes from 0-30 seconds were averaged to give an initial velocity at each metHb concentration.

2.14 Heme transfer from metHb to IsdB\textsuperscript{N2} through IsdB\textsuperscript{N1-L}

Heme uptake from metHb by a combination of IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} was monitored by electronic spectroscopy. 2 μM metHb was mixed with 20 μM IsdB\textsuperscript{N2} and 0.2-20 μM IsdB\textsuperscript{N1-L} and spectra from 250-750 nm were collected every 12 sec for 1 min, then every 1 min until 5 min. Complete transfer results in a characteristic holo-IsdB\textsuperscript{N2} spectrum.

2.15 Stopped-flow spectroscopic analysis of heme transfer from metHb to IsdB

2.15.1 Multi-wavelength data collection

An SX.18MV stopped-flow reaction analyzer (Applied Photophysics Ltd., Leatherhead, UK) was used to investigate the possibility of heme-coordination intermediates in the transfer process between metHb and IsdB\textsuperscript{N1N2}. The temperature of the optical cell and drive syringe chamber was maintained at 25 °C using a circulating water bath. Multiple wavelength data from 180 to 730
nm were collected using the system’s photodiode array (PDA) detector directly coupled to the Xe light source (the practical range was from 250 nm to 730 nm). 20 μM apo-IsdB\textsuperscript{N1N2} and 2 μN metHb samples were in a buffer composed of 20 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 7.4), 50 mM NaCl. Each acquisition was 15 seconds long with spectra collected at logarithmic intervals; 4 acquisitions were averaged. Data was collected in the same manner for the reaction of metHb and IsdB\textsuperscript{N1N2 Y440A}, except 2 μN metHb and 10 μM IsdB\textsuperscript{N1N2 Y440A} were used, with 4 x 20 sec acquisitions.

2.15.2 Single wavelength data collection

A monochromator was used to collect single-wavelength data at 406 and 428 nm for the reaction of metHb and IsdB\textsuperscript{N1N2}. 20 mM HEPES (pH 7.4), 80 mM NaCl was used as the buffer in order to make a direct comparison with the steady-state kinetics experiments, which were performed in HEPES buffer. The reactions were carried out with 2 μN metHb in one syringe (final concentration 1 μN) and concentrations of apo-IsdB\textsuperscript{N1N2} ranging from 10-80 μM (final concentrations 5-40 μM) in the second syringe; a minimum five-fold excess of the IsdB acceptor was used to attain pseudo-first order conditions. The temperature was again maintained at 25 °C and 5 x 30 second acquisitions were performed at each wavelength for each concentration pair. The first 3 milliseconds were excluded from analysis. At least 4 acquisitions were averaged and curve fitting was performed using the ProDataSX software.

2.16 Hb+IsdB\textsuperscript{N1N2} crystal growth and data collection

Solutions of apo-IsdB\textsuperscript{N1N2} and either oxyHb or HbCO in an equimolar (by monomer) ratio were mixed together at 10 mg/mL and immediately used to set up crystal trays. Crystals grew at a 1:1 ratio of protein:well solution composed of citric acid or malonic acid, pH 5.0-5.5, and 2.1-2.4 M ammonium sulfate, in 96-well sitting drop plates. Crystals were cryoprotected with 10-
30% sodium malonate (pH 7) or 30% xylitol and flash frozen in liquid nitrogen or on the homesource goniometer. Data were collected at the Canadian Light Source beamline 08B1-1.

2.17 **Hb+IsdB\(^{\text{NN2}}\) crystal composition**

Crystals from a well of oxyHb+IsdB\(^{\text{NN2}}\) were looped and soaked in well solution to remove adventitiously bound protein, then dissolved in 5 µL of 20 mM HEPES (pH 7.4), 80 mM NaCl. Five small crystals in total were dissolved together. This sample was run on a 15% SDS-PAGE gel and stained with Coomassie stain to visualize the components of the crystal.

2.18 **IsdB\(^{\text{NN2} \ Y440A}\) heme titration**

Heme binding was tracked by difference absorption spectroscopy in the Soret region, 405 nm, at room temperature. Aliquots of hemin (2 µL) solubilized in 0.1 M NaOH and diluted in 20 mM HEPES pH 7.4, 80 mM NaCl were added to 1 mL of a 5 µM solution of apo-IsdB\(^{\text{NN2} \ Y440A}\) or a reference cuvette containing buffer alone. Spectra (250-750 nm) were recorded 8 minutes after addition of heme and titrations covered a concentration range from 1 to 12 µM heme. Saturation was defined as a plateau in the absorption difference between the reference cuvette and the experimental cuvette. All spectra were recorded using a Cary 50 Bio UV-visible spectrophotometer (Agilent Technologies, Mississauga, Canada) with an optical path length of 1 cm in a quartz cell at room temperature. Results shown are for a single experiment.

2.19 **Hb binding competition assay**

The possibility that IsdH\(^{\text{N1}}\) binding to Hb could block binding by IsdB\(^{\text{NN2}}\) was investigated using a competition pulldown assay. 25 µL aliquots of nickel bead slurry (Chelating Sepharose Fast Flow, GE Healthcare; stored in 20% ethanol) were washed twice with 1 mL of dH\(_2\)O, followed by a 1 mL wash of binding buffer, consisting of 20 mM HEPES (pH 7.4), 80 mM
NaCl, 10 mM imidazole. The small amount of imidazole was included to abrogate non-specific binding by IsdB constructs to the beads, but did not affect binding by oxyHb to the nickel beads. For controls (oxyHb, IsdB^{N1N2} or IsdH^{N1} alone), 50 µL of 20 µM IsdB/H or 20 µN (in heme) oxyHb were added to the beads and kept on ice for the duration of the pulldown experiment. For pulldowns, oxyHb was added to 20 µN to the beads and kept on ice for 10 minutes, followed by adding either 0.1, 1, or 5 molar equivalents of oxyHb (in heme) of IsdB^{N1N2} or IsdH^{N1} and keeping on ice for 10 minutes, followed by adding 1 molar equivalent of oxyHb (in heme) of either IsdH^{N1} or IsdB^{N1N2} and keeping on ice a further 10 minutes. All samples were gently agitated occasionally. After incubation, the 50 µL supernatant was removed and beads with bound protein were washed twice with 500 µL of binding buffer, then eluted with 25 µL of 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 mM imidazole. 8 µL of each eluate was run on a 15% SDS-PAGE gel at 150 V for 1 hour, 15 minutes and stained using Coomassie Blue.

An additional pulldown was performed following a similar protocol except that 20 µN oxyHb was used to pull down multiple equivalents of IsdH^{N1} (0.25-4 equivalents in heme) or IsdB^{N1N2} (0.5-4 equivalents in heme), or His_{6}-IsdB^{N1N2} was used to pull down IsdH^{N1}.

### 2.20 Spectra of metHb+IsdB^{N1N2} with increasing IsdH^{N1}

Resultant spectra of mixtures of metHb, IsdH^{N1} and IsdB^{N1N2} were examined. 2 µN metHb was pre-incubated with either 1, 2 or 5 µM IsdH^{N1}, then mixed with 10 µM IsdB^{N1N2}. Spectra (250-750 nm) were recorded immediately after mixing, in a conventional spectrophotometer (Cary50) with an optical path length of 1 cm in a quartz cell at room temperature, 22 °C.
2.21 Endpoint spectrum of metHb+IsdB^{NIN2} with excess Hp

Human haptoglobin (Hp) of mixed serotype purified from plasma was purchased as a lyophilized solid (Athens Research and Technology). 1 mg of Hp was dissolved in 100 μL 20 mM HEPES (pH 7.4), 80 mM NaCl to give a concentration of 10 mg/mL. Hp molecules from different serotypes are a range of sizes (Hp 1-1: 86 kDa, Hp 2-1: 200 kDa, Hp 2-2: 400 kDa; values provided by the manufacturer); the 10 mg/mL solution could therefore be a minimum of 25 μM (all Hp 2-2) to a maximum of 116 μM (all Hp 1-1). The molar concentration was thus taken to be 25 μM, which was likely an underestimate of the actual Hp concentration. Spectra of 2 μN metHb alone or combined with 10 μM IsdB^{NIN2} and/or 5 μM Hp (pre-combined with metHb) were taken from 250-750 nm in a conventional spectrophotometer (Cary50) with an optical path length of 1 cm in a quartz cell at room temperature, 22 °C.

Subsequently, the minimum inhibitory concentration of Hp was determined by adding decreasing amounts of Hp under the same conditions (beginning with 2 mg/mL as above) until the heme transfer reaction from metHb to IsdB^{NIN2} was observed to proceed. Spectra (250-750 nm) were recorded immediately after mixing, as before. The reaction was monitored by recording additional spectra at 2 and 5 min after the initial spectrum.

2.22 Assay for effect of IsdH^{N1} addition on heme uptake by IsdB^{NIN2} of heme from metHb-Hp complex

The effect of IsdH^{N1} on the reaction was investigated. 2 μN metHb was pre-incubated with the minimum inhibitory concentration of Hp, 0.1 mg/mL, as well as 1, 5, or 25 μM IsdH^{N1} before mixing with 10 μM IsdB^{NIN2}. Spectra (250-750 nm) were recorded immediately after mixing with IsdB^{NIN2} as well as at 2 and 5 min afterwards.
2.23  IsdB^{N1N2}-Hb-Hp pulldown assay

The ability of nickel bead-bound His_{6-IsdB}^{N1N2} to pull down metHb-Hp complexes was tested. 6 x 25 μL aliquots of nickel bead slurry (Chelating Sepharose Fast Flow, GE Healthcare; stored in 20% ethanol) were washed twice with 1 mL of dH_{2}O, followed by a 1 mL wash of binding buffer, consisting of 20 mM HEPES (pH 7.4), 80 mM NaCl, 75 mM imidazole. The moderate amount of imidazole was a concentration at which His_{6-IsdB} could bind the nickel beads but Hb could not. To each aliquot of washed beads, 50 μL of 20 μM His_{6-IsdB}^{N1N2}, metHb or Hp (alone for controls or together for pulldowns, as indicated) were added to the beads and kept at room temperature on the benchtop. Samples were incubated for 15 minutes and gently agitated occasionally. After incubation, the 50 μL supernatant was removed and beads with bound protein were washed twice with 500 μL of binding buffer, then eluted with 1 M imidazole, pH 7.5. 4 μL of each eluate was run on a 15% SDS-PAGE gel at 200 V for 1 hour, 5 minutes and stained using Coomassie Blue.
Chapter 3: Unique heme-iron coordination by the hemoglobin receptor IsdB

3.1 Introduction

*S. aureus* exhibits a preference for heme as an iron source during infection (47) and expresses the Iron-responsive surface determinant (Isd) system to exploit the heme and hemoprotein resources of the host (50). IsdB is a cell-wall anchored protein composed of two NEAT domains and is the predominant Hb receptor of *S. aureus* (54,58). The NEAT domains of the surface Isd proteins had been demonstrated to mediate either heme binding (52,59-64) or hemoprotein binding (53,59,65). In the case of IsdB, the N-terminal NEAT domain (IsdB\(^{N1}\)) was thought to bind Hb and the C-terminal NEAT domain (IsdB\(^{N2}\)) to bind and relay heme to IsdA for transport into the cell. Full-length IsdB was known to extract heme from metHb and IsdB\(^{N2}\) was known to transfer heme to IsdA\(^{N1}\) or IsdC\(^{N1}\), which then transfer it to IsdE for internalization through the membrane transporter (51,52). At the time of writing, crystal structures of the NEAT domains of IsdA, IsdC and the C-terminal NEAT domain of IsdH, IsdH\(^{N3}\), had been published and revealed a common 8-stranded β-sandwich fold (60,62,64).

I sought to characterize heme binding and transfer to IsdA\(^{N1}\) by IsdB\(^{N2}\) (Figure 3-1) as a first step towards understanding Hb reception and heme transfer by the Isd system. I solved the 1.45 Å resolution crystal structure of heme-bound IsdB\(^{N2}\) and discovered a new mode of heme-iron coordination, with protein ligands provided by both a Met and a Tyr. I further showed that iron coordination by the Met residue is dispensable for high-affinity heme binding, but has a role in facilitating heme transfer to IsdA\(^{N1}\). The kinetics of heme transfer from IsdB\(^{N2}\) to IsdA\(^{N1}\) could be modelled as a two-step process, and the rate of heme transfer between the isolated NEAT domains (82 s\(^{-1}\)) was found to be similar to that measured for the full length proteins.
3.2 Results

3.2.1 Crystal structure of IsdB\textsuperscript{N2}

The holo structure of IsdB\textsuperscript{N2} was solved to 1.45 Å resolution. The structure of Se-Met labelled IsdB\textsuperscript{N2} was also solved to 1.7 Å, but was inappropriate for interpretation of heme binding due to the artefact introduced by the selenomethionine substitution, discussed in a later section. There were four molecules in the asymmetric unit which overlaid with an average r.m.s.d. of 0.49 Å over all Ca atoms, with the main differences occurring at the N-termini. The final model consists of residues 341-452 for Chain A, 341-458 for Chains B and C, and 341-456 for Chain D, and includes an N-terminal Gly-Ser cloning artefact leftover from the thrombin cleavage site. Data collection and refinement statistics are shown in Table 3-1.
Table 3-1. X-ray data collection and refinement statistics for IsdB<sup>N2</sup> structures.

<table>
<thead>
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<th></th>
<th>Native</th>
<th>Se-Met</th>
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<td>$P2_12_1\overline{2}$</td>
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<td>Unit cell dimensions</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>0.05 (0.31)</td>
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<td>$I/\sigma I$</td>
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<td>24.2 (3.8)</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
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<td>$R_{work}/R_{free}$</td>
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<td>0.193/0.232</td>
</tr>
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<td>No. atoms</td>
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<td>4793</td>
</tr>
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<td>Protein/heme</td>
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<td>4033</td>
</tr>
<tr>
<td>Ions</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
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<td>755</td>
</tr>
<tr>
<td>$B$-factors (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>19.5</td>
<td>26.7</td>
</tr>
<tr>
<td>Heme</td>
<td>15.0</td>
<td>22.8</td>
</tr>
<tr>
<td>Ions</td>
<td>17.1</td>
<td>23.5</td>
</tr>
<tr>
<td>Water</td>
<td>28.3</td>
<td>42.0</td>
</tr>
<tr>
<td>R.m.s. deviations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
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<td>0.013</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.38</td>
<td>1.48</td>
</tr>
</tbody>
</table>

<sup>a</sup>values in parentheses represent highest resolution shell

IsdB<sup>N2</sup> adopts the characteristic eight-stranded immunoglobulin-like β-sandwich fold observed in other NEAT domains whose structures are known (Figure 3-2A) (59,60,62,64,101).

In addition to short α-helices between β1- β2 and β3- β4, there is a C-terminal α-helix of approximately 1.5 turns immediately following β8 which is not observed in other NEAT domain structures.
Figure 3-2. Crystal structure of the heme-bound C-terminal NEAT domain of IsdB.
(A) Overall structure of holo-IsdB$^{N2}$ (chain A) viewed down the heme binding pocket. The backbone is shown as a cartoon with helices in purple, $\beta$-strands in cyan and loops in grey. Heme is shown protruding from the pocket as sticks, with carbon atoms in dark blue and oxygen atoms in red. One conformation of heme is shown for clarity. The N- and C-termini and helices are labelled. (B) A closer view of the heme pocket. Residues directly involved in binding the heme molecule (Tyr440, Met362 and Ser361) or indirectly involved in binding (Tyr444) are shown in sticks and labelled. Protein carbon, oxygen and sulfur atoms are shown in purple, red and yellow, respectively. Heme carbon, oxygen, nitrogen and iron atoms are shown in blue, red, dark blue and dark red, respectively. (C) and (D) The $F_o-F_c$ omit maps (contour level = $3\sigma$) for Tyr440, Met362 and heme of chains A and B.
3.2.2 Structure of the heme binding pocket

Heme is bound in a highly hydrophobic pocket and is modelled at equal occupancy in two orientations when rotated by 180° along the Cα–Cγ axis (Figure 3-2B). Approximately 330 Å² (38%) of the heme surface area was found to be exposed to solvent as calculated using AreaIMol (90). Crystal packing interactions occurred across the face of the heme pocket resulting in vinyl groups of heme molecules being ~3.3 Å apart (Figure 3-3). The heme propionates are extended out from the pocket and one forms a hydrogen bond with the hydroxyl group of Ser361 and the backbone nitrogen of Met362. Tyr440, Tyr444 and Phe366 contribute to π-stacking with a buried heme pyrrole ring whereas Val431, Val433, Val446, Val435, Tyr391, Trp392 and Met363 contribute to hydrophobic contacts.

Figure 3-3. Heme pockets in adjacent IsdB N2 molecules are closely juxtaposed in the crystal structure. Due to close contacts between heme pocket faces, the vinyl groups of protoporphyrin IX are as little as 3 Å apart. The α2 helices are also close; the Met362 residue on each is in a complementary configuration to the other, such that one mainly juts inwards to coordinate heme-iron, and one mainly juts outwards, towards the other molecule. Chains A (cyan) and B (green) are shown as representative of the asymmetric unit.
The heme iron is coordinated by Tyr440 which forms a hydrogen bond with the phenolate of Tyr444, an interaction which is absolutely conserved among heme binding NEAT domains of known structure (60,62,64). The heme-iron exists in a mixture of hexacoordinate and pentacoordinate states, with the sulfur from Met362 occupying the sixth-coordinate position. The electron density is best modeled with Met362 in both coordinating and non-coordinating conformations in the structure, and the occupancy of the conformations is unequal and dependent upon the molecule examined. In chains A and D, inspection of $F_o-F_c$ maps indicates that Met362 exists in a coordinating position at 75% occupancy (sulfur atom 2.5-2.6 Å from heme-iron) and 25% occupancy in a non-coordinating position (sulfur atom 4.9-5.0 Å from heme-iron) (Figure 3-2C). The opposite is true for chains B and C, where the sulfur atom models away from the pocket at 70% occupancy (4.8 Å from heme-iron) and the sulfur atom is directed towards the heme iron at 30% occupancy (2.6 Å from heme-iron) (Figure 3-2D). Furthermore, these conformations appear to be complementary to one another, such that the heme pocket containing a mainly-coordinating Met362 (A or D) is tightly packed against a heme pocket containing a mainly-non-coordinating Met362 (B or C). Lastly, the heme-iron is pulled out of the plane of the porphyrin ring and closer to Tyr440 in chains B and C (2.1 to 2.2 Å Fe-O bond length; 0.4 to 0.5 Å from planarity), where Met362 is mainly non-coordinating, whereas the iron is pulled away from Tyr440 and lies closer to the porphyrin ring plane in chains A and D (2.2 to 2.3 Å Fe-O bond length, less than 0.2 Å from planarity), where Met362 is mainly coordinating.

The variability in Met362 coordination state was first observed in the 1.7 Å Se-Met structure, motivating the determination of the native structure. Although it is well-documented that substitution of a Met heme-iron ligand for Se-Met has little effect on heme iron coordination (55), I was concerned that the slightly larger Van der Waals radius of selenium or an incomplete
substitution of Se-Met for Met might have caused the mixture of coordination states observed in the structure.

### 3.2.3 Visible absorption spectra of heme pocket variants

Based on the residues which were observed to directly participate in heme binding in the IsdB<sup>N2</sup> crystal structure, several variants of the wild-type (WT) protein were constructed using site-directed mutagenesis: Y440A, Y444A, S361A and M362L. The visible absorption spectra of the variants reconstituted with equimolar hemin were compared to that of the WT protein, as the spectrum is indicative of the heme environment. Mutation of any one of these residues was found to significantly affect the shape, height and maximum wavelength of the Soret peak, indicating a significant change in the environment experienced by the heme (Figure 3-4A). Furthermore, the spectra of the Y444A, Y440A and S361A variants more closely resembled free heme than WT IsdB<sup>N2</sup>, indicating that loss of Tyr444, Tyr440 or Ser361 resulted in severe heme binding disruption. The spectrum of M362L resembled most closely that of the wild-type hemoprotein rather than free heme, with a less pronounced shoulder at 380 nm and more pronounced α/β bands (Figure 3-4B). However, the Soret peak shifted from 404 nm for WT to 398 nm for M362L and the Soret shape and height were also significantly different, indicating different heme environments.

### 3.2.4 Heme titration of IsdB<sup>N2</sup> and variants

Although a single heme molecule is bound per monomer of IsdH<sup>N3</sup> in the crystal structure, investigators found that a monomer could bind up to 4 molecules of heme in solution (64). Though the crystal structure of IsdB<sup>N2</sup> also bound one heme per monomer, to confirm that 1:1 stoichiometry was recapitulated in solution apoprotein was titrated with increasing amounts of heme and difference spectra were generated by subtracting spectra from buffer titrated with the
Figure 3-4. Visible spectra of holo-IsdB$^{N2}$ WT and variants. 10 μM heme was added to 10 μM apoprotein and allowed to reach equilibrium at room temperature. 10 μM heme alone in buffer and 10 μM heme added to transferrin, a non-heme binding protein, are also shown for comparison. (A) Spectra of all proteins recorded between 250 and 700 nm. Absorbance at 280 nm is similar for all IsdB$^{N2}$ proteins (~14 kDa), whereas that of transferrin (~80 kDa) is significantly higher, and heme absorbs much less at 280 nm. The spectrum in the Soret range (~400 nm) is noticeably different for most variants, except S361A and Y444A, which are largely indistinguishable. (B) A close-up of the spectra between 400 and 700 nm reveals characteristic markers of the heme-iron environment; WT and M362L are most similar to each other, whereas Y440A, Y444A and S361A resemble the spectrum of free heme.

same amounts of heme (Figure 3-5A-C). IsdB$^{N2}$ WT and M362L (Figure 3-5D,E) both demonstrated a binding stoichiometry of approximately 1:1. However, Y444A, Y440A and S361A difference spectra did not follow a single-site, specific binding curve under the same conditions and resembled that of a non-heme binding protein, transferrin, indicating weak heme-binding (Figure 3-6A-D). Interestingly, at heme concentrations greater than 10-fold below equimolar, Y444A, Y440A and S361A variants displayed a more characteristic Soret peak (Figure 3-7A,C,E). However, addition of increasing heme caused the Soret peak to both broaden and blue-shift for each variant, suggesting an increasing absorbance signal from free heme (Figure 3-7B,D,F). Overall, these variants can still bind heme weakly, but a large excess of apoprotein is required to drive the equilibrium towards a heme-protein complex.
Figure 3-5. IsdB\textsuperscript{N2} WT and M362L variant follow a similar heme binding curve. Titration of 5 \( \mu \)M apo IsdB\textsuperscript{N2} wild-type protein (B) or M362L (C) with heme results in an increasing characteristic Soret peak, whereas titration of buffer (A) with heme results in an increasing plateau between \( \sim 350-400 \) nm, characteristic of free heme. IsdB\textsuperscript{N2} WT (D) and IsdB\textsuperscript{N2 M362L} (E) display a binding stoichiometry of \( \sim 1:1 \), as the increase in absorbance at respective Soret peaks when compared to heme alone plateaus at approximately 6 \( \mu \)M.
Figure 3-6. Heme titration of other IsdB<sup>N2</sup> variants.
Titration of 5 μM apo IsdB<sup>N2</sup> Y440A, Y444A or S361A (B, C and D, respectively) with 0.1-10 μM heme compared to the heme titration of 5 μM transferrin (A), a non-heme binding protein. The absorption differences at the wavelengths of greatest difference between heme titration of each protein and heme titration of buffer do not result in an observable plateau, indicating no saturation occurred.
Figure 3-7. Apo IsdB\textsuperscript{N2} variants mixed with sub-equimolar heme. The spectra resulting from the first 4 heme titration points for 5 \( \mu \)M Y444A, Y440A and S361A variants are examined in more detail. The Soret maxima of the first two titration points (A,C,E) are indicated by dotted lines for each variant. The Soret maximum blue-shifts beginning at the third titration point, with the shoulder at \( \sim380 \) nm becoming more prominent in each case (B,D,F).
IsdB\textsuperscript{N2} contains one Trp residue (W392), which resides at the base of the heme-binding pocket. I was able to exploit this fact to develop a fluorescence-based measurement of heme binding as heme addition causes quenching of Trp fluorescence. I observed a concentration-dependant and saturable quenching of Trp fluorescence by both WT IsdB\textsuperscript{N2} and the M362L variant (Figure 3-8A-C). At a concentration of 1 \(\mu\)M, IsdB\textsuperscript{N2} WT and M362L variant had a very similar \(K_D\) (0.38±0.06 and 0.49±0.09 \(\mu\)M, respectively) when fit to an equation accounting for ligand depletion; however, as I was working at a protein concentration close to the calculated \(K_D\) (necessarily high for a reproducible fluorescence signal), these values can only be considered an upper bound, with the actual \(K_D\) likely being lower. I can conclude that both the native and M362L variant bind heme with high nanomolar or better affinity. By comparison, the variant proteins which displayed weak heme binding by visible spectroscopy (Y440A, Y444A and S361A) required much greater (at least ten-fold higher) heme concentrations for maximal quenching and curve-fitting suggested dissociation constants at least 25-fold weaker (Figure 3-8D-F). Due to the apparent weak binding for these variants they were deemed unsuitable for performing transfer assays. Dissociation constants are listed in Error! Reference source not found.

Table 3-2. Kinetic parameters of heme binding and transfer.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(K_D \pm SE) (heme; (\mu)M)</th>
<th>Off-rate (\pm SE) (x (10^3) s(^{-1}))</th>
<th>(k_{\text{transfer}} \pm SE) (s(^{-1}))</th>
<th>(K_D^{-1} \pm SE) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsdB\textsuperscript{N2} WT</td>
<td>(&lt; 0.38 \pm 0.06)</td>
<td>28 (\pm) 3</td>
<td>4.84 (\pm) 0.02</td>
<td>82 (\pm) 6</td>
</tr>
<tr>
<td>IsdB\textsuperscript{N2} M362L</td>
<td>(&lt; 0.49 \pm 0.09)</td>
<td>130 (\pm) 10</td>
<td>12.65 (\pm) 0.04</td>
<td>34 (\pm) 2</td>
</tr>
<tr>
<td>IsdB\textsuperscript{N2} Y440A</td>
<td>17 (\pm) 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IsdB\textsuperscript{N2} Y444A</td>
<td>15 (\pm) 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IsdB\textsuperscript{N2} S361A</td>
<td>13 (\pm) 1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(1\)Estimated dissociation constant for the protein complexed with IsdA\textsuperscript{NT}. ND: not determined.
Figure 3-8. Trp fluorescence quenching of IsdB\textsuperscript{N2} and variants.

(A) Titration of 1 μM apo IsdB\textsuperscript{N2} wild-type protein with 0.1-15 μM heme results in a decrease in Trp fluorescence with increasing heme concentration. (B-F) One-site, specific binding curves generated by plotting total fluorescence intensity (calculated as the area under the fluorescence curve) at each heme concentration for wild-type and variants. (B,C) 1 μM apo IsdB\textsuperscript{N2} wild-type and M362L variant, respectively, titrated with with 0.1-15 μM heme. (D-F) 5 μM apo IsdB\textsuperscript{N2} Y440A, S361A and Y444A variants, as indicated, titrated with 1-150 μM heme. A graph of the residuals for all curves is inset.
3.2.5 Rate of heme transfer to apomyoglobin

Heme transfer from the binding pockets of IsdB$^{N2}$ WT and M362L variant to apomyoglobin (apoMb) was followed by visible stopped-flow spectroscopy at 408 nm, the Soret maximum for holomyoglobin. ApoMb has an affinity for heme in the picomolar range (102), does not directly bind to IsdB (54) and has been used to characterize heme binding by Isd proteins previously (51). The rate of heme transfer into apoMb is independent of the concentration of apoMb, therefore the observed rate is assumed to be the rate of heme release (off-rate) from IsdB$^{N2}$ (98). The transfer rate data are best fit by a double exponential curve, as judged by the greater randomness of the residual plot than that for a single-exponential curve. The initial fast phase accounted for only 2.8 % of the curve for WT and 3.5 % for M362L and may represent the presence of a minor extraneous species in solution, such as improperly folded holo-IsdB$^{N2}$ or IsdB$^{N2}$ with non-specifically bound surface heme. The slow phase, describing the vast majority of the absorption change, yielded an off-rate value of $4.8 \times 10^{-3} \text{s}^{-1}$ for WT IsdB$^{N2}$ (Figure 3-9A), and the substitution of Met362 for Leu (Figure 3-9B) increased the off-rate by 2.7-fold, to $1.3 \times 10^{-2} \text{s}^{-1}$.

A summary of kinetic and equilibrium binding characteristics can be found in Error! Reference source not found..

3.2.6 Rate of heme transfer to IsdA$^{N1}$

Using stopped-flow spectroscopy the observed rates ($k_{obs}$) of heme transfer from holo-IsdB$^{N2}$ to apo-IsdA$^{N1}$ were determined by the difference in the Soret region of their visible spectra at the wavelength of maximal change over ~0.3 seconds (for WT) or ~1.0 seconds (for M362L), equivalent to a minimum of 15 half-lives. The change in absorbance at 418.9 nm (WT) or 425.3 nm (M362L) was plotted against time and fit by a single exponential to determine a value for the
observed rate constant \( (k_{\text{obs}}) \). The \( k_{\text{obs}} \) for 2 \( \mu \text{M} \) IsdB\textsuperscript{N2} was determined under pseudo-first order conditions for concentrations of IsdA\textsuperscript{N1} from 10-50 \( \mu \text{M} \). The \( k_{\text{obs}} \) vary hyperbolically with

\[
\text{Figure 3-9. Heme transfer from IsdB\textsuperscript{N2} to apomyoglobin.}
\]

There is a considerable change in the visible spectrum as heme is released from holo-IsdB\textsuperscript{N2} and bound by apomyoglobin (apoMb). The increase in absorbance at 408 nm is characteristic of holomyoglobin formation; a plot of the absorbance at 408 nm vs. time fits a two-phase association function in which the rate of holomyoglobin formation is equal to the off-rate from IsdB\textsuperscript{N2}. The dotted black line is a double exponential curve fit to the data, and the grey band represents the standard error of 3 replicates for WT and 2 replicates for M362L. A graph of the residuals is inset and displays a relatively random distribution. (A) IsdB\textsuperscript{N2} WT. (B) IsdB\textsuperscript{N2} M362L.

respect to the concentration of IsdA\textsuperscript{N1}, suggesting a two-step transfer mechanism. A model for heme transfer has been proposed by Liu et al. \textit{(103)}, which states that the transfer of heme from the holo-NEAT domain to the apo-NEAT domain has two observable steps characterized by rapid protein-protein complexation followed by rate limiting heme transfer between the proteins (Figure 3-10).

\[
\begin{align*}
\text{holoIsdB\textsuperscript{N2}} + \text{apoIsdA\textsuperscript{N1}} & \overset{k_1}{\rightarrow} \text{holoIsdB\textsuperscript{N2}} + \text{apoIsdA\textsuperscript{N1}} \\
\text{holoIsdB\textsuperscript{N2}} + \text{apoIsdA\textsuperscript{N1}} & \overset{k_{\text{transfer}}}{\rightarrow} \text{apoIsdB\textsuperscript{N2}} + \text{holoIsdA\textsuperscript{N1}}
\end{align*}
\]

\text{Figure 3-10. Reaction scheme for transfer of heme from IsdB\textsuperscript{N2} to IsdA\textsuperscript{N1}.}
Using this model, the rate constant ($k_{\text{transfer}}$) for transfer of heme from wild-type holo-IsdB$^{N2}$ to apo-IsdA$^{N1}$ was found to be $82 \pm 6 \text{ s}^{-1}$ (Figure 3-11A). For IsdB$^{N2}$ M362L to IsdA$^{N1}$, the rate constant was found to decrease by more than two-fold, at $34 \pm 3 \text{ s}^{-1}$ (Figure 3-11B). The calculated $K_D$ for the protein-protein complexes was similar for both WT and M362L transfer reactions (18 and 16 μM, respectively). The results are summarized in Error! Reference source of found.

![Figure 3-11](image)

**Figure 3-11.** Kinetics of heme transfer from holo-IsdB$^{N2}$ to apo-IsdA$^{N1}$. The observed transfer rate ($k_{\text{obs}}$) varies hyperbolically with IsdA concentration, characteristic of a two-step reaction. Each point represents the mean and standard error of 4 replicates of the 2 μM IsdB$^{N2}$ to IsdA$^{N1}$ heme transfer experiment. Observed transfer rates at each concentration of IsdA$^{N1}$ were determined by plotting the change in absorbance at a given wavelength (418.9 nm for WT and 425.3 nm for M362L) over time. The residuals for the curves are shown inset. (A) Transfer from holo WT IsdB$^{N2}$ to apo-IsdA$^{N1}$. (B) Transfer from holo M362L IsdB$^{N2}$ to apo-IsdA$^{N1}$.

### 3.3 Discussion

Investigating the molecular basis for heme uptake by *S. aureus* is a crucial step towards understanding host-pathogen interactions. IsdB is the dominant Hb-binding protein of *S. aureus* (54). I have found that the heme-binding NEAT domain (IsdB$^{N2}$) of IsdB adopts a similar fold to those observed in other NEAT domain structures (53,59,60,62,64). Additionally, heme-iron is coordinated by a conserved Tyr residue as predicted by multiple sequence alignments (60) and observed in other heme-binding NEAT domains in *S. aureus* (60,62,64). However, a unique
feature of IsdB$^{N2}$ is a distal heme-iron ligand, Met362. The equivalent position in IsdA$^{N1}$ is taken by a His, which is a common iron-ligand but does not coordinate to the heme iron in the crystal structure (60) or in solution in the absence of a strong reductant (61). In the heme binding NEAT domains of IsdC (IsdC$^{N1}$) and IsdH (IsdH$^{N3}$), there is an Ile and a Val, respectively, in the sixth position, neither of which have the capacity for heme-iron coordination (62,64). As well as being unprecedented in the Isd system of S. aureus, Tyr-Met heme-iron coordination has not been reported in the literature for any heme-binding protein to date.

The protein Shp from Streptococcus pyogenes uses an unusual bis-methionyl heme-iron coordination as part of a heme uptake system with some components distantly related to the Isd system of S. aureus (101). The crystal structure of the NEAT-like domain of Shp, denoted Shp180, superposes on IsdB$^{N2}$ with a core r.m.s.d. of 2.5 Å over 94 C$\alpha$ atoms using the SSM Superposition program (104). The average Fe-S bond length between the sulfur of the coordinating Met residues of Shp and the heme-iron was found to be 2.4 Å (101), less than the Fe-S bond length of 2.5-2.6 Å found here. By comparison, the Fe-S bond length seen in IsdE, which coordinates heme-iron using Met and His, is shorter at 2.3 Å (55). The Fe-O bond length (Tyr-heme-iron) observed in IsdB$^{N2}$, which ranges from 2.1-2.3 Å depending on the molecule, is typical of those seen in other NEAT domain structures: 2.1 Å for IsdA$^{N1}$ (55), 2.2 Å for IsdH$^{N3}$ (64) and 2.0 – 2.1 Å for IsdC$^{N1}$ (62).

It is noteworthy that previous work using electronic and magnetic circular dichroism (MCD) spectroscopy did not identify Met as a heme-iron ligand in IsdB$^{N2}$, though Tyr was accurately predicted to be a ligand (52,61,105). The discrepancy may be explained by photoreduction of the heme-iron to Fe$^{2+}$ in the crystal by the x-ray radiation, resulting in a preference for ligation by methionine; beamline photoreduction is a well-known issue when studying hemoproteins and can
result in conformational changes as well as changes in coordination state (106). However, IsdA\textsuperscript{N1} contains a potential distal heme-iron ligand (His83) which was not observed to coordinate heme-iron in the crystal structure (60). Moreover, it has been shown that reduction of the heme-iron in IsdA results in heme iron coordination solely by the distal His83 (63,107). Given that Met362 clearly occupies both coordinating and non-coordinating conformations in the IsdB\textsuperscript{N2} crystal structure, I suggest that it may do the same in solution, consistent with spectra indicating a predominantly five coordinate heme iron with the sixth position readily available to bind exogenous CN\textsuperscript{-} (105).

Aside from Tyr440 and Met362, two other residues form important interactions to secure heme: Ser361, which hydrogen bonds to one propionate, and Tyr444, which hydrogen bonds with Tyr440. All four residues were mutated separately to probe their involvement in stable heme binding by IsdB\textsuperscript{N2}, resulting in the Y440A, Y444A, M362L and S361A variants. Of these four, only the M362L variant was proficient at heme binding: a gel filtration step intended to purify hemoprotein after heme reconstitution resulted in mainly apoprotein in the cases of the Y440A, Y444A and S361A variants (data not shown). The apparent deficiency in heme binding of these variants was further characterized by examining their visible electronic spectra, which more closely resembled free heme than hemoprotein. The loss of high affinity heme binding by S361A variant suggests that the interaction with the heme propionate is crucial for stability of the loop that includes Met362 and forms one side of the heme binding pocket. Similarly, Tyr444 is absolutely conserved in all NEAT domains of the Isd system, heme binding or not; the data suggest that it is a critical heme binding residue as well, although it does not directly interact with the heme. Tyr444 may be required to stabilize the fold of NEAT domains as well as to position Tyr440 for heme-iron coordination or to mediate the phenolate-iron bond.
To investigate the significance of Met362 with regard to potential functional roles in the heme pocket, I carried out a series of spectroscopic and kinetic characterizations of the WT IsdB\textsuperscript{N2} protein in comparison with a M362L variant. I found that the variant retained nanomolar affinity for heme, as measured by tryptophan fluorescence quenching. The 1:1 stoichiometry of heme binding was not altered either; however, the off rate of heme from the pocket increased by 2.7-fold. This may be explained by considering that loss of a heme-iron ligand weakens heme binding, and may also destabilize the distal loop, allowing increased solvation of the heme in the pocket. In contrast, the M362L substitution causes a decrease in the first order rate constant for heme transfer to IsdA\textsuperscript{N1} by half, from 81 s\textsuperscript{-1} to 34 s\textsuperscript{-1}. The combined increase in heme off rate by the M362L variant paired with a decreased rate of transfer to IsdA\textsuperscript{N1} results in a ratio of catalyzed heme transfer to heme release of 6.4-fold less for the M362L variant (transfer rate \(\sim 2600\)-fold greater than off-rate) than the wild-type protein (transfer rate \(\sim 17,000\)-fold greater than off-rate). The implication is that Met362 plays a role in passage of heme between the IsdA and IsdB NEAT domains. Heme transfer between the NEAT domain-containing hemophore IsdX1 and cell wall-anchored IsdC of \textit{Bacillus anthracis} was observed to be biphasic and slightly slower than seen here (fast phase of 13 s\textsuperscript{-1}) but once again was at least 10,000-fold faster than the observed off-rate (108). The mechanism of inter-NEAT domain heme transfer is not yet known; however, in the case of IsdB\textsuperscript{N2}, Met362 may act to pull the heme-iron away from Tyr440, weakening the bond and thus facilitating transfer to IsdA. The observed bond-lengthening between Tyr440 and the heme-iron in chains A and D, where Met362 mainly coordinates, supports this hypothesis.

A comparison with the streptococcal protein Shp again reveals surprising parallels. As previously mentioned, Shp uses two methionines for heme-iron coordination, namely Met66 and
Met153, located in structurally analogous positions to IsdB$^{N2}$ Met362 and Tyr440, respectively (101). Mutation of the Met66 or Met153 heme-iron ligand to Ala was found to have dramatically different effects on heme binding and kinetic parameters of the protein (109). A M66A variant caused minimal change in heme dissociation constant (22 µM) compared with the wild-type protein (22 µM); conversely, mutation of the other heme-iron ligand, Met153, resulted in a 3-fold increase in heme dissociation constant (62 µM) (109). An investigation of the effect of Met153 and Met66 mutation on transfer rates to streptococcal HtsA, the cognate lipoprotein receptor for Shp-heme, also revealed striking differences between the two variants. Whereas mutation of Met66 to Ala resulted in a 7.5-fold decrease in the rate-limiting transfer step (0.4 s$^{-1}$, versus 2.9 s$^{-1}$ for wild-type), mutation of Met153 to Ala resulted in very little change in the rate-limiting transfer step (2.5 s$^{-1}$) (109). Parallels between IsdB$^{N2}$ and Shp reveal that abolishing a heme-iron ligand can have highly variable effects on the heme binding and transfer characteristics of the protein.

Nonetheless, the extent of the effects of the loss of Met362 as a heme iron ligand in IsdB is likely not yet fully characterized. As ferric iron (Fe$^{3+}$) displays a preference for the phenolate of Tyr and ferrous iron (Fe$^{2+}$) prefers Met or His, possessing both Met and Tyr heme-iron ligands may provide IsdB the flexibility to bind heme-iron in both oxidation states. For instance, S. aureus secretes hemolysins during infection which lyse red blood cells, releasing the stored Hb in a reduced state (110). Hb heme-iron quickly oxidizes in the bloodstream and is subsequently lost from Hb at an accelerated rate (110). If heme-iron is not completely oxidized, being able to bind ferrous heme-iron through Met362 may be advantageous for S. aureus. Also possible is that Met362 plays a role in heme extraction from Hb; however, I have found that mixing IsdB$^{N2}$ with
Hb did not result in observable heme transfer (data not shown), which is consistent with published observations that IsdB$_{N2}$ alone does not interact with Hb (111).

Though IsdB$_{N2}$ and IsdH$_{N3}$ (the heme-binding NEAT domain of IsdH) share 56% sequence identity (59) and both IsdB and IsdH are known to bind Hb (53,54,67), only the loss of IsdB significantly hampers the cells’ utilization of Hb as a sole iron source (54). Furthermore, a solution of IsdH$_{N1}$ (Hb binding NEAT domain) and IsdH$_{N3}$ takes up heme from Hb at a rate slightly faster than heme dissociation from Hb, 11 h$^{-1}$ (67), whereas full-length IsdB takes up heme from Hb ~100 times faster, at a rate of 0.31 s$^{-1}$ (51). While regions outside the individual NEAT domains may be required for efficient heme extraction by IsdH, it is also possible that the unique Met362 residue somehow participates in Hb recognition or heme extraction and expedites the process. A M362L variant of full-length IsdB is thus planned in order to explore this possibility.

A binding stoichiometry of 1:1 for IsdB$_{N2}$ and IsdH$_{N3}$ was estimated from reconstituted samples by using MCD (105). Similar stoichiometry would be expected based on the high sequence identity (56%) and similar folds (rmsd of 0.77 Å over 110 Cα atoms) of IsdB$_{N2}$ and IsdH$_{N3}$ and binding is 1:1 in both crystal structures (64). In contrast to IsdB$_{N2}$, a heme titration experiment with IsdH$_{N3}$ indicated a molar ratio of 4:1 leading to a model of heme stacking in the active site to provide some biological advantage (64), although it is unclear at this time what that may be, as each NEAT domain heme pocket is built to coordinate one heme at a time. An investigation of transfer kinetics between IsdH$_{N3}$ and IsdA or IsdC may help to resolve this question.

A comparison of spectroscopic and kinetic results between full-length IsdB and IsdB$_{N2}$ reveals highly similar heme-binding and transfer characteristics. Zhu et al. were able to
determine an off-rate of $1.3 \times 10^{-3} \text{s}^{-1}$ for recombinant full-length IsdB by heme transfer to apoMb, close to our value of $4.8 \times 10^{-3} \text{s}^{-1}$ (51). Heme transfer between IsdB and IsdA also appears to be closely mirrored by the NEAT domains alone. A solution of 3 μM full-length holo IsdB transferred heme to a solution of 30 μM full-length apo IsdA at a rate of 114 s$^{-1}$ (51); whereas using 2 μM holo IsdB$^{N2}$, I observed a transfer rate to 30 μM apo IsdA$^{N1}$ of ~50 s$^{-1}$. Since the buffers, temperatures and protein concentrations of the experiments differ, these rates are not directly comparable; nonetheless, they are of the same order of magnitude supporting the hypothesis that NEAT domains alone are sufficient for heme transfer. Finally, the close similarity in heme transfer rate from IsdB to IsdA, and IsdB$^{N2}$ to IsdA$^{N1}$, demonstrates the likelihood that the unusual Tyr-Met heme-iron coordination observed in the NEAT domain crystal structure is recapitulated in the full-length IsdB protein.

In summary, I have defined the structural basis for heme binding by IsdB. The second NEAT domain of IsdB is structurally similar to other heme binding NEAT domains and heme-iron is coordinated by a tyrosine residue as predicted; however, I have also shown that Met362 coordinates the heme-iron on the distal side, resulting in an unprecedented mode of heme-iron coordination. I also demonstrated that Met362 plays a role in heme trafficking into and out of the heme pocket, but is dispensable for stable heme binding. Lastly, I propose that the unusual nature of the heme-iron coordination suggests the possibility of a new functional role for IsdB$^{N2}$, perhaps in heme extraction from Hb.
Chapter 4: Hemoglobin binding and catalytic heme extraction by IsdB NEAT domains

4.1 Introduction

The role of IsdB in the S. aureus Isd system 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 (51,52,54). IsdH is also able to bind Hb, but does not appear to be dominant for the use of Hb as an iron source during infection (54).

IsdB-N1 shares 46% and 65% amino acid sequence identity with the IsdH-N1 and IsdH-N2 domains, respectively, and IsdB-N2 shares 56% identity with IsdH-N3 (59). Furthermore, both IsdB-N2 and IsdH-N3 have been shown to bind heme (67,83) and isolated IsdH-N1 and IsdH-N2 were shown to bind Hb as isolated domains (53,67-69). A motif rich in aromatic residues that is conserved between IsdB-N1, IsdH-N1 and IsdH-N2 was shown to be required for Hb binding by IsdH-N1 (67) and is part of the binding interface between αHb and IsdH-N1 (68). Moreover, IsdH-N2 and IsdH-N3 must be present in a contiguous unit with the linker region intact to perform rapid heme extraction from metHb (69).

In contrast, little is understood about IsdB-Hb interactions beyond the observation that full-length IsdB does bind Hb (50,51,53) and remove heme from metHb (51). Owing to the high sequence identity between IsdB and IsdH domains, the presence of the conserved aromatic motif and their apparent similarity in overall function, the function of IsdB-N1 has been speculated to bind Hb in a fashion analogous to IsdH-N1. Using a series of recombinant IsdB constructs which included at least one NEAT domain (Figure 4-1), I demonstrated that both domains are required to bind Hb with high affinity ($K_D = 0.42 \pm 0.05 \mu M$) and to extract heme from Hb. Moreover, IsdB only extracted heme 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 heme transfer from metHb to IsdA with a $K_m$ for metHb of $0.75 \pm 0.07 \mu N$ and a $k_{cat}$ of $0.22 \pm 0.01 s^{-1}$. The latter is consistent with the transfer of heme from metHb to IsdB as 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 carried out, representing all known Hb-heme uptake functions of the full-length IsdB protein.

Figure 4-1. Sequence features of Isd surface proteins 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. High sequence identity between NEAT domains is indicated by similar NEAT domain coloring.
4.2 Results

4.2.1 Hb purification

Hb was purified from blood graciously provided by a donor. Spectra of prepared 5 µN Hb in varying states are shown in Figure 4-2 and show the typical spectral features associated with these forms of Hb. OxyHb was exclusively found in tetrameric form (~63 kDa), and metHb was a mixture of tetrameric and dimeric forms, consistent with its higher tetramer-dimer dissociation constant (Figure 4-3). Due to the multiple potential oligomerization states, for clarity, hemoglobin concentrations are reported on a heme basis and thus by normality rather than molarity.

Figure 4-2. Absorption spectra of oxyHb, metHb and HbCO. (A) The major Soret peak wavelength (5 µN of each by heme) differs according to the heme-iron ligand: O2 for oxyHb (415 nm), CO for HbCO (419) or H2O for metHb (406 nm). (B) The wavelength of the α and β bands for oxyHb are at 542 and 577 nm, for HbCO the peaks are at 540 and 569 nm; and metHb lacks significant features in this region.
Figure 4-3. SEC-MALS plots showing the oligomerization states of Hb. (A) The tetramer of oxyHb at 63 kDa (64 kDa predicted) and (B) a mixture of dimers and tetramers of metHb at an average molecular weight of 65 kDa at the peak and 47 kDa on the peak shoulder.

4.2.2 Regions of IsdB required to bind Hb

Recombinant IsdB constructs (Figure 4-1) containing a NEAT domain or a NEAT domain plus the interdomain linker (L) were tested for Hb binding. I used the well-known property of Hb to bind to Ni-NTA beads (112) 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 pulled down by either oxyHb or metHb (Figure 4-4). Interestingly, this result 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 less IsdB$^{N1N2}$ pulled down, suggesting that the affinity of the interaction is decreased possibly due to a conformational change that occurs in IsdB upon heme binding (Figure 4-5A).
Figure 4-4. Hemoglobin pulldown assay of IsdB constructs.
20 μN oxyHb (O) or metHb (M) was immobilized on nickel beads, followed by incubation with 20 μM IsdB. Bound protein was eluted with buffer + 500 mM imidazole. The positive controls (+) show Hb binding to the beads in the absence of IsdB. The negative controls (-) show 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\textsuperscript{L-N2}, in which the reference is to the right of the ladder).
Figure 4-5. Nickel bead pulldown assays of oxyHb or metHb and IsdB constructs. 
(A and D) Hb was immobilized to the nickel beads. (B and C) His₆-tagged IsdB constructs were immobilized to the nickel beads. OxyHb is signified by (O); metHb by (M).
(A) Hb pulldowns of 20 μM holo IsdB(NO₂); much less IsdB(NO₂) is pulled down than in Figure 4-4, where IsdB(NO₂) is in the apo state.
(B) His₆-tagged IsdB(NO₂) (lanes 1, 6, 7) and His₆-tagged IsdB(N1-L) (lanes 2, 8-12) pulldowns of Hb. Lanes 1-2: His₆ IsdB(NO₂) and IsdB(N1-L) bind nickel beads alone. Lanes 3-5: oxyHb, metHb and IsdB(NO₂) cannot bind nickel beads alone. Lanes 6-7: His₆ IsdB(NO₂) pulls down both oxyHb and metHb. Lane 8: His₆ IsdB(N1-L) does not pull down IsdB(NO₂). Lanes 9-10: His₆ IsdB(N1-L) pulls down both oxyHb and metHb. Lanes 11-12: His₆ IsdB(N1-L) pulls down some IsdB(NO₂) in the presence of oxyHb, and much more IsdB(NO₂) in the presence of metHb. Lane 13: 1 μg of IsdB(NO₂) for reference.
(C) His₆ IsdB(L-N2) pulldown of Hb. Lane 1: His₆ IsdB(L-N2) binds nickel beads alone. Lane 2: IsdB(N1) does not bind nickel beads alone. Lanes 3-4: His₆ IsdB(L-N2) does not pull down oxyHb, but pulls down a small amount of metHb. Lanes 5-6: His₆ IsdB(L-N2) does not pull down IsdB(N1) in the presence of Hb. IsdB(N1) runs at approximately the 15 kDa mark, for reference.
(D) Hb pulldowns of IsdB(N1)/IsdB(N1-L) and IsdB(NO₂). Lanes 1-2: Hb does not pull down a combination of IsdB(N1) and IsdB(NO₂). Lanes 3-4: Hb pulls down a small amount of IsdB(N1-L) when in combination with IsdB(NO₂).
Thermodynamics of Hb binding by IsdB constructs was characterized by ITC. Titration of IsdB\textsuperscript{N1N2} into HbCO resulted in an exothermic reaction (similarly to oxyHb, IsdB\textsuperscript{N1N2} does not take up heme from HbCO) (Figure 4-6A). Analysis of the data with a single site binding model indicated high-affinity binding ($K_D = 420 \pm 50$ nM; $\Delta H: -6.5 \pm 0.2$ kcal mol\textsuperscript{-1}; $\Delta S: 7.5 \pm 0.8$ cal mol\textsuperscript{-1} K\textsuperscript{-1}; average of 3 runs). The stoichiometry of binding was $0.76 \pm 0.02$, which is equivalent to approximately three IsdB\textsuperscript{N1N2} molecules per HbCO tetramer. Similar results were obtained for the opposite reaction, in which HbCO was titrated into IsdB\textsuperscript{N1N2}, including the stoichiometry. Note that Hb possesses two unequal subunits and IsdB\textsuperscript{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 $K_D = 50$ nM was reported for full-length IsdB with Hb from an unidentified source using surface plasmon resonance (53).

Under the same conditions and concentrations of IsdB\textsuperscript{N1} (Figure 4-6C), IsdB\textsuperscript{N1-L} (Figure 4-7A), or IsdB\textsuperscript{N2} (Figure 4-7B) no interaction could be observed with HbCO by ITC, confirming the lack of a high affinity interaction observed in the pulldown reactions. When the concentrations of reactants were significantly increased, no interaction between IsdB\textsuperscript{L-N2} and HbCO was observed (Figure 4-6D); however, a lower affinity interaction between IsdB\textsuperscript{N1-L} and HbCO was observed (Figure 4-6B). The heats of titration did not approach zero over the course of the experiment indicating that saturation of HbCO by IsdB\textsuperscript{N1-L} did not occur. Thus, the fit to a single-site binding model was poor but gave an estimated $K_D \sim 130$ µM (average of two runs), approximately 300-fold weaker than the interaction between IsdB\textsuperscript{N1N2} and HbCO.
Figure 4-6. Representative ITC data for titration of IsdB constructs into HbCO. (A) 250 μM IsdB^{N1N2} into 25 μN HbCO; (B) 700 μM IsdB^{N1-L} into 70 μN HbCO; (C) 250 μM IsdB^{N1} into 25 μN HbCO; (D) 700 μM IsdB^{L-N2} into 70 μN HbCO. 20 x 2 μL injections at 180 s intervals in 20 mM NaH_2PO_4 (pH 7.4), 50 mM NaCl at 25 °C.
4.2.3 *IsdB*<sup>N1N2</sup> cannot remove heme from oxyHb, but removes all heme groups from metHb

Using the same panel of *IsdB* constructs, the overall competency in heme extraction from Hb was investigated by electronic spectroscopy, exploiting the large visible spectral differences between Hb and heme-bound *IsdB* (51). Initially, heme uptake from oxyHb was assayed; however, when *IsdB*<sup>N1N2</sup> (or any *IsdB* construct) was mixed with oxyHb, minimal spectral changes were observed after 60 minutes, indicating no significant heme transfer (Figure 4-8). 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 (26,28). Given that *IsdH* can bind haptoglobin and both *IsdH* and *IsdB* can bind haptoglobin:Hb, 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 (113). Altogether, metHb is likely the most physiologically relevant Hb heme source for S. aureus during human colonization or infection.

Figure 4-8. Electronic spectra of oxyHb mixed with IsdB^{N1N2}. 4 μN oxyHb was mixed with 5 μM IsdB^{N1N2}. The spectra did not change from that of oxyHb over 60 minutes incubation indicating that negligible heme extraction by IsdB^{N1N2} occurred.

When an excess of IsdB^{N1N2} was mixed with metHb, the visible spectrum resembled that of the holo-IsdB, which binds heme through the IsdB-N2 domain in a 1:1 stoichiometry; the IsdB-N1 domain does not bind heme (83). Indeed, the spectrum overlayed well with that of IsdB^{N1N2} reconstituted with the same amount of heme, indicating that quantitative heme removal from Hb occurred (Figure 4-9A). Heme transfer to IsdB^{N1N2} occurred within the sample mixing and spectra collection time (< 20 s). When IsdB^{N1-L}+IsdB^{N2} were mixed with metHb, an immediate spectral shift to the holo-IsdB spectrum was also observed (Figure 4-9B). When IsdB^{N1}+IsdB^{N2} or IsdB^{N1}+IsdB^{L-N2} were mixed with metHb, slow spectral changes were observed, estimated to be at approximately equivalent to the heme off-rate for metHb (Figure 4-9C,D). Thus, both IsdB NEAT domains and the linker region were all required for rapid heme uptake from metHb. Also, connectivity between IsdB^{N1} and the linker region was essential for rapid heme transfer to IsdB^{N2}, as the same domains are present in IsdB^{N1}+IsdB^{L-N2} but rapid heme uptake did not occur.
Figure 4-9. Electronic absorption spectra of metHb mixed with IsdB constructs. 2 μN metHb was mixed with 20 μM of IsdB constructs (as indicated). A decrease in the Soret band is associated with heme transfer. Heme-reconstituted IsdB apoprotein, diluted to 2 μM by heme concentration, is shown for comparison.

High affinity binding by IsdB^{N1-L} or IsdB^{N2} to Hb was not essential for rapid heme uptake under these conditions. To determine if IsdB^{N1-L} and IsdB^{N2} could form a complex in the presence of Hb, reconstituting the IsdB^{N1N2} protein, further pulldown assays were employed. Hb did not pull down either IsdB^{N1} or IsdB^{N2} when presented with both (Figure 4-5D, lanes 1-2); but Hb did pull down some IsdB^{N1-L} in the presence of IsdB^{N2} (Figure 4-5D, lanes 3-4). In the reverse pulldown, immobilized His_{6}-IsdB^{N1-L} did not pull down IsdB^{N2} (Figure 4-5B, lane 8). However, in the presence of Hb, His_{6}-IsdB^{N1-L} did pull down Hb as well as IsdB^{N2} when presented together (Figure 4-5B, lanes 11-12). Conversely, His_{6}-IsdB^{L-N2} did not pull down IsdB^{N1} in the presence of Hb (Figure 4-5C, lanes 5-6).
4.2.4 IsdB\textsuperscript{N1N2} is a catalyst for heme transfer between metHb and IsdA\textsuperscript{N1}

Heme extraction from Hb at the cell surface was modeled by incubating a catalytic amount of IsdB construct in the presence of excess Hb and IsdA\textsuperscript{N1}, the sole NEAT domain of IsdA. The steady state rate of heme transfer by IsdB\textsuperscript{N1N2} from metHb to IsdA\textsuperscript{N1} was monitored using visible electronic spectroscopy (Figure 4-10A). IsdB\textsuperscript{N1N2} catalyzed complete heme transfer from metHb, as judged by comparison of the final spectrum to that of IsdA\textsuperscript{N1} loaded with a similar amount of heme. As a control, the same experiment but without IsdA\textsuperscript{N1} resulted in no change to the spectrum of metHb (Figure 4-11A). Also, the absence of IsdB\textsuperscript{N1N2} resulted in slow heme transfer to IsdA\textsuperscript{N1}, consistent with solution-based heme transfer occurring at the metHb heme off-rate (Figure 4-11B). Furthermore, the spectra of IsdB and IsdA when bound to heme are sufficiently different (Figure 4-11C) that heme transfer kinetics between these proteins were defined previously (83,114). Kinetics of the IsdB\textsuperscript{N1N2}-mediated heme transfer reaction were followed at a single wavelength (408 nm) for one minute under steady-state conditions at 25 °C. The initial velocities obtained were linearly dependent on IsdB\textsuperscript{N1N2} concentration and independent of IsdA\textsuperscript{N1} concentration. Reactions were performed with varying metHb concentration and the data were fit by 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\textsuperscript{-1} (Figure 4-10B).
Figure 4-10. Steady-state transfer of heme from metHb to IsdA\textsuperscript{N1} by IsdB\textsuperscript{N1N2}.
(A) Large-scale spectral changes consistent with heme transfer occur when a catalytic amount of IsdB\textsuperscript{N1N2} (50 nM) was mixed with 2 µM metHb and an excess of acceptor IsdA\textsuperscript{N1} (50 µM). Heme-reconstituted IsdA\textsuperscript{N1}, diluted to 2 µM by heme concentration, is shown for comparison. (B) Plot of initial heme transfer rates from metHb to IsdA\textsuperscript{N1} via a catalytic amount of IsdB\textsuperscript{N1N2} (50 nM) as a function of metHb concentration. The curve is a fit of Michaelis-Menten kinetics.
Figure 4-11. Control experiments for the metHb-IsdB-IsdA steady state reaction. 50 nM IsdB$^{N1N2}$, 50 µM IsdA$^{N1}$, 2 µM metHb in 20 mM HEPES pH 7.4, 80 mM NaCl, at 25 °C. (A) In the absence of the heme acceptor, IsdA$^{N1}$, the catalytic amount of IsdB$^{N1N2}$ present did not result in observable spectral changes characteristic of heme transfer. (B) In the absence of catalytic amounts of IsdB$^{N1N2}$, the heme acceptor IsdA$^{N1}$ removed heme from metHb slowly through solution-based transfer, as IsdA$^{N1}$ is unable to bind Hb. (C) Reference spectra of heme-reconstituted IsdB$^{N1N2}$ and IsdA$^{N1}$. 
The combination of catalytic amounts of IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} did not catalyze rapid heme transfer from metHb to IsdA\textsuperscript{N1}. This result is in contrast to the observed extraction of heme from metHb by the same combination of IsdB fragments at excess concentration (Figure 4-9B). Possibly, a ternary complex is formed when heme is relayed from metHb to IsdA\textsuperscript{N1} through IsdB\textsuperscript{N1N2} which is not formed for the “cleaved” polypeptide. Alternatively, the reduced affinity of IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{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\textsuperscript{N1N2}, IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} are unable to perform multiple turnovers of heme extraction from metHb.

This latter hypothesis was investigated by testing the potential of varying concentrations of IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} to carry out heme uptake from metHb. Heme transfer from metHb (2 \( \mu \)M) to IsdB\textsuperscript{N2} (20 \( \mu \)M) was assayed by electronic spectroscopy in the presence of 0.2 to 20 \( \mu \)M of IsdB\textsuperscript{N1-L} (Figure 4-12). Only in the presence of equimolar (2 \( \mu \)M) or greater IsdB\textsuperscript{N1-L} was near-complete heme transfer from metHb observed. With a 10-fold lower amount of IsdB\textsuperscript{N1-L}, 0.2 \( \mu \)M, less than half of the metHb heme was transferred to IsdB\textsuperscript{N2} after 5 min. Under these conditions, IsdB\textsuperscript{N1-L} alone is unable to act as a partner for heme uptake from metHb to IsdB\textsuperscript{N2}. Therefore, under the conditions of the steady-state experiment, the combination of IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} was not fully catalytic. An explanation for the limited turnover in the IsdB\textsuperscript{N1-L}+IsdB\textsuperscript{N2} reaction may be that once heme extraction from metHb has occurred, IsdB\textsuperscript{N1-L} and IsdB\textsuperscript{N2} are bound together in a non-productive complex. This hypothesis is supported by the observation that IsdB\textsuperscript{N1-L} did not pull down IsdB\textsuperscript{N2} alone but did pull down IsdB\textsuperscript{N2} in the presence of metHb (Figure 4-5B, lanes 11-12).
Figure 4-12. Heme uptake from metHb by a combination of IsdB$^{N1-L}$ and IsdB$^{N2}$.

(A) The spectrum of 2 μN metHb alone is compared with the spectra at 5 min for the combination of IsdB$^{N1-L}$ (concentrations as indicated), 2 μN metHb and 20 μM IsdB$^{N2}$. Complete heme transfer resulted in a characteristic holo-IsdB$^{N2}$ spectrum, as observed for 20, 10 and 2 μM IsdB$^{N1-L}$. (B) The decrease in absorbance at 406 nm over time indicated heme transfer from metHb to IsdB$^{N2}$; the initial absorbance at 406 nm by metHb is given by the dots at $t=0$, on the left axis. Heme transfer using 20 or 10 μM IsdB$^{N1-L}$ was complete within mixing time. Heme transfer using 2 μM IsdB$^{N1-L}$ occurred more slowly but was complete within 60 seconds. Heme transfer using 0.2 μM IsdB$^{N1-L}$ also occurred more slowly and was incomplete within the time frame of the experiment.

4.2.5 Kinetic parameters of metHb heme uptake by IsdB$^{N1N2}$

Heme transfer from metHb to IsdB$^{N1N2}$ or IsdB$^{N1-L}$+IsdB$^{N2}$ was completed within the sample mixing and spectra collection time, approximately 20 sec. Therefore, stopped-flow spectroscopy was employed to follow the rapid changes in spectral characteristics.

Firstly, the changes over the entire spectrum (180-730 nm) were followed for 15 seconds using a photodiode array (PDA) coupled to the stopped-flow apparatus. The changes were complete within approximately 10 seconds, consistent with previous reports (51). Overall, the Soret peak height decreased in intensity and broadened over that time period (Figure 4-13A); however, for approximately the first 60 ms of the heme transfer reaction, the Soret peak intensity increased (Figure 4-13B). After 60 ms, the large-scale spectral shift associated with the complete reaction and previously observed by conventional spectroscopy occurred (Figure 4-13C).
Figure 4-13. Rapid spectral changes due to heme transfer from metHb to IsdB.
(A) Mixing of 2 μN metHb with 20 μM IsdB^{N1N2} results in a rapid shift in the electronic spectrum; the Soret peak briefly increases in intensity before decreasing. (B) A brief increase in Soret peak intensity occurs for ~60 ms, from ~350-420 nm. (C) After the initial increase, the Soret peak decreases in intensity while the peak shoulders increase in intensity.
Two wavelengths (406 and 428 nm) were chosen to undergo single-wavelength stopped-flow spectroscopy using a monochromator in order to characterize the observed changes more accurately. The heme transfer reaction was carried out under pseudo-first order conditions, with metHb held constant at 1 µN and IsdB\textsuperscript{N1N2} increasing from 5 to 40 µM. Four phases were consistently observed to occur at 406 nm, with one phase at 428 nm.

At 428 nm, a single phase was observed (Figure 4-14A). This wavelength was outside the range of wavelengths whose intensity increased in the first 60 ms (~350 – 420 nm), thus the kinetics were far simpler. The change in absorbance was plotted against time and fit by a single exponential to determine a value for the observed rate constant ($k_{obs}$). The $k_{obs}$ varied hyperbolically with IsdB\textsuperscript{N1N2} concentration, suggesting a two-step transfer mechanism (as seen for the IsdB\textsuperscript{N2} $\rightarrow$ IsdA\textsuperscript{N1} heme transfer reaction in Chapter 3) (Figure 4-14B). Again using the two-step model of heme transfer, the rate constant for heme transfer from metHb to IsdB\textsuperscript{N1N2} was 0.35 ± 0.02 s\textsuperscript{-1}.

Figure 4-14. Kinetics of heme transfer from metHb to IsdB\textsuperscript{N1N2} at 428 nm. (A) 1 µN metHb and 17 µM IsdB\textsuperscript{N1N2}; the grey bars represent the standard error of 4 replicates, and the black curve is a fit to a single exponential equation. The residuals for the experiment are inset. (B) The observed transfer rate ($k_{obs}$) varies hyperbolically with IsdB\textsuperscript{N1N2} concentration, characteristic of a two-step reaction. Each point represents the mean and standard error of 4 replicates of the 1 µN metHb to IsdB\textsuperscript{N1N2} heme transfer experiment. The residuals are inset.
At 406 nm, the kinetics were far more complex; this wavelength was in the range of wavelengths whose intensities increased in the first 60 ms. Curve fitting yielded four phases of differing amplitudes, rates and concentration dependencies (Error! Reference source not found., Figure 4-15A). The first phase rate was linearly dependent on IsdB<sub>N1N2</sub> concentration, and thus likely reflected the increased collision events in more concentrated solution. Although the rate of the first phase displayed concentration dependence, the amplitudes of all phases were entirely concentration-independent (Error! Reference source not found.). Phase 1 accounted or 13% of the reaction (judged by the total absolute change in absorbance over the course of the reaction) and the rate ranged from 11 – 108 s<sup>-1</sup> (Figure 4-15C). Phases 2-4 did not display any obvious concentration dependence of their rates. Phase 2 accounted for 17% of the change in absorbance with a rate ranging from 1.9 – 4.8 s<sup>-1</sup> (Figure 4-15D). Phase 3 described the largest absorbance change with an amplitude of 48%, with a rate ranging from 0.41 – 0.53 s<sup>-1</sup> (Figure 4-15E). Phase 4 described 22% of the absorbance change, with a rate ranging from 0.12 – 0.15 s<sup>-1</sup> (Figure 4-15F).

Table 4-1. Summary of stopped-flow spectroscopy results at 406 nm for heme transfer from metHb to IsdB<sub>N1N2</sub>. The amplitude is given as a fractional quantity. All values represent the mean and standard error of 4 replicates.

<table>
<thead>
<tr>
<th></th>
<th>5 μM IsdB&lt;sub&gt;N1N2&lt;/sub&gt;</th>
<th>10 μM IsdB&lt;sub&gt;N1N2&lt;/sub&gt;</th>
<th>17 μM IsdB&lt;sub&gt;N1N2&lt;/sub&gt;</th>
<th>25 μM IsdB&lt;sub&gt;N1N2&lt;/sub&gt;</th>
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Figure 4-15. Kinetics of heme transfer from metHb to IsdB<sup>N1N2</sup> at 406 nm. (A) Heme transfer from 1 µN metHb and 17 µM IsdB<sup>N1N2</sup>; the grey bars represent the standard error of 4 replicates. A plot of the residuals for the average minus the fit is inset. (B) A closer look at the first 1 s of the reaction. A plot of the residuals for the average minus the fit is inset. (C-F) Plots of $k_{\text{obs}}$ vs IsdB<sup>N1N2</sup> for each of the four phases of the transfer reaction, respectively. Each point represents the mean and standard error of 4 replicates of the 1 µN metHb to IsdB<sup>N1N2</sup> heme transfer experiment.

4.2.6 Crystals of the IsdB-Hb complex

Crystals of the IsdB<sup>N1N2</sup>-oxyHb complex were grown in citric acid or malonic acid, pH 5.0-5.5, and 2.1-2.4 M ammonium sulfate (Figure 4-16A). Crystals appeared after 2-4 weeks and were consistently small (~0.05-0.1 µm). Neither IsdB<sup>N1N2</sup> nor Hb crystallized alone under these conditions; the crystals were reddish, though not as strongly red as Hb crystals alone (crystallized under different conditions). Furthermore, several washed crystals were dissolved and run on an SDS-PAGE gel confirming that both components were present in the dissolved crystals (Figure 4-16B).
Figure 4.16. Crystals of the IsdB^{N1N2}-Hb complex. (A) Red crystals from a protein solution of IsdB^{N1N2} and oxyHb. (B) Five crystals from a single well were washed and dissolved in water, then run on a 15% SDS-PAGE gel. Both protein components can be seen on the stained gel, as well as a third unknown band.

X-ray diffraction data from the crystals was low resolution (highest resolution data set: 5.1 Å), likely due in part to the consistently small size of the crystals. Preliminary diffraction statistics suggest a space group of C2 with a large unit cell of 133 x 217 x 70 Å. Attempts to solve the structure by molecular replacement were unsuccessful. Optimization of the crystal growth and cryoprotection conditions is underway in order to obtain larger crystals of higher quality.

4.3 Discussion

Both IsdB NEAT domains and the intervening linker region must be present and contiguous for high affinity Hb binding (Figure 4-4, Figure 4-6). No single NEAT domain of IsdB tested was competent for Hb binding on its own, and 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 which differs from ours by the addition of only 3 amino acids (I omitted the N-terminal Leu125 and C-terminal Glu271-Asp272) (115). 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 IsdBN1 construct is likely properly folded. IsdBN1 was also found to be required for Hb binding to S. aureus cells in vivo, as cells expressing a version of IsdB lacking the NEAT1 domain could not bind Hb (74). This is consistent with our data, which conclude that IsdBN1 is essential for high-affinity Hb binding since IsdBN1, the linker region and IsdBN2 must all be present and contiguous for Hb binding to occur. Although no interaction between IsdBN1 or IsdBN1-L with Hb was demonstrated with Hb immobilized on nickel beads, immobilized His6-tagged IsdBN1-L did pull down significant amounts of Hb (Figure 4-5B) and weak binding of non-His6-tagged IsdBN1-L for HbCO could be shown by ITC (Figure 4-6B). The preferred interaction interface of Hb with nickel beads may have limited binding of IsdBN1-L.

In contrast, a single NEAT domain of IsdH, IsdH-N1, binds Hb with high affinity (68). Based on 46% amino acid sequence identity between IsdBN1 and IsdH-N1, these domains were proposed to share the same function. This difference in binding affinity may be related to the pI for IsdBN1N2 (8.5), which is markedly higher than that of the analogous construct in IsdH, IsdH-N2N3 (5.0) as calculated using the Compute pI/Mw tool on ExPASy.org (116). 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 IsdBN1, IsdH-N1 and IsdH-N2 share a conserved 5 amino acid aromatic motif (FYHYA in IsdB-N1, IsdH-N2; YYHFF in IsdH-N1) in a sequence alignment (59,67). 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 (67). The motif was also found to be intimately associated with the Hb α-chain in the crystal structure of an IsdH\textsuperscript{N1}-metHb complex (68). In the IsdB\textsuperscript{N1} NMR structure, the backbone resonances of only 10 residues could not be assigned; four of these were Phe164 – Tyr167, within the aromatic motif (115). In the determination of the NMR structure of IsdH\textsuperscript{N1}, the equivalent residues of the aromatic motif were also not assigned to resonances (53,59). Recently, a study demonstrated that mutation of residues in the IsdB aromatic motif abrogated binding of Hb to \textit{S. aureus} cells, and slowed the rate of heme transfer from metHb to IsdB \textit{in vitro} (74). Also, swapping out the IsdB\textsuperscript{N1} aromatic motif for the IsdH\textsuperscript{N1} aromatic motif resulted in cells that were unable to bind Hb, and IsdB with the chimeric aromatic motif was significantly impaired at removing heme from metHb (74). 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 Hb binding to the N-terminal (IsdB\textsubscript{N}) and C-terminal (IsdB\textsubscript{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\textsuperscript{N1} within this construct (111). Since the IsdB\textsubscript{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\textsuperscript{N1} construct, the extra ~125 N-terminal residues may contribute to Hb-binding. Thus, the region N-terminal to IsdB-N1 may be involved in binding to Hb, but appears to be dispensable for high-affinity Hb binding and heme extraction by IsdB\textsuperscript{N1N2}.

Though ITC was unable to provide an unambiguous stoichiometry of the interaction between IsdB\textsuperscript{N1N2} and Hb, based on the ability to remove all four heme groups from tetrameric Hb, IsdB\textsuperscript{N1N2} could interact with both α and β chains. IsdH\textsuperscript{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 (68). 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 4-7C). Thus, the interaction with α and β chains decreases apparent stoichiometry for IsdH and potentially, the observed stoichiometry of IsdB N1N2 may differ when 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, was recently shown to quantitatively remove heme from metHb as well (70). IsdH N2N3 bears 64% amino acid sequence identity to IsdB N1N2 over the entire 333 amino acid construct, with no insertions or deletions (69). A crystal structure of IsdH N2N3 in complex with tetrameric metHb clearly demonstrates that a IsdH N2 domain interacts with each of the α and β Hb chains (70).

Although the entire IsdB N1N2 polypeptide was required to be contiguous for high affinity binding to metHb, rapid heme extraction from metHb did not display the same requirement. Both IsdB N1N2 and the mixture of IsdB N1-L+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 (51). Rapid heme uptake by the mixture of IsdB N1-L+IsdB N2 contrasts with findings for the analogous construct in IsdH. IsdH N2N3 could rapidly extract heme from metHb, but IsdH N2-L+IsdB N3 was unable to perform rapid heme extraction (69). The ability of IsdB N1-L+IsdB N2 to perform rapid heme extraction 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+IsdB N2 did not perform heme relay from metHb to IsdA N1. Furthermore, IsdB N1-L did not
perform multiple turnovers of heme transfer 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 which acts as a rigid spacer holding the NEAT domains ~40 Å apart, resulting in a rigid, elongated dumbbell shape (69). As the IsdB and IsdH linker regions share 70% amino acid sequence identity and give similar circular dichroism spectra (69), IsdB likely also shares this overall shape. The crystal structure of IsdH^{N2N3}:metHb revealed that IsdH^{N2} interacted closely with the Hb chains as previously shown (68) and that the linker region and IsdH^{N3} did not interact with Hb, although IsdH^{N3} was poised directly over the Hb heme pockets (70). The domains of IsdH (N2, linker, N3) fold independently and do not make extensive interdomain interactions (69,117). Similar NMR data is not available for IsdB, and interdomain interactions may differ, which may explain the requirement of all three IsdB domains (N1, linker, N2) for hemoglobin binding and heme uptake 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 heme extraction 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 a rate of heme transfer observed from metHb to full-length IsdB (0.3 s\(^{-1}\)) (51) and it is conceivable that increasing the temperature of the reaction to human body temperature, 37 C, would increase the transfer rates further. The rate of the overall heme transfer reaction from metHb to IsdA^{N1} through IsdB^{N1N2} is > 400-fold slower than the rate of heme transfer from IsdB^{N2} to IsdA^{N1} (82 s\(^{-1}\)) (83) indicating that the heme extraction process from metHb is rate-limiting for the overall heme transfer reaction. IsdB^{N1N2} has a specificity constant ($k_{cat}/K_m$) of $3.1 \pm 0.2 \times 10^5$ M\(^{-1}\) s\(^{-1}\), comparable to 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 between metHb, IsdB$^{NIN2}$ and IsdA$^{N1}$. IsdB and IsdA are colocalized on the cell wall and can be co-immunoprecipitated \textit{in vivo} (118), but efforts to show an interaction with recombinant protein \textit{in vitro} have not been successful. Noteworthy is 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 $\mu$M (73), three times the measured $K_m$ for the metHb in the heme transfer reaction, suggesting that IsdB on the \textit{S. aureus} cell surface could be greater 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.

The kinetics of the heme transfer reaction from metHb to IsdB$^{NIN2}$ were examined at two wavelengths, 406 nm (within the Soret peak) and 428 nm (Soret peak shoulder). The number of phases present, as well as their rates, differed between the two wavelengths; at 406 nm, there were four phases, whereas 428 nm displayed only one phase. This suggests that at some wavelengths, intermediates in the heme transfer complex are observable. The single phase observed at 428 nm with a rate constant of 0.35 s$^{-1}$ closely matched both the $k_{cat}$ for the overall heme transfer reaction described above (0.22 s$^{-1}$), as well as the rate of heme transfer from metHb to full-length IsdB previously described, 0.3 s$^{-1}$ (51). However, at 406 nm, our data differed considerably from the published data for metHb and full-length IsdB; whereas a single phase of 0.3 s$^{-1}$ was seen at 406 nm, I observed four distinct phases at the same wavelength. A concentration-dependent phase ~36 – 360-fold faster than the published rate of 0.3 s$^{-1}$ was consistently observed to occur first, followed by two concentration-independent phases which were also faster than the published rate. Curiously, the fourth phase was actually slower (0.13 s$^{-1}$)
than the $k_{\text{cat}}$ for IsdB$^{N1N2}$ catalyzing heme transfer from metHb to IsdA$^{N1}$, 0.22 s$^{-1}$. Ordinarily, the $k_{\text{cat}}$ describes the rate-limiting step for the reaction; no step in the transfer process should be slower than $k_{\text{cat}}$. Phase 4 therefore may represent an internal rearrangement of heme coordination within the IsdB heme binding site (for instance, equilibrium heme-iron coordination by Tyr440 and Met362) which is necessary for stable heme binding, but dispensable for heme relay. The extra phases observed at 406 nm may be a result of the use of a truncated construct rather than a construct comprising the entire mature IsdB protein (residues 40-613), which interacts with metHb slightly differently; however, in spite of these differences, the total time for the completion of the reaction was approximately 10 seconds, the same as previously published.

In summary, I 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 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 present in the IsdB$^{N1N2}$ polypeptide. The linker region in IsdB proved to be essential for 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 rapid heme transfer to IsdB$^{N2}$, although with one or few turnovers. Interestingly, addition of the linker region to IsdB$^{N2}$ resulted in a non-functional 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 heme loss to IsdB$^{N2}$. Furthermore, although IsdB can bind oxyHb, metHb and HbCO, only metHb serves as a substrate for heme extraction. The interaction between IsdB$^{N1N2}$ and oxyHb is sufficiently stable
that crystals can be produced. Lastly, IsdB\textsuperscript{N1N2} can serve as a catalyst for a heme transfer reaction that reconstitutes metHb, IsdB\textsuperscript{N1N2} and IsdA\textsuperscript{N1} under physiologically relevant infection conditions. The observed $K_m$ of IsdB\textsuperscript{N1N2} for metHb provides a mechanism for \textit{S. aureus} colonization in such an iron-restricted environment as the human body.
Chapter 5: Perturbing the metHb-IsdB\textsuperscript{N1N2} heme transfer reaction

5.1 Introduction

Heme extraction from Hb by IsdB can be considered as a two-component reaction. However, on the surface of \textit{S. aureus} during human infection, IsdB and Hb would not be the only macromolecules present. Other proteins may have an impact on IsdB function. Firstly, IsdH is also known to interact with Hb. However, the interplay between IsdB and IsdH in terms of Hb binding and heme extraction is completely unknown; they may be competitors for Hb binding and heme extraction, or act synergistically, or have no impact on each other. More IsdB than IsdH is likely to be on the surface of the cell (41,72), but IsdH\textsuperscript{N1} has been reported to have a four-fold higher affinity for Hb (68) than IsdB\textsuperscript{N1N2}. Hp is present in concentrations of at least 10 mg/mL (34) in the blood and has an affinity for dimeric Hb that has been described as “immeasurably strong” with an estimated dissociation constant of $10^{-15}$ M (119). Therefore, metHb present in the bloodstream is likely to exist as a complex with Hp; while both IsdB and IsdH bind Hb-Hp complexes with low nanomolar affinity (53), the impact of Hp on heme uptake from Hb has not been described.

I investigated the effects of IsdH\textsuperscript{N1} and Hp, separately and together, on heme uptake from metHb by IsdB\textsuperscript{N1N2}. I found that IsdB\textsuperscript{N1N2} was capable of binding Hb-Hp complexes, but the presence of Hp blocked heme uptake from metHb by IsdB\textsuperscript{N1N2}. The presence of IsdH\textsuperscript{N1}, either alone or in addition to Hp, did not appear to have an effect on heme uptake by IsdB\textsuperscript{N1N2}.

Lastly, two of the same heme pocket point variants characterized in the previous IsdB\textsuperscript{N2} study, Y440A and M362L, were produced in the IsdB\textsuperscript{N1N2} construct. I hypothesized that such constructs would bind metHb but be defective in heme transfer, thus trapping the metHb-IsdB\textsuperscript{N1N2} complex for crystallization. However, IsdB\textsuperscript{N1N2} Y440A was defective not only in
performing heme uptake from metHb, but also in Hb binding. Characterization of IsdB\textsubscript{N1N2 Y440A} revealed unforeseen differences between equivalent mutations in the IsdB\textsubscript{N2} and IsdB\textsubscript{N1N2} recombinant proteins which raise the possibility of interdomain interactions that play a role in how heme and Hb are bound by IsdB.

5.2 Results

5.2.1 IsdB\textsuperscript{N1N2} heme pocket variants

Two single point variants at Tyr440 and Met362 were created with site-directed mutagenesis: IsdB\textsuperscript{N1N2 Y440A} and IsdB\textsuperscript{N1N2 M362L}. These residues coordinate to the iron of heme bound to IsdB\textsuperscript{N2} (Chapter 3). Apo and holo IsdB\textsuperscript{N1N2} protein elute separately while being run over a nickel column during recombinant protein purification from \textit{E. coli} lysate; the major proportion of recombinant protein for each variant eluted with some Soret absorbance (~ A\textsubscript{405 nm}), indicating that both variants could bind and retain heme during the purification process. However, when additional heme was added to purified apo IsdB\textsuperscript{N1N2 M362L}, or when the protein was concentrated above ~ 5 mg/mL, the protein precipitated. Purified apo IsdB\textsuperscript{N1N2 Y440A} was stable upon concentrating and upon addition of exogenous heme; therefore, based on the requirement for protein which was stable in solution, the characterization of only the IsdB\textsuperscript{N1N2 Y440A} variant was pursued.

5.2.2 IsdB\textsuperscript{N1N2 Y440A} heme binding characteristics

The visible absorption spectrum of IsdB\textsuperscript{N1N2 Y440A} reconstituted with equimolar hemin was compared to that of IsdB\textsuperscript{N2 Y440A} and wild-type IsdB\textsuperscript{N2}, as the spectrum is indicative of the heme environment. The heme-reconstituted spectrum of IsdB\textsuperscript{N1N2 Y440A} was significantly different from that of wild-type, with the shape, height and maximum wavelength of the Soret peak all affected,
indicating a significant change in the environment experienced by the heme (Figure 5-1A).

Interestingly, the spectrum of IsdB$^{N1N2 \ Y440A}$ was also significantly different from that of IsdB$^{N2 \ Y440A}$, which more closely resembled the spectrum of free heme in buffer at equimolar heme and protein concentrations (Figure 3-7). This suggested that IsdB$^{N1N2 \ Y440A}$ may bind heme more tightly than IsdB$^{N2 \ Y440A}$. To address this possibility, apo-IsdB$^{N1N2 \ Y440A}$ was titrated with increasing amounts of heme and difference spectra were generated by subtracting spectra from buffer titrated with the same amounts of heme. A slight plateauing of the absorption difference at ~ 1:1 stoichiometry occurred (Figure 5-1B), unlike the results for an equivalent titration of IsdB$^{N2 \ Y440A}$ (Figure 3-6B), although the plateau was not as striking as for wild-type IsdB$^{N2}$ and IsdB$^{N2 \ M362L}$ (Figure 3-5D,E). A preliminary dissociation constant could be estimated by fitting the single-wavelength titration points with a hyperbolic curve, yielding a $K_D = 13 \pm 2 \ \mu M$.

Earlier experiments showed that IsdB$^{N2 \ Y440A}$ heme affinity is $17 \pm 1 \ \mu M$, meaning that addition of the N1 and linker domains increased the heme affinity by ~ 30%.

Figure 5-1. Electronic spectrum of the IsdB$^{N1N2 \ Y440A}$ variant with heme. (A) A comparison of the Soret peaks of 5 \mu M wild-type IsdB$^{N2}$ with 5 \mu M heme added, 5 \mu M IsdB$^{N2 \ Y440A}$ with 5 \mu M heme added, and 5 \mu M IsdB$^{N1N2 \ Y440A}$ with 5 \mu M heme added. 5 \mu M heme in buffer alone is also shown for reference. The holo spectrum of IsdB$^{N1N2}$ is the same as that of holo IsdB$^{N2}$ and was not included for clarity. (B) Titration of 5 \mu M apo-IsdB$^{N1N2 \ Y440A}$ with heme demonstrates a binding stoichiometry of ~ 1:1, as the increase in absorbance at respective Soret peaks when compared to heme alone plateaus at approximately 6 \mu M. The data represent a single titration experiment.
5.2.3 Addition of IsdB\textsuperscript{N1N2 Y440A} to metHb results in a novel electronic absorption spectrum

Interestingly, upon adding IsdB\textsuperscript{N1N2 Y440A} to metHb, the resultant spectrum was significantly red-shifted with a Soret peak maximum of 409 nm (Figure 5-2). Thus, the spectrum resembled neither that of metHb nor that of heme-bound IsdB\textsuperscript{N1N2 Y440A} suggesting that complete heme transfer did not occur. One explanation for such a phenomenon is that the resulting electronic spectrum represents an intermediate in the heme transfer process between wild-type IsdB\textsuperscript{N1N2} and metHb that has a much longer lifetime in the Y440A variant; however, this intermediate was not detected in spectra collected for the transfer reaction with IsdB\textsuperscript{N1N2} by stopped-flow (Figure 4-13).

Figure 5-2. Resultant electronic spectra of addition of IsdB\textsuperscript{N1N2 Y440A} to metHb. 2 µN metHb was added to 10 µM IsdB\textsuperscript{N1N2 Y440A}; the resultant spectral shift was immediate (< 20 s) and did not change over time. 2 µN metHb alone and 10 µM IsdB\textsuperscript{N1N2 Y440A} with an equal amount of heme added are shown for comparison.

To better characterize the unusual spectral shift, stopped-flow spectroscopy experiments with a photodiode array were performed with the variant. At a single concentration of metHb and IsdB\textsuperscript{N1N2 Y440A}, spectra were collected from 180-730 nm for a total of 20 seconds (Figure 5-3). The majority of the absorption change occurred in the first 5 seconds and was complete by 15
seconds. Overall, the Soret peak was reduced and red-shifted, as observed in the endpoint spectrum (Figure 5-3A,B). A closer look at the area around the α/β bands (500-700 nm) revealed that although there was little overall change in spectrum in this range from first to last time point, transient changes occurred (Figure 5-3C). Inspecting single wavelength data from the PDA data set revealed that numerous phases occurred within the time frame of the experiment suggesting kinetics more complex than observed for wild-type IsdB^{N1N2}. However, at 402 nm (the wavelength of greatest change, as judged by a difference spectrum between the 5 ms and 15 sec spectra), the kinetics were reasonably fit by a double exponential equation (Figure 5-3D). The $k_{\text{fast}} = 1.44 \pm 0.03$ s$^{-1}$ accounted for 74% of the change in absorbance, with $k_{\text{slow}} = 0.20 \pm 0.02$ s$^{-1}$.

Figure 5-3. Time course of spectral changes that occur upon mixing IsdB^{N1N2 Y440A} with metHb. (A) Electronic spectra after 2 μN metHb is mixed with 10 μM IsdB^{N1N2 Y440A}. (B) Expansion of the Soret peak region; the Soret peak is diminished and is red shifted. (C) Expansion of the α/β region. (D) Time course of absorption data at 402 nm, the wavelength of largest change. The grey error bars represent the standard error of 4 replicates, and the black curve is a fit to a double-exponential equation, for which the residuals are inset.
5.2.4 **IsdB^{N1N2 Y440A} does not bind Hb or binds Hb weakly**

To determine if the Y440A variant altered the binding affinity of IsdB^{N1N2} for Hb, ITC was carried out. No heats resulting from binding were observed (Figure 5-4) under conditions for which wild-type IsdB^{N1N2} and HbCO did result in a measureable interaction (Figure 4-6A). If Hb binding to variant does occur, it is with a much weaker affinity than the wild-type interaction.

![Figure 5-4](image)

Figure 5-4. ITC data for titration of IsdB^{N1N2 Y440A} into HbCO. When 250 μM IsdB^{N1N2 Y440A} was injected into 25 μN HbCO, no significant heats were observed implying no interaction. This data is representative of 3 runs performed. 20 x 2 μL injections at 180 s intervals in 20 mM NaH₂PO₄ (pH 7.4), 50 mM NaCl at 25 °C.

5.2.5 **IsdH^{N1} binding Hb does not interfere with IsdB^{N1N2} binding Hb**

At present, the IsdB^{N1N2} binding site on Hb is unknown. However, the binding site for IsdH^{N1} on αHb has been defined from a crystal structure of the complex (68). Also, the affinity of IsdH^{N1} for αHb (K_D=100 nM) (68) is approximately four-fold higher than that of IsdB^{N1N2} for
HbCO ($K_D=420$ nM). Thus, if IsdB$^{N1N2}$ and IsdH$^{N1}$ share a common binding face on Hb, the binding of IsdH$^{N1}$ by oxyHb would inhibit subsequent binding of IsdB$^{N1N2}$.

A competition assay was performed in which oxyHb was immobilized to nickel beads and pre-incubated with either 0.1, 1 or 5 molar equivalents of either IsdH$^{N1}$ or IsdB$^{N1N2}$; subsequently, one molar equivalent (to Hb in heme) of the other Isd protein was added to the mixture. Since IsdH$^{N1}$ only binds to $\alpha$Hb with high affinity (68), if IsdH$^{N1}$ is added to Hb first to completely block the $\alpha$Hb sites, only the $\beta$ subunits of Hb are expected to be available for binding by IsdB$^{N1N2}$. Although I have not directly shown that IsdB$^{N1N2}$ binds to both $\alpha$Hb and $\beta$Hb, IsdB$^{N1N2}$ removes the heme groups from both Hb chain types, suggesting interaction occurs with both (Chapter 4). Conversely, when IsdB$^{N1N2}$ is added to Hb first, if IsdB$^{N1N2}$ binds both Hb chains, and if IsdH$^{N1}$ requires those sites for binding, either no IsdH$^{N1}$ would bind (if IsdB$^{N1N2}$ remains bound to Hb) or some IsdH$^{N1}$ would be bound with less IsdB$^{N1N2}$ bound (if the superior affinity of IsdH$^{N1}$ for $\alpha$Hb displaces some IsdB$^{N1N2}$).

The results of the competition assay did not fit a simple model of binding (Figure 5-5). Pre-incubating Hb-bound beads with IsdH$^{N1}$ did indeed result in less IsdB$^{N1N2}$ being pulled down, as would be expected if they shared a common Hb-binding site (Figure 5-5, lanes 4-6); however, Hb-bound beads preincubated with IsdB$^{N1N2}$ did not result in less of either IsdH$^{N1}$ or IsdB$^{N1N2}$ being pulled down (Figure 5-5, lanes 9-11). In fact, larger quantities of IsdB$^{N1N2}$ were pulled down by Hb when IsdH$^{N1}$ was added. A subsequent Hb pulldown assay demonstrated that oxyHb could not pull down more than one molar equivalent of IsdB$^{N1N2}$ or IsdH$^{N1}$ (Figure 5-6). Furthermore, His$_6$-tagged IsdB$^{N1N2}$ could not pull down IsdH$^{N1}$ alone, confirming that the two proteins do not interact in the absence of Hb (Figure 5-6). Overall, the results suggest that
IsdB^{N1N2} and IsdH^{N1} may be able to bind Hb simultaneously, and binding of one may enhance binding of the other.

Figure 5-5. Competition pulldown between IsdH^{N1} and IsdB^{N1N2}. Hb binds to nickel beads alone (lane 1). IsdH^{N1} (lane 2) and IsdB^{N1N2} (lane 7) do not bind nickel beads alone. 20 μN oxyHb incubated with 1 molar equivalent (20 μM) IsdH^{N1} (lane 3) or IsdB^{N1N2} (lane 8) are shown for comparison. Lanes 4-6: Hb-bound beads were incubated with the indicated molar equivalents of IsdH^{N1}, then mixed with 1 molar equivalent (to Hb-heme) of IsdB^{N1N2}. Lanes 9-11: Hb-bound beads were incubated with the indicated molar equivalents of IsdB^{N1N2}, then mixed with 1 molar equivalent of IsdH^{N1}.

Figure 5-6. Hb pulldown of increasing molar equivalents of IsdB^{N1N2} and IsdH^{N1}. 20 μN oxyHb bound to nickel beads was incubated with increasing molar equivalents of IsdH^{N1} (lanes 1-5) or IsdB^{N1N2} (lanes 6-9). Alternatively, His_{6}-tagged IsdB^{N1N2} was used to pull down IsdH^{N1} (lane 10). This gel photo was taken while in a plastic carrying box resulting in the “recycle” symbol in the top half.
5.2.6 IsdH\textsuperscript{N1} does not prevent heme transfer from metHb to IsdB\textsuperscript{N1N2}

Apart from a potential effect on Hb binding by IsdB\textsuperscript{N1N2}, the presence of IsdH\textsuperscript{N1} may also impact the function of heme uptake from metHb by IsdB\textsuperscript{N1N2}. MetHb was mixed with an excess of IsdB\textsuperscript{N1N2} and varying amounts of IsdH\textsuperscript{N1} and the resulting spectra were immediately recorded (within ~20 seconds of reaction initiation), as before (Figure 5-7). Adding 1 μM IsdH\textsuperscript{N1} to 2 μN metHb (one molar equivalent of αHb) without IsdB\textsuperscript{N1N2} present did not result in any spectral changes, as previously noted by other researchers (68). When metHb was pre-incubated with 0.5 or 1 molar equivalents of IsdH\textsuperscript{N1}, upon adding excess IsdB\textsuperscript{N1N2} the spectrum changed to that of holo-IsdB\textsuperscript{N1N2} within 20 s (Figure 5-7), the same time frame as experiments in the absence of IsdH\textsuperscript{N1} (Figure 4-9A). However, when 2.5 molar equivalents of IsdH\textsuperscript{N1} were pre-incubated with metHb, the spectral changes upon addition of excess IsdB\textsuperscript{N1N2} were intermediate within the same time frame.

![Figure 5-7. Electronic spectra of the metHb+IsdB\textsuperscript{N1N2} heme transfer reaction in the presence of IsdH\textsuperscript{N1}. 2 μN metHb was pre-incubated with increasing amounts of IsdH\textsuperscript{N1} (as indicated) and then 10 μM IsdB\textsuperscript{N1N2} was added to initiate the heme transfer reaction. Spectra were recorded within 20 seconds of mixing.](image)
5.2.7 Haptoglobin prevents heme uptake from metHb by IsdB\textsuperscript{NIN2}

Human Hp is a tetrameric molecule consisting of two light chains (\(\alpha_1\) or \(\alpha_2\)) and two heavy chains (\(\beta\)); the light \(\alpha\) chains form disulfide bonds to each other and to the heavy \(\beta\) chains, resulting in a linear \(\beta-\alpha-\alpha-\beta\) complex in which only the heavy \(\beta\) chains can bind Hb dimers (120). Within the human host, normal levels of plasma Hb (less than 5 \(\mu\)N) would largely be bound by the normal levels of Hp present (around 25 \(\mu\)M).

Therefore, in an effort to investigate heme uptake by IsdB\textsuperscript{NIN2} under biologically relevant conditions, I tested the ability of IsdB\textsuperscript{NIN2} to take up heme from metHb pre-incubated with a physiologically relevant level (2 mg/mL) of mixed-serotype Hp. Electronic spectra were monitored for 5 minutes, during which time the spectra did not change from that of metHb-Hp, indicating that heme transfer to IsdB\textsuperscript{NIN2} had not occurred (Figure 5-8A). To further characterize this inhibition, metHb was pre-incubated with decreasing concentrations of Hp in order to find a minimal inhibitory concentration (Figure 5-8B). Only at Hp concentrations as low as 20 \(\mu\)g/mL (500-fold lower than serum concentrations, normally 10 mg/mL) did heme transfer proceed, and the transfer was incomplete within the time frame of the experiment (approximately 85% of heme was transferred based on \(\Delta A_{405\text{ nm}}\)). This suggests that the Hp-Hb complex is not an accessible heme source for the IsdB\textsuperscript{NIN2} construct, and IsdB\textsuperscript{NIN2} was only able to remove heme once the Hp concentration was low enough that most metHb was unbound by Hp.
Figure 5-8. Electronic spectra of Hp-metHb combined with IsdB^{N1N2}.
(A) Pre-incubating 2 μN metHb with 2 mg/mL mixed-serotype Hp results in a metHb-like spectrum even after addition of IsdB^{N1N2}; the reaction of metHb with IsdB^{N1N2} (in the absence of Hp) is shown for comparison. (B) 2 μN metHb was pre-incubated with decreasing amounts of Hp until a concentration was reached whereby IsdB^{N1N2} could successfully remove the heme from metHb. Spectra for all were recorded within 20 seconds of mixing and did not change within 5 minutes.

5.2.8  IsdB^{N1} and IsdB^{N1N2} do not interact synergistically to remove heme from the metHb-Hp complex

IsdB and IsdH can both bind Hb-Hp complexes, but IsdB cannot bind Hp alone, whereas IsdH can (53,65,67). Therefore, I reasoned that IsdH^{N1} interacting with Hp may allow IsdB^{N1N2} to perform heme uptake from the Hp-Hb complex. MetHb was pre-incubated with the minimum inhibitory concentration of Hp determined from the previous experiment, 100 μg/mL Hp, and
then varying amounts of IsdH$^N_1$ were added (from 1 to 25 μM). Finally, excess IsdB$^{N1N2}$ was added to initiate the heme transfer reaction and spectra were recorded immediately after mixing (within 20 s) and again at two and five minutes afterward. Even at the highest concentration of IsdH$^N_1$ (25 μM) no effect was observed on the ability of IsdB$^{N1N2}$ to remove heme from the Hp-Hb complex (Figure 5-9).

![Figure 5-9. Effect of IsdH$^N_1$ on electronic spectra of metHb-Hp mixed with IsdB$^{N1N2}$. 2 μN metHb was pre-incubated with 0.1 mg/mL mixed-serotype Hp, and IsdH$^N_1$ was added as indicated. Excess IsdB$^{N1N2}$ was then added to initiate the heme transfer reaction (if any) and spectra were recorded within 20 seconds of mixing. No further spectral changes occurred after 5 more minutes of monitoring.](image)

5.2.9 **IsdB$^{N1N2}$ binds the metHb-Hp complex**

A possible explanation for the inability of IsdB$^{N1N2}$ to remove heme from metHb when Hp is present is that the IsdB$^{N1N2}$ construct is unable to bind the Hb-Hp complex. This possibility was investigated by pulldown assay using 20 μM His$_6$-tagged IsdB$^{N1N2}$ as the bait protein. Sufficient Hp was included to bind 20 μN metHb, i.e. ~ 20 μM Hp (which would bind 40 μN metHb; estimation of Hp molar concentration is discussed below). The results demonstrated that
IsdB^{N1N2} did not interact with Hp alone, but pulled down both metHb and Hp when presented together (Figure 5-10).

Figure 5-10. Nickel bead pulldown of His_{6}-IsdB^{N1N2}, Hp and metHb. 20 μM His_{6}-IsdB^{N1N2} was used as bait to pull down 20 μN metHb and/or ~20 μM Hp. His_{6}-IsdB^{N1N2} could bind nickel beads alone (Lane 1), whereas metHb and Hp could not (Lanes 2 and 3, respectively). His_{6}-IsdB^{N1N2} pulls down metHb (Lane 4), but not Hp (Lane 5). When metHb is added to nickel beads mixed with His_{6}-IsdB^{N1N2} and Hp, all three species are pulled down (Lane 6). 1 μg of Hp is shown in Lane 7, for reference. Although Hp runs at nearly the same position on the gel as His_{6}-IsdB^{N1N2}, two separate bands in Lane 6 are distinguished, largely due to their differential staining (Hp is heavily glycosylated, affecting staining by Coomassie dye).

5.3 Discussion

Three domains of IsdB (IsdB-N1, linker, IsdB-N2) were required for high affinity binding of Hb and multiple turnovers of heme uptake (Chapter 4). Although none of the domains bound Hb alone, likely all played a role in the interaction with Hb. Initially, two heme pocket variants (Y440A and M362L) were produced in the IsdB^{N1N2} construct based on the hypothesis that one or both variant proteins would bind Hb but be heme-transfer incompetent, thus trapping the complex and aiding in crystallization. However, IsdB^{N1N2 M362L} was unstable in solution, and IsdB^{N1N2 Y440A} was not only impaired for heme uptake from metHb, the amino acid substitution also rendered the construct deficient for Hb binding. Tyr440 is within the heme binding pocket...
of IsdB-N2 and thus was not expected to be part of the interaction surface between IsdB and Hb. An inspection of the IsdB-N2 structure reveals that Tyr440 is partially exposed to the solvent (Figure 5-11). When heme is bound in the pocket, the accessible surface area of Tyr440 as calculated by ArealMol (90) was 22.3 Å², or 10% accessible (Figure 5-11A,C). However, when the heme molecule is deleted from the file (rendering the protein apo in silico) the accessible surface area of Tyr440 increases to 66.3 Å², or 30% accessible (Figure 5-11B,D). Thus, Tyr440 may be directly involved in the interaction interface with Hb.

Figure 5-11. Inspection of the IsdB-N2 heme binding pocket reveals that Tyr440 is solvent-exposed. (A and C) Chain C of the IsdB-N2 structure with heme bound in the pocket. Tyr440 (blue sticks) and heme (orange sticks) are shown for orientation in panel A, with a space-filling model in the same orientation in panel C. Tyr440 is 10% solvent-exposed in holo-IsdB-N2. (B and D) Chain C of the IsdB-N2 structure in which the heme coordinates have been deleted. Tyr440 appears highly solvent-exposed in the cartoon model in panel B; the space-filling model (same orientation as panel B) in panel D confirms this. Tyr440 is 30% solvent-exposed in apo-IsdB-N2.
Another possible explanation for the dramatic effect of the Y440A substitution on the ability of IsdB<sup>N1N2</sup> to bind Hb is that the substitution destabilizes the IsdB<sup>N2</sup> domain, weakening the Hb interaction surface. Evidence of interaction with Hb remains in that mixing IsdB<sup>N1N2 Y440A</sup> with Hb produces a significant spectral shift. Moreover, IsdB<sup>N1N2 Y440A</sup> retains heme-binding ability, suggesting that the IsdB<sup>N2</sup> domain is more-or-less intact. Stability of IsdB<sup>N1N2 Y440A</sup> could be assessed and compared to that of wild-type by heat denaturation experiments using differential scanning calorimetry (DSC) or dynamic light scattering. The loss of high-affinity Hb binding upon mutation of Tyr440 in IsdB<sup>N1N2</sup> could be due to increased protein instability or loss of a directly interacting residue; regardless, this variant provides further evidence that IsdB-N2 is involved in Hb binding.

Interestingly, an equivalent amino acid substitution in the IsdH<sup>N2N3</sup> construct, Y642A, resulted in a construct that was deficient in heme binding but could still readily disrupt Hb tetramers (69) and was also crystallized in a complex with metHb (70). Hb-binding affinity of IsdH<sup>N2N3 Y642A</sup> was not reported. However, IsdH<sup>N1</sup> and IsdH<sup>N2</sup> alone were shown to have a high affinity for Hb (53,68); therefore it is likely that the IsdH Y642A mutation did not have as strong an effect on Hb binding as the IsdB Y440A mutation.

The holo-IsdB<sup>N1N2 Y440A</sup> spectrum differed greatly from that of IsdB<sup>N1N2 Y440A</sup> mixed with metHb. As the IsdB<sup>N1N2 Y440A</sup> spectrum when mixed with metHb resembles a metHb spectrum that has been red-shifted, a possible explanation for this phenomenon is that IsdB<sup>N1N2 Y440A</sup> induces an initial conformational change in the Hb heme binding pocket, slightly changing the spectrum, but the heme remains in the Hb pocket rather than being transferred into the IsdB<sup>N1N2 Y440A</sup> heme pocket. Likely Tyr440 is essential for heme transfer into the IsdB pocket.
One unexpected aspect of the Y440A and M362L heme-pocket substitutions is their vastly differing properties in the IsdB^{N1N2} construct versus the IsdB^{N2} construct. Wild-type IsdB^{N2} and IsdB^{N1N2} displayed comparable heme binding: their holo-protein spectra were identical and both could rapidly transfer heme to IsdA^{N1}. As reported in Chapter 3, IsdB^{N2 M362L} was highly stable in solution and retained heme-binding characteristics that were comparable to the wild-type protein; IsdB^{N2 Y440A} was unstable in solution and bound heme weakly. However, the same amino acid substitutions in the IsdB^{N1N2} construct produced different effects. IsdB^{N1N2 Y440A} was highly stable in solution and exhibited a holo-protein spectrum upon titration with heme, whereas IsdB^{N1N2 M362L} was unstable and precipitated upon addition of heme. This suggests that interactions between the NEAT domains are present within IsdB, which alter the outcome of amino acid substitutions under different contexts; the presence of the N1 and linker domains significantly stabilized heme binding by IsdB^{N2} with a Y440A mutation. The implications of this can be further explored using the current array of IsdB^{N2} heme pocket variants (Chapter 3) with the IsdB^{N1} or IsdB^{N1-L} constructs to examine impact on heme uptake from metHb.

No compelling evidence was obtained for either synergy or competition between IsdB^{N1N2} and IsdH^{N1}, whether in Hb binding or in heme extraction from metHb. The impact of IsdH^{N1} on steady-state heme transfer from metHb to IsdA through a catalytic amount of IsdB^{N1N2} was investigated; unfortunately, the preliminary kinetic results could not be readily modeled. Further experimentation could be undertaken to explore the possibility that presence of IsdH^{N1} slows down heme uptake from metHb by IsdB^{N1N2}. However, IsdB is highly upregulated on the surface of the cell under iron-restriction, whereas IsdH is not upregulated (41,72). A proteomic analysis of exoproteins produced by wild-type or Δfur S. aureus under iron-rich conditions revealed that at least 33-fold more IsdB than IsdH is produced by the cell (by mass spectra count), in the
absence or presence of Fur (43). On a surface greatly enriched for IsdB, a low degree of competition between IsdB and IsdH for Hb substrates would likely not make a large impact on the import of heme into the cell.

The effect of Hp on heme uptake from metHb by IsdB^{N1N2} was also investigated. Full-length IsdB is known to bind to Hb and Hp-Hb complexes, but not free Hp (53). Thus, IsdB binding of the Hp-Hb complex is likely to occur through binding of the Hb molecule itself which then binds Hp at a distinct site. Notably, Hp-Hb complexes were not an accessible heme source for IsdB^{N1N2} in this study; heme uptake only occurred when the concentration of mixed-serotype was decreased to 20 μg/mL, which is 500-fold lower than human serum concentrations of Hp.

Considering that S. aureus successfully infects human blood and tissue, and that loss of IsdB (but not IsdH) impairs S. aureus growth in a mouse model by ten-fold (54), it seems unlikely that S. aureus is unable to use Hp-Hb as an iron source in vivo. In fact, S. aureus has been shown to prefer Hp-Hb over Hb alone as an iron source (121).

The ability of IsdB^{N1N2} to take up heme from metHb when only 20 μg/mL Hp was present likely reflected an increased proportion of free metHb. Due to the polymorphic nature of human Hp phenotypes, the molar concentration of Hp at 20 μg/mL can only be estimated. Regardless of the polymeric state of the Hp, the basic functional unit is the α₁β or α₂β dimer, which binds one Hb dimer. The mass of the β chain is 34 kDa, the α₁ chain is 9 kDa and the α₂ chain is 16 kDa (122,123). Therefore, the average size of the Hb-binding unit is 46 kDa. At 2 mg/mL Hp, approximately 43 μM of Hb dimer binding sites are present. At 0.02 mg/mL Hp, the concentration is 0.43 μM of Hb dimer binding sites, which would therefore bind approximately 0.86 μN Hb. Since 2 μN metHb was used in this study, at 0.02 mg/mL the molar concentration
of Hp was low enough to result in > 1 μM free Hb, which likely explains why heme uptake by IsdB^N1N2 was seen under these conditions.

There are several possible explanations for our observation in vitro that IsdB^N1N2 cannot remove heme from Hp-Hb complexes, the first of which is that IsdB^N1N2 lacks the regions of IsdB required to extract heme from Hp-Hb complexes. Binding of Hp to Hb stabilizes the binding of heme in the Hb pocket (124); inhibition of heme uptake from the Hp-Hb complex may therefore reflect the increased stability of heme in the pocket, rather than a more intuitive direct blocking of the IsdB^N1N2 binding site on Hb by Hp. IsdB^N1N2 is a truncated version of the mature protein, and it may be that regions N-terminal or C-terminal to the construct are required for obtaining heme from Hp-Hb complexes. Large portions of IsdH were also not included in the construct investigated in this study; again, regions outside IsdH^N1 may act synergistically with IsdB in order to promote heme uptake from Hp-Hb complexes. However, as deletion of isdH does not affect *S. aureus* growth in vivo (54), it is unlikely that IsdB relies on a synergistic reaction with IsdH in order to carry out heme uptake from Hp-Hb. *S. aureus* may also rely on secreted proteases or other molecules to cleave apart the Hp-Hb complex; the secreted V8 protease specifically cleaves helices of Hb around the heme pocket (125), although expression of the V8 protease is downregulated during incubation in human blood or serum (72). Lastly, although *S. aureus* itself may use Hp-Hb as a preferred iron source, heme uptake from Hp-Hb has never been shown for either IsdB or IsdH. It is possible that IsdB indeed cannot remove heme from Hp-Hb, and relies on hemolysin production and local hemolysis to increase the Hb concentration, thereby saturating the circulating Hp stores and allowing additional Hb to remain unbound.
As the interaction interface between IsdB and Hb is still unknown, I attempted to infer its approximate position based on knowledge of where IsdH<sup>N2N3</sup> and Hp bind to the Hb molecule. A superposition of the structures of IsdH<sup>N2N3 Y642A</sup>-metHb (Figure 1-7) (70) and Hp-Hb (Figure 1-2) (37) revealed completely distinct binding interfaces, as previously suggested (70) (Figure 5-12A). The heme groups of the Hb dimer are distant from the Hp binding site, with at least 10 Å between any heme atom and any Hp residue, with a Hb helix separating the heme and Hp (Figure 5-12B). Conversely, IsdH binds the Hb flanks where the heme resides and there is only a 3 Å gap between the hydroxyl group of Tyr646 and a heme propionate group, with both heme pockets open to each other (Figure 5-12B). It is likely that the IsdB<sup>N1N2</sup> binding site on Hb overlaps at least partially with the IsdH<sup>N2N3</sup> binding site, in order to access the heme molecules and avoid displacement by Hp.

In summary, I have shown that mutation of the heme-iron coordinating residue Tyr440 in the context of IsdB<sup>N1N2</sup> produces severe defects in Hb binding and heme uptake from metHb, providing further evidence that IsdB-N2 is involved in Hb binding. Furthermore, the different effects produced by equivalent mutations in IsdB<sup>N1N2</sup> and IsdB<sup>N2</sup> raise the intriguing possibility of intraprotein interactions in IsdB. The presence of IsdH<sup>N1</sup> did not have an impact on IsdB<sup>N1N2</sup> function in Hb binding or heme uptake. However, IsdB<sup>N1N2</sup> bound Hp-Hb complexes, but was unable to access heme in these complexes, opening an entire new area of research into IsdB function in vitro as well as in vivo.
Figure 5-12. Superposition of the IsdH\textsuperscript{N2N3 Y642A}-metHb and Hp-Hb crystal structures. Porcine Hp is a dimer, each monomer of which binds one Hb (αβ) dimer (PDB ID: 4F4O); for simplicity, one Hp monomer (deep purple) binding one biologically relevant Hb dimer (light blue) is shown. The IsdH\textsuperscript{N2N3 Y642A}-metHb structure (PDB ID: 4IJ2) showed a Hb tetramer surrounded by four IsdH\textsuperscript{N2N3 Y642A} polypeptides; for simplicity, one Hb dimer (light orange) binding two IsdH\textsuperscript{N2N3 Y642A} molecules (dark orange) is shown. Heme molecules are shown in green. The superposition was performed in Coot and the image was made in PyMOL. (A) The superposed complex is shown from the side; Hp binds in between the Hb dimer, whereas IsdH flanks the Hb dimer in a completely separate binding site. The heme moiety are at a distance from the Hp molecule. (B) The area surrounding one Hb heme pocket of the superposed complex is expanded, with Tyr646 from the IsdH\textsuperscript{N3} heme pocket drawn in sticks to demonstrate its proximity to the heme propionate. Hp (deep purple) is seen in the foreground.
Chapter 6: Overview and future directions

The Gram-positive pathogen *Staphylococcus aureus* uses an elaborate system of cell-wall anchored proteins to extract heme from host hemoglobin (Hb) and transport it into the cell in order to fulfill its iron needs. The structure and function of the major *S. aureus* Hb receptor, IsdB, was investigated in this study.

In Chapter 3, I studied the structure and function of the heme binding NEAT domain of IsdB, IsdB-N2. I found that IsdB$^{N2}$ was structurally similar to other heme binding NEAT domains and heme-iron was coordinated by a tyrosine residue as predicted; however, I also found that Met362 coordinates the heme-iron on the distal side, resulting in an unprecedented mode of heme-iron coordination. I determined that heme is bound with equimolar stoichiometry in solution, as seen in the crystal structure, and found an upper bound for the affinity of IsdB$^{N2}$ for heme ($K_D$<0.4 μM). Point mutations of heme-interacting residues in the heme pocket demonstrated that Met362 played a role in heme trafficking into and out of the heme pocket, but was dispensable for stable heme binding, whereas variants of Tyr440, Tyr444 and Ser361 were each impaired in heme binding. Lastly, I proposed that the unusual nature of the heme-iron coordination suggested the possibility of a new functional role for IsdB-N2, perhaps in heme extraction from Hb.

In Chapters 4 and 5, I investigated aspects of Hb binding and heme extraction from Hb with numerous constructs comprising different regions of IsdB. I found that only a construct comprising IsdB-N1, the linker domain and IsdB-N2 together (IsdB$^{N1N2}$) was competent for both high affinity Hb binding and heme uptake. Moreover, IsdB$^{N1N2}$ removed all heme groups from metHb, but could not remove heme from oxyHb or HbCO. I determined the affinity of IsdB$^{N1N2}$ for Hb ($K_D$=0.42 μM) and investigated the kinetics of the heme transfer process. I also demonstrated that IsdB$^{N1N2}$, in catalytic amounts, could catalyze the transfer of heme from
metHb to IsdA\textsuperscript{N1} with a \( k_{\text{cat}}/K_m \) that places it in the category of moderately efficient enzymes, and hypothesized that the system would work with comparable efficiency on the cell surface during infection. I also investigated the effects of the Hb-binding serum protein haptoglobin (Hp) and another Hb-binding \textit{S. aureus} surface protein (IsdH\textsuperscript{N1}) on the heme transfer process and found that while IsdB\textsuperscript{N1N2} could bind the Hp-Hb complex, Hp blocked heme uptake from metHb by IsdB\textsuperscript{N1N2}. IsdB\textsuperscript{N1N2} and IsdH\textsuperscript{N1} did not interact in the absence of Hb and IsdH\textsuperscript{N1} did not appear to have an effect on heme uptake from metHb by IsdB\textsuperscript{N1N2}. Lastly, I investigated the effects of two of the same point variants as in the IsdB\textsuperscript{N2} study, Y440A and M362L, on IsdB\textsuperscript{N1N2} Hb binding, heme binding and heme uptake parameters, and found notable differences between the same mutations in the different constructs, suggesting that there are interdomain interactions that play a role in how heme is bound by IsdB. A schematic of heme transfer between metHb, IsdB and IsdA derived from the work in this thesis is presented in Figure 6-1.

Figure 6-1. A schematic of heme transfer between metHb, IsdB and IsdA.
The process of heme uptake from the host by \textit{S. aureus} can be thought of as two distinct parts: the recognition and binding of Hb followed by heme uptake, and subsequent heme transfer between surface receptors to the cellular membrane.

6.1 Hemoglobin binding and heme uptake by the Isd system and future directions

For some time IsdB has been known to be a Hb receptor for the Isd system (50,54) and that full-length IsdB is capable of not only binding Hb, but also removing its heme (51). I have now shown that a polypeptide containing the two IsdB NEAT domains and the intervening linker region, IsdB\textsuperscript{N1N2}, is the minimum unit possible for carrying out these functions (Chapter 4). Interestingly, IsdB\textsuperscript{N1N2} is unable to remove heme from Hb that is in complex with Hp (Chapter 5). It is known that full-length IsdB binds the Hp-Hb complex (53), but heme uptake from Hp-Hb by either IsdB or IsdH has not been previously shown; presumably, this function must occur \textit{in vivo} as free Hb is unlikely to be available due to the presence of excess Hp. The IsdB\textsuperscript{N1N2} construct does not include the regions N-terminal of the IsdB-N1 domain (~83 amino acids) or C-terminal of the IsdB-N2 domain (~154 amino acids); it is possible that inclusion of one or both of these regions would permit IsdB to remove heme from Hp-Hb complexes. The region C-terminal to IsdB-N2 is predicted to be highly disordered by the program DISOPRED (126) but constructs with C-terminal extensions could be explored. I produced a full-length construct of IsdB (residues 43-609) which was unstable in solution and did not exhibit a NEAT domain-like heme-binding spectrum upon addition of heme (data not shown). A construct of IsdB\textsuperscript{N1N2} with a 20 amino acid N-terminal extension (residues 106-459) has been produced and is soluble. This construct could be tested for heme uptake from Hb-Hp complexes.

Alternatively, \textit{S. aureus} may express an additional factor \textit{in vivo} which leads to dissociation of the Hp-Hb complex or degrades Hp. It is unlikely that IsdB and IsdH necessarily work
together on the surface of the cell to remove heme from Hp-Hb complexes; single gene deletions of *isdB* or *isdH* were only partially impaired for growth in a mouse infection model, with the *isdB* deletion significantly more impaired (54). Most *S. aureus* growth studies thus far have focused on the use of heme or Hb as the sole iron source; growth of *S. aureus* (and Δ*isdB* or Δ*isdH* strains) using Hp-Hb as the sole iron source may yield some answers as to the ability of each of these proteins to use Hp-Hb *in vivo*.

The effect of addition of IsdH*N1* on Hb binding and heme uptake by IsdB*N1N2* was explored (Chapter 5), but IsdB-N1 has a significantly higher amino acid sequence identity to IsdH-N2 (65%) than IsdH-N1 (46%). Moreover, IsdB-N1 and IsdH-N2 share an identical aromatic motif (FYHYA) which differs from the motif in IsdH-N1, YYHFF. *In vivo* studies published this year showed that swapping out the IsdB-N1 aromatic motif for the IsdH-N1 aromatic motif resulted in *S. aureus* cells that were unable to bind Hb, and IsdB with the chimeric aromatic motif was significantly impaired at removing heme from metHb (74). Thus, IsdB*N1* clearly has more in common with IsdH*N2* than IsdH*N1*, and performing the above experiments with IsdH*N2* instead of IsdH*N1* may produce different results with regard to competition or synergy. IsdH*N1* was used in this study because it has been well-characterized in numerous studies and is highly soluble, whereas relatively little work has been done on IsdH*N2*. IsdH*N2N3* has a high degree of sequence identity to IsdB*N1N2* (64%) and a structure of IsdH*N2N3 Y642A* in complex with metHb was also published this year. A direct biochemical comparison of IsdB*N1* and IsdH*N2*, and IsdB*N1N2* and IsdH*N2N3*, should be pursued in order to assess their similarities and differences in Hb binding, as well as heme uptake from Hb and Hp-Hb complexes.

It has also been shown that *S. aureus* cannot grow on hemopexin (54), a heme-binding serum protein induced after a host inflammatory event which reportedly has an affinity for heme of
approximately 1 pM (127). It is unknown whether hemopexin could prevent IsdB from taking up heme from Hb; addition of hemopexin to Hb-IsdB$^{N1N2}$ heme uptake assays is a new avenue for exploration. Additionally, *in vivo* studies on application of exogenous hemopexin during mouse infection with *S. aureus* would inform on its use as a potential therapeutic for *S. aureus* infections.

Finally, the mechanism of heme extraction from Hb by IsdB$^{N1N2}$ remains unknown and a crystal structure of the Hb-IsdB$^{N1N2}$ complex will continue to be pursued. Additionally, IsdB$^{N1N2}$ constructs with longer N- or C-terminal tails should be tested for Hb binding by ITC and heme uptake from metHb and compared to IsdB$^{N1N2}$; it is possible that a different construct may yield higher affinity binding or a more stable complex, which would aid in crystallization. I have shown that Hp inhibits heme uptake from metHb by IsdB$^{N1N2}$ but does not affect binding of IsdB$^{N1N2}$ to Hp-Hb (Chapter 5); this characteristic is highly desirable for trapping a complex and could potentially yield a crystal of improved quality due to the increased stability of the complex. Polymorphic human Hp was used in this study; Hp of a single serotype and thus uniform size and composition, Hp 1-1, would be required for crystallization trials. However, human Hp or Hp-Hb has thwarted crystallization attempts for over 50 years. The first crystal structure of Hp-Hb was reported in 2012 and used proteins isolated from porcine blood and serum (37). Thus, in order to increase the chances of successful crystallization, porcine Hp and Hb may be used instead. Experiments characterizing IsdB$^{N1N2}$ binding to porcine Hb, heme uptake, binding to porcine Hp-Hb complexes, and the effect of porcine Hp on heme uptake from porcine Hb by IsdB$^{N1N2}$ would first need to be carried out and confirmed to be similar to those characteristics using human Hp and Hb.
6.1.1 Regulation of IsdB expression

Although all Isd genes and operons have a Fur-binding region in their promoter region, all genes are not equivalently expressed. For instance, IsdG and IsdI, the homologous intracellular heme-degrading enzymes, are differentially regulated by heme: under iron-restriction conditions, *isdG* expression is significantly upregulated upon exposure to hemin, whereas *isdI* expression is unaffected (128). There is post-translational control of protein levels for IsdG and IsdI as well: pulse-chase experiments showed that the half-life of IsdG in the cell is one-fifth that of IsdI, and that the half-life of IsdG (but not IsdI) increases when heme is present in the media (128). Furthermore, *isdH* expression has not been shown to be upregulated under any iron-restriction conditions tested (41,72), but it is upregulated 18-fold in a Δ*fur* mutant (D. Heinrichs, personal communication); conversely, *isdB* is greatly upregulated in those same studies. Additional levels of transcriptional and post-transcriptional control appear to be operating for most, if not all, members of the Isd system.

Recently, an intriguing new finding has come to light: IsdB expression is regulated at the transcriptional level by the ClpXP protease (129). The Clp proteolytic system is the major intracellular protease of *S. aureus* and is composed of a regulatory recognition ATPase (e.g. ClpB, ClpC or ClpX) paired with the ClpP protease. Deletion of *clpP* significantly reduced the amount of IsdB protein visualized on the cell surface, with a concomitant drop in Hb binding and ability to grow on Hb as a sole iron source; deletion of *clpX* nearly eliminates cell surface IsdB. However, this effect is indirect, and occurs at the transcriptional level: a deletion of *clpX* or *clpP* reduces transcripts of *isdB*. Levels of IsdA, IsdC and IsdE proteins were also decreased in the *clpP* mutant strain, although less dramatically. Curiously, this effect was independent of Fur: deletion of *fur* failed to rescue expression of *isdB*. The Clp system is known to regulate the
abundance of numerous other transcriptional regulators, but none tested could regulate \textit{isdB} abundance in a Clp-dependent manner. Thus, the evidence points to a potential positive regulator of \textit{isdB} transcription that is activated by ClpXP in the absence of Fur.

6.2 Heme transfer between Isd surface proteins and future directions

Cell-wall bound Isd proteins transfer heme inward from the extracellular milieu to the cellular membrane. It is thought that their relative positions within the thick cell wall help to achieve this goal: a proteinase K digestion of the intact cell resulted in complete digestion of IsdB, partial digestion of IsdA, and no digestion of IsdC, implying that they are anchored at different depths (IsdH was not tested) (50). The differential anchoring is likely due to differences in their sortase and signal sequences: IsdC has a sortase B signal (NPQTN) whereas IsdABH have a sortase A signal (LPXTG) (49). Moreover, IsdB and IsdH carry a YSIRK-G/S motif in their signal sequence, whereas IsdA and IsdC do not (118,130); this motif is known to direct the protein to be secreted at the site of cell division, whereas the absence of the motif directs protein secretion to the cell pole (131,132). The YSIRK-G/S motif may therefore play a further role in directing anchoring depth.

Heme transfer between Isd surface NEAT domains is unidirectional and coincides with transfer deeper into the cell wall (52). In terms of heme binding and transfer, the C-terminal NEAT domains alone are sufficient and closely resemble the full-length proteins in these functions (Chapter 3) (51,83,103,114). Recently, the basis for the unidirectionality of heme transfer was established: heme affinity increases from IsdH\textsuperscript{N3} (34 nM) to IsdA\textsuperscript{N1} (14 nM) to IsdC\textsuperscript{N1} (6 nM) (82). Heme is therefore transferred up the affinity gradient to IsdC. Heme transfer from IsdC to IsdE, the lipoprotein component of the membrane transporter and a non-NEAT domain protein, is significantly slower than inter-NEAT domain transfer (52,133). However,
full-length IsdC is the only Isd cell wall protein that is known to transfer heme to IsdE (51); IsdC\textsuperscript{N1}, IsdB\textsuperscript{N2} and IsdH\textsuperscript{N3} can transfer heme to IsdE as isolated NEAT domains (52), but full-length IsdB and IsdA cannot (it is unknown whether full-length IsdH can transfer heme directly to IsdE) (51). It has been suggested that transfer of heme from IsdC to IsdE is adaptive, in order to control the rate of influx of heme into the cell (133). Heme overload in the \textit{S. aureus} cell is toxic, and \textit{S. aureus} expresses a two-component heme sensing system, HssRS, which controls the expression of a dedicated heme efflux pump, HrtAB (134,135).

I found that heme transfer from IsdB\textsuperscript{N2} to IsdA\textsuperscript{N1} was 17,000X faster than the IsdB\textsuperscript{N2} heme off-rate, indicating formation of an active transfer complex (Chapter 3); in spite of this, a complex between IsdB\textsuperscript{N2} and IsdA\textsuperscript{N1} has not yet been detected. In fact, stable complexes between any NEAT domains have yet to be reported. However, formation of “ultra-weak affinity” complexes (mM or weaker affinity) has been detected by paramagnetic relaxation enhancement (PRE) techniques (136). In the absence of protoporphyrin molecules, IsdA\textsuperscript{N1} and IsdC\textsuperscript{N1} do not associate, whereas introduction of Zn-PPIX triggers a weak association. The authors predicted that complexation induced structural distortions of the heme binding pocket due to steric strain from residues from IsdA\textsuperscript{N1} and IsdC\textsuperscript{N1} coming into close contact, leading to heme transfer; mutation of the predicted sterically distorting residues resulted in wild-type heme binding affinity by IsdA\textsuperscript{N1}, but association with IsdC\textsuperscript{N1} and heme transfer were greatly reduced.

However, it is unknown whether these “ultra-weak affinity” complexes are physiologically relevant, or are an artifact of using only the NEAT domains. Again, regions outside the NEAT domains may be necessary for more stable inter-NEAT domain complexation, and constructs containing these regions could be tested for stable binding in order to confirm that high affinity
complexes do not form *in vivo*. There is evidence that IsdB and IsdA form a stable complex on the cell surface, as pulling down IsdB from a cell extract also pulled down IsdA (118).

Regardless, it is likely that a stable IsdB/IsdA or IsdA/IsdC complex would resemble the predicted structures of NEAT domain heme transfer complexes: a predicted transfer complex model in which the heme molecule is coordinated by one Tyr from each heme pocket (114) bore remarkable similarity to the “handclasp” model of the heme transfer complex developed by PRE methods (136).

### 6.3 IsdB variants: future directions

As noted in Chapter 5, the same single amino acid substitutions (Y440A and M362L) in IsdB$^{N1N2}$ as opposed to IsdB$^{N2}$ resulted in dramatically different effects. The Y440A mutation in the IsdB$^{N2}$ domain is destabilizing and prevents heme binding, whereas the same mutation in the IsdB$^{N1N2}$ domain is not destabilizing and heme binding occurs. This strongly suggests that there are interactions between IsdB domains that could not have been predicted in the IsdB$^{N2}$ domain alone. Moreover, mixing of IsdB$^{N1N2 \ Y440A}$ with metHb resulted in an electronic spectrum that did not overlay either holo metHb or holo IsdB$^{N1N2 \ Y440A}$. I hypothesize that binding of IsdB$^{N1N2 \ Y440A}$ to metHb alters the heme environment of metHb, resulting in a shifting of the hemoprotein spectrum, but without successful transfer of heme from metHb to the IsdB heme pocket. This hypothesis can be tested a number of ways; here I suggest two potential methods. First, separation of the two proteins after mixing in order to determine which protein now bears the heme component. Second, a crystal structure of the complex would inform on which heme pocket the heme moiety resides in.
6.4 Concluding remarks

This thesis described heme transfer pathways from metHb to IsdA, using IsdB as a necessary intermediary, in order for *Staphylococcus aureus* to obtain heme-iron from a human host. While the experiments were carried out *in vitro*, they generated testable hypotheses for how the system may operate *in vivo* and during infection. A framework for Hb binding and heme extraction from metHb by IsdB was elucidated. Considering that NEAT domains are conserved across several Gram positive pathogens, the mechanisms uncovered may be conserved as well, and may inform rational drug design in order to inhibit these processes in clinically important Gram positive infections. Studies of this nature increase awareness of the biological significance of biochemical mechanisms we explore *in vitro*, and in this case revealed intriguing new areas of study.
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