FUNCTION OF NON-HEME-BINDING DOMAINS OF THE *STAPHYLOCOCCUS AUREUS* ISDB PROTEIN IN HEME ASSIMILATION FROM METHEMOGLOBIN

by

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NOMENCLATURE

IsdB, iron-regulated surface determinant B; ND, N1, MD, N1, and CD, the N-terminal, NEAT 1, middle, NEAT 2, and C-terminal domains of IsdB; metHb, ferric hemoglobin.

ABSTRACT

As a hemoglobin acceptor, IsdB rapidly and efficiently acquires heme from methemoglobin (metHb) in the heme acquisition pathway of *Staphylococcus aureus*. The pathway of heme assimilation in *S. aureus* involving IsdB has been established; however, the mechanism of rapid and efficient heme assimilation of metHb heme by IsdB remains unclear. IsdB consists of five major domains: the N-terminal (ND), NEAr Transporter 1 (N1), middle (MD), heme binding NEAr Transporter 2 (N2), and C-terminal (CD) domains. The goal of this study is to elucidate the roles of these IsdB domains in the metHb-to-IsdB heme transfer reaction. Deletion of the CD region does not alter the kinetics and equilibrium of the reaction. Sequential deletions of ND and N1 of ND-N1-MD-N2 progressively reduce heme transfer rates but have no effect on the reaction equilibrium. Further deletion of MD decreases the efficiency of heme transfer from metHb to N2. The MD domain reduces heme dissociation from holo-N2 and drives the metHb/N2 reaction to the formation of holo-N2. ND-N1-MD and N2 fragments, but not ND-N1, MD, and N2, reconstitute the rapid metHb/IsdB reaction, indicating an MD/N2 interaction. Analyses of MD, N2, and MD-N2 mixture by size exclusion chromatography support an interaction between MD and N2. These results indicate that ND-N1 and MD domains critically contribute to the kinetics and equilibrium of the metHb-to-IsdB heme transfer reaction, respectively. The results also suggest that CD functions as a spacer to position IsdB in the cell wall envelope for heme relay through the cell wall. These findings support a mechanism of direct extraction of metHb heme by IsdB that involves the four structural domains of IsdB.

INTRODUCTION

Staphylococcus aureus

Staphylococcus aureus was first identified in 1880 in Aberdeen, United Kingdom (1). Since then, it has been shown to be a potential pathogenic Gram-positive bacterium. *S. aureus* can cause various infections ranging from minor skin infections, such as impetigo, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, toxic shock syndrome (TSS), bacteremia, and sepsis (2). It is estimated that *S. aureus* causes about 18,500 deaths annually in the United States.

Antibiotic-Resistant *Staphylococcus aureus*

S. aureus infections are treated by antibiotics (3). Penicillin is the first antibiotic used to treat *S. aureus* infections. However, most strains of pathogenic *S. aureus* are resistant to penicillin to date (4). The inability of penicillin to treat *S. aureus* infections led to the introduction of methicillin. However, not long after its introduction, strains that resist methicillin emerged. Methicillin-resistant *staphylococcus aureus* (MRSA) is becoming prevalent rapidly. A study in 2010 found that the overall MRSA prevalence rate was 66.4 per 1,000 inpatients in USA (5, 6). MRSA usually resists to commonly used antibiotics, and vancomycin is the last available for treatment of MRSA infections. Unfortunately, an initial case of reduced vancomycin susceptibility in clinical isolates of *S. aureus* was reported in Japan in 1997, generating a huge alarm in the medical

community (7). Lack of antibiotics to treat vancomycin-resistant MRSA highlights the urgency of developing new antibiotics to treat *S. aureus* infections.

Essentiality of Iron for Growth of Bacteria

Iron is an essential nutrient for most of bacteria and other living organisms. Iron is a cofactor for many proteins that are involved in a variety of cellular processes, such as DNA biosynthesis, the tricarboxylic acid cycle (Krebs cycle), respiration, oxygen transport, and gene regulation (8, 9, 10, 11). Deprivation of iron is bacteriostatic and extended period of iron deficiency often is lethal to bacteria (12). During growth, a continuing assimilation of iron from the host is required for bacteria to survive in hosts.

Major Iron Sources in Mammals for Bacterial Pathogens

The ferrous and ferric forms of iron have different availabilities. Ferrous iron is highly soluble in water but tends to be oxidized in an aerobic environment. Therefore, ferric iron is a dominant form of free iron in the host but, has an extremely low solubility at a neutral pH (10^{-18} M at pH 7 and 10^{-24} M at pH 7.4) (13). Consequently, the concentration of free iron in mammals is far below the levels that are required for the growth of bacteria. In addition, more than 99.9% of total iron in the host is sequestered as forms of iron-protein complexes (14). Thus, free iron is not the major iron source in mammals for bacteria

Transferrin and lactoferrin are extracellular proteins with high affinity for ferric in serum and mucosal secretion, respectively. Both proteins share a similar structure, and

have two similar but not identical binding sites for iron (15). Some gram-negative bacterial species, such as *Neisseria meningitides,* are believed to be able to directly acquire iron from the complex of ferric with transferrin or lactoferrin (16). S. aureus is also able to use transferrin iron as a source for iron.

Most of iron in mammals is complexed with protoporphyrin, which is called heme. Heme is the prosthetic group of hemoglobin in red blood cells and many other hemoproteins. To grow in mammals, bacterial pathogens are able to extract heme from host hemoproteins for iron. Hemoglobin, haptoglobin-hemoglobin complex, and hemopexin have an ample supply of heme. Hemoglobin exists as an intracellular form within cells. However, most bacteria can release hemoglobin by producing hemolysins to lyse red blood cells and utilize it as an iron source (17). Hemoglobin is a preferred iron source for *S. aureus* in the host (18).

Iron and Heme Acquisition in Bacteria

There are two kinds of the iron/heme acquisition systems in bacteria. One kind of acquisition system consists of proteins called receptors that directly capture iron- or heme-protein complexes and subsequently extracts iron or heme from the captured host complex. In another system, bacteria produce small iron chelators called siderophores or secrete proteins called hemophores that scavenge iron or heme from various sources (17).

Siderophore

Siderophore is a Fe^{3+} chelator produced by many pathogenic bacteria that enables the extraction and solubilization of iron from the host for utilization by microbes. It is a small compound with an extreme high affinity for ferric iron. The affinity of siderophore for gallium is also high, but is substantially less than ferric. Thus, the siderophore ligand can be said to be "virtually specific" for Fe^{3+}among the naturally occurring metal ions of abundance (19). Ferric iron-siderophore complexes are recognized by specific transporters of bacteria to transport them inside bacteria (17).

Hemophore

Hemophore is the scavenging protein for heme which is found in both gram-negative bacteria, *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens, Yersinia pestis* and *Yersinia enterocolitica* (20), and the gram-positive bacterium *Bacillus anthracis* (21). Secreted hemophore has a high affinity for heme and acquires heme from hemoglobin. The heme-hemophore complex is captured by a specific receptor on the bacterial surface, and heme in the complex is then extracted by the receptor and subsequently transported into bacteria.

Iron and Heme Acquisition in Gram-Negative Bacteria

Gram- negative bacteria are different from gram-positive bacteria by having the outer membrane and periplasmic space. Thus, acquisition of iron and heme in gram-negative bacteria involves the translocation of captured iron and heme across the outer membrane into the periplasmic space and then transport across the cytoplasmic

membrane into the cells. The porous feature of the outer membrane serves as a molecular sieve, with an exclusion size of 550-650 Dalton (22); this prevents the penetration of the ferric-siderophore complex or heme-hemophore complex, which have a molecular weights larger than 700 Dalton (23). Meanwhile, it obstructs the utilization and transformation of chemical energy, such as ATP hydrolysis for the membrane transport, since the energy sources are not presented in this region due to the outer membrane's porous nature (24).

Transport of Iron and Heme Through the Outer Membrane

Gram-negative bacteria overcome these obstacles by utilizing a special system for the transportation process. After assimilation of iron or heme from host proteins or siderophore- or hemophore-complexes by a specific receptor in the outer membrane, captured iron or heme is translocated into the periplasmic space using the TonB-dependent system (25). The TonB system comprises of the membrane protein ExbB and the membrane anchored periplasmic proteins ExbD and TonB. The TonB protein interacts with the outer membrane receptor and undergoes conformational changes that are driven by the proton motive force, bringing iron-siderophore or heme into the periplasmic space.

Transport of Iron and Heme Across the Cytoplasmic Membrane

After iron-siderophore or heme is transported into the periplasmic space, it is then translocated across the cytoplasmic membrane by specific ATP-binding cassette (ABC)

transporters. ABC transporters consist of a periplasmic substrate binding protein, a membrane protein called permease, and an intracellular ATPase. ABC transporters use energy from ATPase-catalyzed hydrolysis of ATP to translocate iron-siderophore or heme across the cytoplasmic membrane.

ATP-Binding Cassette (ABC) Transporters

ABC transporters are key component systems for nutrient uptake in both Gram-negative and Gram-positive bacteria, including the uptake of iron-siderophore and heme. An ABC transporter is usually a complex of three proteins: substrate binding protein, permease, and ATPase. The substrate binding protein is located in the periplasmic space in Gram-negative bacteria and is anchored as a lipoprotein in the cytoplasmic membrane in Gram-positive bacteria, and this protein specifically binds a nutrient molecule. The permease usually has multiple segments in the membrane, forming a tunnel-like structure for solute translocation. The ATPase energizes the transportation system by hydrolyzing ATP in the cytoplasm. The detailed mechanism of transportation by ABC transporter still remains to be unveiled. It is believed that the binding of a solute by the substrate binding protein triggers the whole transport process in which substrates are released to the permease by protein-protein interaction, and the substrate binding of permease induces a conformational change which is then transmitted to the ATPase to initiate ATP hydrolysis for energy supply. The answer to how the conformational change causes substance transportation is not available, yet it has been proposed that the transporters have two binding sites, an intracellular one is one with higher affinity and a second one

that is extracellular with lower affinity. The two sites alternate in affinity and sidedness in a “two-cylinder engine” model (26). As a whole, the interaction among substrate-binding protein, permease and ATPase facilitates the transportation of solutes across the cytoplasmic membrane in bacteria.

Iron and Heme Acquisition in Gram-Positive Bacteria

Gram-positive bacteria do not have the outer membrane but have the thick cell wall that is composed of murein sacculus, polysaccharides, teichoic acids, and cell wall proteins. Iron-siderophore complexes can usually diffuse through the cell wall envelope to research their specific ABC transporters, and, thus, ABC transporters are sufficient for uptake of iron-siderophore complexes. However, heme cannot diffuse through the cell wall. Thus, surface proteins in addition to ABC transporters are required for heme acquisition in Gram-positive bacteria. These surface proteins are usually linked to the cell wall by sortases which can recognize a conserved LPXTG motif at the C- terminus of these proteins and covalently link them to the peptidoglycan (27). These proteins are needed to relay heme from the surface through the cell wall envelope to the ABC transporter (28).

Iron and Heme Acquisition Systems in *S. aureus*

Iron Acquisition

S. aureus produces siderophores staphyloferrin A and B that can remove iron from iron transferrin complex. *S. aureus* can also utilize a variety of siderophores produced by other bacteria, including enterobactin, ferrichrome, and aerobactin (29, 30). *S. aureus* produces ABC transporters, HtsABC, SirABC, FhuCBG-D1-D2, and SstABCD, which are involved in uptake of iron-siderophore complexes. HtsABC and SirABC share a common ATPase named FhuC and take up the iron complexes with staphyloferrin A and B, respectively (30, 31, 32). Fhu D1 and D2 undergo a modest conformational change during the binding of iron siderophore complexes and facilitate a broad specificity for iron-xenosiderophore complexes, playing an important role in stealing xenosiderophores from other bacteria by *S. aureus* (30). The substrate for another ABC transporter SstABCD still remains unclear (33).

Heme Acquisition

S. aureus has evolved a delicate system for heme acquisition from the host. This system contains nine proteins and is regulated by iron and is designated as the iron-regulated surface determinants or Isd system. This system consists of cell wall-linked proteins (IsdB, IsdH, IsdA, and IsdC, ABC transporter IsdDEF), and heme monooxygenases IsdG and IsdI (34). Our laboratory has established the pathway of heme acquisition by this system (35). As shown in Fig. 1, IsdB captures metHb and extracts its heme from it and IsdA and IsdC then relay heme from IsdB to IsdE, where heme is

transported across the cytoplasmic membrane by IsdDF. IsdG and IsdI degrade protoporphyrin to release iron for intracellular use (36).

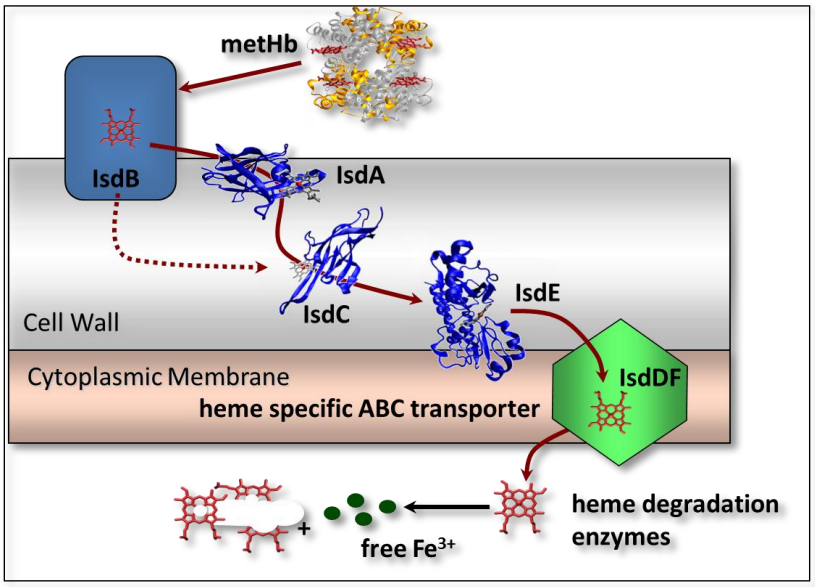


Figure 1: The heme acquisition pathway by the Isd system in S. aureus (Zhu et al., J Biol Chem 2008).

Assimilation of Hemoglobin Heme by the *S. aureus* Hemogolobin Receptor IsdB

IsdB is the key protein in the heme acquisition pathway of the *S. aureus* Isd system. IsdB is a receptor of human metHb on the S. aureus surface (18). This protein can rapidly and efficiently acquire heme from metHb in vitro and donate its heme directly to IsdA and IsdC but not to IsdE (36). This protein has two NEAT (NEAr Transporter) domains that are divided into 5 domains or regions: the N-terminal domain (ND), NEAT 1 domain (N1), middle domain (MD), NEAT 2 domain (N2), and C-terminal domain (CD). How these domains contribute to the rapid and efficient heme acquisition of the metHb heme by IsdB is not well understood.

Hypothesis and Objectives

I hypothesize that the different domains of IsdB contribute to the kinetics and or equilibrium of the metHb-to-IsdB heme transfer reaction. The objective of this study is to test this hypothesis by measuring the kinetics and equilibrium of the reactions of metHb with truncated IsdB fragments to identify the minimum region of IsdB for rapid and/or efficient heme transfer. Reconstitution of rapid and/or efficient heme transfer reactions with different combinations of IsdB fragments will be used to determine the roles of IsdB domains in the kinetics and thermodynamics of the reaction.

MATERIALS AND METHODS

Gene Cloning

IsdB protein is divided by two NEAT domains into five domains, ND, N1, MD, N2, and CD. The DNA fragments of the *isdB* gene encoding one or more of these domains were PCR amplified from the *isdB* gene of S.*aureus* strain MW2 with the primers listed in Table 1. PCR products were inserted into pET-21d at the NcoI and BamHI or EcoRI sites (Table. 1). Ligated recombinant plasmids were introduced into competent cells of *Escherichia coli* strain NovaBlue. *E. coli* transformants were screened by PCR, and desired clones were confirmed by digesting relevant plasmids and by PCR using plasmids as templates.

Table 1 Primers and Clone sites Used for PCR Cloning *isdB* Fragments

Fragment	Forward primer	Reverse primer	Clone site
ND-N1	TACCATGGAAGCAGC AGCTGAAGAAACA	TGGATCCTTATTCAGT TTTGAATTTATCTGC	NcoI, BamHI
ND-N1-MD	TACCATGGAAGCAGC AGCTGAAGAAACA	TGGATCCTTATGTTGG TTGTACATTTTG	NcoI, BamHI
N1-MD-N2	ACCATGGGCGCACCA AACTCTCGTC	TCGAATTCTTAATTGG CTTTTGTAAATGC	NcoI, EcoRI
N1-MD	ACCATGGGCGCACCA AACTCTCGTC	TGGATCCTTATGTTGG TTGTACATTTTG	NcoI, BamHI
MD-N2	TACCATGGAAGATTAT AAAGCTGAAAA	TCGAATTCTTAATTGG CTTTTGTAAATGC	NcoI, EcoRI
MD	TACCATGGAAGATTAT AAAGCTGAAAA	TGGATCCTTATGTTGG TTGTACATTTTG	NcoI, BamHI

Table 1 Continued

N2	TACCATGGCAAATGAA AAAATGACTGAT	TCGAATTCTTAATTGG CTTTTGTAAATGC	NcoI, EcoRI
N1	ACCATGGGCGCACCA AACTCTCGTC	TGGATCCTTATTCAGT TTTGAATTTATCTGC	NcoI, BamHI
ND-N1-MD-N2	TACCATGGAAGCAGC AGCTGAAGAAACA	TCGAATTCTTAATTGG CTTTTGTAAATGC	NcoI, EcoRI

Protein Purification

General Procedure

Plasmid for each IsdB fragment was introduced into *E. coli* BL21, and a resulting transformant of *E. coli* BL21 was streaked on LB plates with ampicillin (100 mg/L), incubated at 37 ℃ overnight, and inoculated into 4 L of Luria-Bertani broth supplemented with 80 mg/liter ampicillin to optical density at 600 nm of 0.5. Next, 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression for 6 more hours. *E. coli* cells were harvested by centrifuge at 1372 RCF for 10 min. Cell pellets were stored at -20 ℃. Upon purification, cell walls were broken by sonication for 20 min on ice in Tris-HCl buffer at different concentrations or pH for different IsdB fragment proteins (details in below). Soluble proteins were separated from cell debris by centrifuge at 25200 RCF for 20 min. All IsdB fragment proteins were contained in the supernatant in soluble forms. Iron-exchange and hydrophobic chromatography were applied to isolate the IsdB fragment proteins based on each IsdB fragment (details in below). After purification, proteins were dialyzed against 3 L Tris-

HCl buffer (20 mM, pH=8.0) overnight. All of the proteins did not have any tag at the amino or carboxyl termini and were purified to >80 % purity.

Purification of MD

The supernatant (80 ml) containing the MD domain was loaded onto an SP column (2.5 × 10 cm). The column was washed with 100 ml of Tris-HCl buffer (20 mM, pH=8.0) until UV absorbance reached baseline and then eluted with a 340-ml linear gradient of 0-0.1 M NaCl. Fractions containing MD were pooled, and $(NH_4)_2SO_4$ was added into the sample to 2 M. The sample was loaded onto a phenyl sepharose column (2.5 x 2.5 cm). The column was washed with 100 ml of 2 M $(NH_4)_2SO_4$ and eluted with a 50-ml gradient of 2 to 0 M $(NH_4)_2SO_4$. Fractions containing MD with >80% purity were pooled and dialyzed against 20 mM Tri-HCL buffer.

Purification of N1

N1 was purified from inclusion bodies. The inclusion bodies were dissolved in 8 M urea, and the denatured N1 was loaded onto an SP sepharose column (1.5 x 5 cm) that was pre-equilibrated with 6 M urea. The column was washed with 30 ml 6 M urea and eluted with a 60-ml gradient of 0 to 100 mM NaCl in 6 M urea. Fractions containing N1 with >90% purity were pooled, and the pooled sample was dialyzed against 20 mM Tris-HCl.

Purification of N2

N2 lysate was loaded onto an SP sepharose column (2.5 x 10 cm), washed with 20 mM Tris HCl, and the flow through was collected. The flow through was then loaded

onto a DEAE sepharose column and eluted with a 120-ml gradient of 0 to 0.2 M NaCl. Fractions containing N2 were pooled and dialyzed against 20 mM Tri-HCL buffer.

Purification of ND-N1

ND-N1 lysate was loaded onto an SP sepharose column (2.5 x 5 cm). The column was washed with 150 ml 20 mM Tris-HCl and eluted with a 200-ml gradient of 0-75 mM NaCl and 40 ml 75 mM NaCl. Fractions containing ND-N1 was pooled, and $(NH_4)_2SO_4$ was added to the pool to 1.5 M. The sample was loaded onto a phenyl sepharose column (1.0 x 5 cm). The column was washed with 60 ml 1.5 M $(NH_4)_2SO_4$ and eluted with a 120-ml gradient of 1.5 to 0.5 M $(NH_4)_2SO_4$. Fractions containing ND-N1 with >90% purity were pooled and dialyzed against 20 mM Tri-HCL buffer.

Purification of N1-MD

The lysate of N1-MD containing the amino acids 122-338 (40 ml) was loaded onto a SP column (2.5 × 10 cm). The column was washed with 200 ml Tris-HCl buffer (20 mM, pH=8.0) until the A_{280} reading dropped to baseline and then eluted with a 250-ml linear gradient of 0.0 to 0.1 M NaCl. Fractions containing N1-MD were pooled and dialyzed against 20 mM Tris-HCl overnight.

Purification of MD-N2

MD-N2 was expressed as a mixture of the heme-binding form (holo-MD-N2) and the heme-free form (apo-MD-N2). MD-N2 lysate was loaded onto a DEAE sepharose column (1.5 x 7 cm) and washed with 20 ml 20 mM Tris-HCl to collect the flowthrough. The flowthrough was loaded onto an SP sepharose column (2.5 x 8 cm), and the column

was washed with 100 ml Tris-HCl and eluted with a 100-ml gradient of 0 to 100 mM NaCl. Fractions containing apo-MD-N2 were pooled, and the pool was adjusted to 2 M $(NH_4)_2SO_4$ and loaded onto a phenyl sepharose column (1.5 x 7 cm). The column was washed with 50 ml 2M $(NH_4)_2SO_4$ and then eluted with a 100-ml gradient of 2.0 to 1.0 M $(NH_4)_2SO_4$. The fractions containing apo-MD-N2 with >90% purity were pooled and dialyzed against 20 mM Tri-HCL buffer.

Purification of ND-N1-MD

ND-N1-MD lysate was dialyzed against 3 l of 10 mM Tris HCl, pH 7.0, for 4 h and then loaded onto a SP sepharose column (2.5 x 5 cm). The column was washed with 100 ml 10 mM Tris HCl, pH 7.0, and then eluted with a 120-ml gradient of 0 to 0.2 M NaCl in Tris-HCl, pH 7.0. Peak fractions containing ND-N1-MD were pooled and dialyzed overnight in 20 mM Tris HCl, pH 8.0. The dialyzed sample was loaded onto a DEAE sepharose column (2.5 x 5 cm). The column was washed with 20 mM Tris HCl, and the protein was eluted with a 60-ml gradient of 0 to 0.04 M NaCl. The flowthrough and peak fractions were pooled, adjusted to 1.5 M $(NH_4)_2SO_4$ and loaded onto to a phenyl sepharose column (1 x 5 cm). The column was eluted with a 50-ml gradient of 1.5 to 0.5 M $(NH_4)_2SO_4$. Fractions containing ND-N1-MD with >90% purity were pooled and dialyzed against 10 mM NaCl, 20 mM Tri-HCL buffer..

Purification of ND-N1-MD-N2

The ND-N1-MD-N2 supernatant was dialyzed against 4 L of 5 mM Tris-HCl, pH 7.0, for 4 hours. The sample was loaded onto an SP column (2.5 × 10 cm). The column

was washed with 100 ml 5 mM Tris-HCl and then eluted with a 250-ml linear gradient of 0.0 to 0.1 M NaCl. Fractions containing ND-N1-MD-N2 were pooled and dialyzed against 20 mM Tris-HCl overnight.

Preparation of Apo-H64Y/V68F Myoglobin

Recombinant H64Y/V68F myoglobin was purified, as previously described (37). Apo-H64Y/V68F myoglobin was prepared by the methyl ethyl ketone method (38). Briefly, a holo-H64Y/V68F myoglobin sample was adjusted to A_{280}=0.5 and to pH 3.0 with HCl and mixed with an equal volume of methyl ethyl ketone by vortex. The sample was centrifuged, and the solution at the bottom layer was collected and then dialyzed against 4 L of water for 2 hours then against 4 L of 20 mM Tris-HCl overnight.

Preparation of Holo-N2

N2 was purified in its heme-free form (apo-N2). Its heme-binding form (holo-N2) was reconstituted from apo-N2 with hemin as described (39). Briefly, apo-N2 was incubated with excess hemin for 15 min, and holo-N2 was separated from free hemin using a G-25 Sephadex column (1 ×30 cm).

Determination of Protein Concentrations and Heme Contents

Protein concentrations were determined using a modified Lowry protein assay kit with bovine serum albumin as a standard. Heme contents of holo-IsdB proteins and

metHb were measured using the pyridine hemochrome assay (ε_{418} = 191.5 mM^{-1} cm^{-1}) (37).

Measurement of Passive Heme Dissociation Using H64Y/V68F Apo-Myoglobin

Passive heme dissociation from holo-N2 or metHb in the absence or presence of MD or ND-N1-MD was measured using H64Y/V68F apomyoglobin as a heme scavenger (31). Absorbance readings at 600 nm (A_{600nm}) was measured as a function of time following the mixing of 2 μM holo-N2 or metHb, MD or ND-N1-MD at the indicated concentrations, and 40 μM H64Y/V68F apomyoglobin. Time-dependent changes in absorption (ΔA_{600}) were used to assess the kinetics of the heme dissociation reaction.

Kinetics of Hemin Transfer

The rates of slow hemin transfer from metHb to apo-N2, MD-N2, or N1-MD-N2, ND-N1-MD-N2, or apo-IsdB were measured by monitoring the absorbance changes using a conventional spectrophotometer (SPECTRAmax 384 Plus, Molecular Devices). Each holo-protein was incubated with apo-protein at concentrations > 5 times the holo-protein concentrations, and the absorbance changes at the indicated wavelengths were monitored for up to 6 h.

The rates of heme disassociation assays from holo-N2 to apo-myoglobin with or without the presence of other IsdB fragments (ND-N1-MD, MD, or ND-N1), and the heme disassociation assays from met-hemoglobin to apo-myoglobin with or without the presence of ND-N1-MD, were examined by monitoring the absorbance changes using a

conventional spectrophotometer (SPECTRAmax 384 Plus, Molecular Devices). Each holo-protein was incubated with apo-protein at concentration > 5 times the holo-protein's concentration, and the absorbance changes at the indicated wavelengths were monitored for up to 6 h.

Estimation of Transferred Heme in Heme Transfer Reactions

Absorption spectra of metHb, apo-IsdB protein constructs, and their mixtures were recorded before mixing or at 30 min and 12 h following mixing to monitor the rapid and slow heme transfer reactions, respectively. Amounts of transferred metHb heme were estimated using extinction coefficients at 406 nm of 1.7×10^5, and 1.15×10^5 M^{-1} cm^{-1} for metHb- and N2-bound heme, respectively.

Size-Exclusive Chromatography

Holo- or apo-N2 (47 μM) was incubated with 30 μM MD for 10 min, and the mixture were loaded on a Tricorn superdex75 10/300 GL column and eluted by 20 mM Tris-HCl buffer (pH 8.0) on a Biologic Duoflow Chromatography System (Bio-Rad). Control experiments were also performed for apo-N2/MD and N1/MD mixtures and each of holo-N2, apo-N2, N1, and MD alone. Elution profiles of A_{280} were measured.

RESULTS

Recombinant IsdB Fragments

The primary sequence of IsdB is comprised of an N-terminal secretion signal sequence, the mature IsdB protein, and a C-terminal end consisting of a transmembrane domain and a short C-terminal stretch rich in positively and negatively charged residues (Figure 2A). The N- and C-terminal ends are both cleaved to generate the mature form of IsdB that is anchored to the cell wall at the newly processed C-terminal ends (40). The mature IsdB protein is comprised of two NEAT domains (N1 and N2) that segment the protein into five regions or domains: N-terminal domain (ND) (amino acids 40-121), N1 (amino acids 122-270), middle domain (MD) (amino acids 271-338), N2 (amino acids 339-458), and C-terminal domain (CD) (amino acids 459-613) (Figure 2A). To identify the domains of IsdB that critically contribute to the kinetics and equilibrium of the apo-IsdB/metHb reaction, we prepared the following recombinant IsdB fragments: N1, MD, N2, ND-N1, N1-MD, MD-N2, ND-N1-MD, N1-MD-N2, and ND-N1-MD-N2. All of these fragments except N1 were expressed in soluble form and were tag-free. The MD-N2, N1-MD-N2, and ND-N1-MD-N2 fragments were produced both as heme-bound holo- and heme-free apo-forms, and the apo-form of each fragment could be separated from its holo-form by chromatography. These fragments were purified to >80% of purity according to SDS-PAGE analysis (Figure 2B).

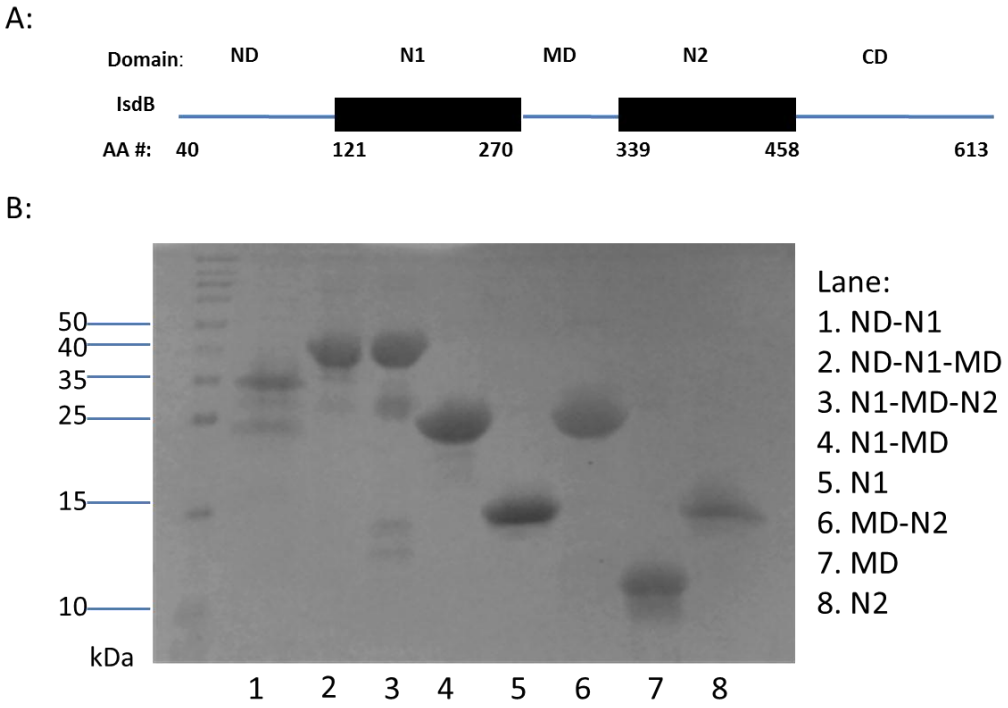


Figure 2: Recombinant IsdB fragments. (A) A schematic diagram showing the structures of IsdB domains. The protein contains the N-terminal (ND), NEAT 1 (N1), middle (MD), NEAT 2 (N2), and C-terminal (CD) domains. Secretion signal domain, cleaved transmembrane domain and charged tail are not shown in the figure. The numbers are the numbers of amino acid residues at the start and end of the indicated domains. (B) SDS-PAGE analysis of purified recombinant IsdB fragments.

N2 is The Heme-Binding Domain of IsdB

To determine whether both ND-N1-MD and N2 are both involved in heme binding, ND-N1-MD and N2 were incubated with excess hemin and separated from free hemin by Sephadex G-25 chromatography. Samples from the elutions were collected. Spectra of treated ND-N1-MD exhibit absorbance at 300-420 nm, which is more similar to the spectrum of free hemin (Figure 3A). The treated N2 displays UV-Vis spectral characteristics that are identical to those of holo-IsdB (Figure 3B). The samples were

subject to SP sepharose chromatography, to see whether ND-N1-MD really loosely binds hemin. The ND-N1-MD sample, but not N2, eluted from the SP sepharose column lost most of the associated heme. These results indicate that recombinant N2 contains the heme-binding site of IsdB, confirming previous findings (19), and that ND-N1-MD can only loosely bind hemin without the formation of axial bonds.

Inefficient and Indirect Heme Acquisition from Methemoglobin by N2

Since N2 is the heme-binding domain of IsdB, we investigated whether N2 alone can efficiently and rapidly acquire heme from metHb. A heme transfer assay from metHb to apo-N2 was performed, and kinetics and equilibrium of the apo-N2/metHb reaction were measured. Since the coefficient extinctions of the Soret peak of metHb and holo-N2 are different, the Soret peak of the reaction mixture should shift from that of metHb to that of holo-N2 if the reaction efficiently occurs. After 12 hours, the spectrum of the apo-N2/metHb mixture exhibited a peak at 405 nm, which was slightly lower than that of metHb and higher than that of holo-N2 (Figure 3C). Calculation indicated that 10% of the metHb heme was transferred to N2. This transfer efficiency was just $1/7^{th}$ of that of the metHb/IsdB reaction (41). Kinetic analysis of the metHb/apo-N2 reaction indicates that the heme transfer process was not only inefficient but also very slow. The observed rate constants of the metHb/apo-N2 reaction were almost identical to those of passive heme dissociation from metHb as measured using apo- H64Y/V68F myoglobin (Figure 3D). Heme can be transferred directly from a donor to an acceptor. Alternatively, a heme donor can release heme first, and the released heme is scavenged by the heme acceptor.

The later mechanism for heme loss from metHb is slow and has two kinetic phases (41). The low and biphasic heme transfer from metHb to apo-N2 indicates an indirect heme transfer reaction. Therefore, we conclude that apo-N2 alone cannot directly and efficiently acquire heme from metHb.

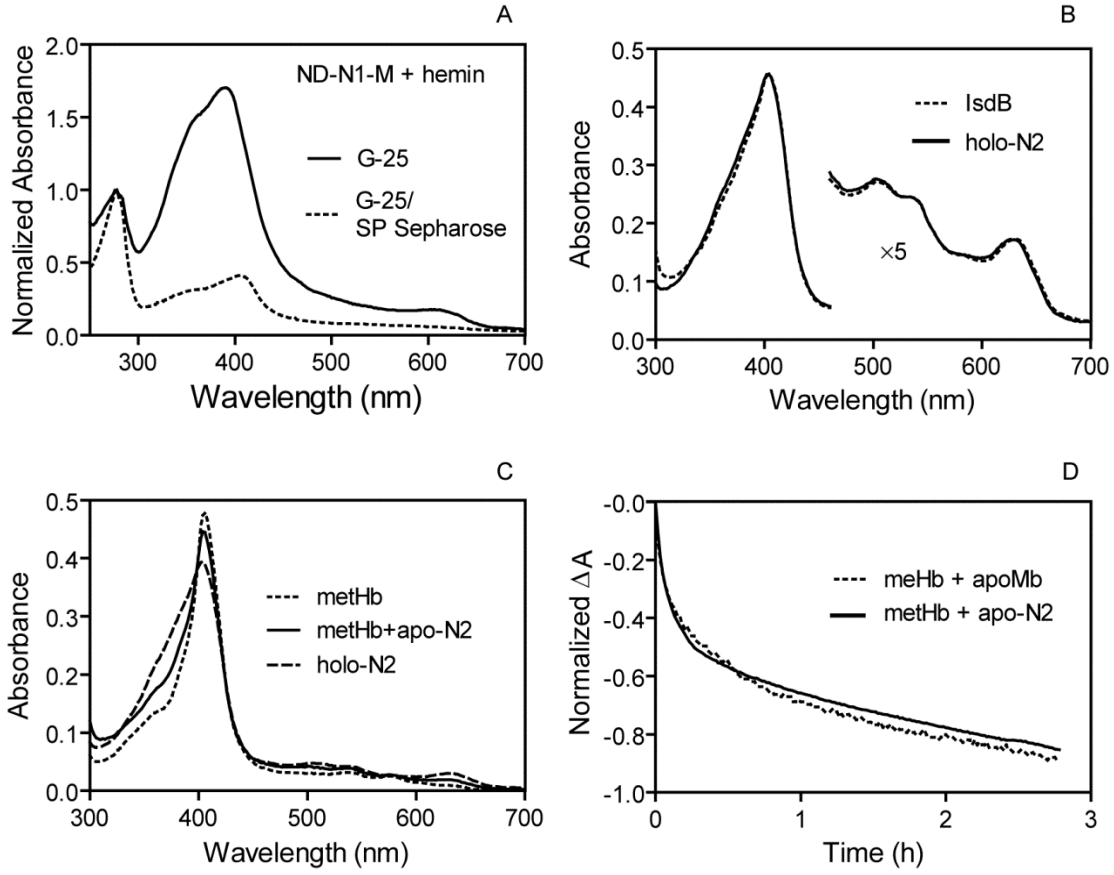


Figure 3: Non-specific and specific binding to heme of N1, N2, and slow, inefficient heme transfer from metHb to N2. (A) Absorption spectra of N1 after passing a N1/excess hemin mixture through G-25 Sephadex column and subsequent SP Sepharose chromatography. (B) Absorption spectrum overlay of holo-IsdB and holo-N2 at 4 μM. (C) Absorption spectrum comparison of metHb (3 μM heme, metHb/30 μM apo-N2 after 12 h incubation, and 3 μM holo-N2. (D) Time course of normalized ΔA at 406 nm associated with partial heme transfer from 3 μM metHb heme to apo-N2 (30 μM). Time course for the passive heme loss of 3 μM metHb to H64V/H68F apo-myoglobin (40 μM) is included for comparison.

The Minimal Region of IsdB for Rapid and Efficient Heme Acquisition from MetHb

Since the metHb/apo-N2 reaction was very slow and inefficient, we hypothesize that the rapid heme transfer from metHb to IsdB needs the assistance of IsdB domains other than N2. We first tested this hypothesis by determing the minimal region of IsdB for rapid and efficient heme acquisition from metHb. MetHb reacted with the apo-form of each of N2, MD-N2, N1-MD-N2, ND-N1-MD-N2, and full-length IsdB. The time course of spectral change in each reaction was fit to an exponential expression to obtain the rate constant for the heme transfer reaction dissociation. The percentage of transferred metHb was also calculated from the spectral changes. Except the metHb/apo-N2 reaction, the percentages of transferred metHb heme in the reactions of metHb with apoMD-N2, N1-MD-N2, and ND-N1-MD-N2 were similar to those of the metHb/IsdB reaction and about 7-fold higher than that of the metHb/apo-N2 reaction, indicating the minimum region for efficient heme transfer was MD-N2. However, the metHb/apo-MD-N2 reaction was biphasic with two observed rate constants, $0.0031s^{-1}$ and $0.00023s^{-1}$, which were similar to those of the passive heme transfer from metHb to apo-H64Y/V68F myoglobin (Table 2). Thus, MD-N2 lost the ability of IsdB to rapidly acquire heme from metHb. The metHb/N1-MD-N2 reaction was still biphasic although the observed rate constants, $0.025\ s^{-1}$ and $0.00191\ s^{-1}$, were about 10-fold greater than those of the metHb/apo-N2 reaction. In contrast, the reaction of metHb with ND-N1-MD-N2 displayed a single kinetic phase with a rate constant of $0.28\ s^{-1}$, which was comparable to that ($0.31\ s^{-1}$) of the metHb/IsdB reaction. Thus, we conclude that ND-N1-MD-N2 is the

minimal region for the rapid heme transfer, while N1 has effects to enhance the reaction's speed, and MD-N2 is the minimal structure required for a high efficiency of the heme transfer reaction.

In Trans Rescue of Rapid and Efficient Heme Transfer from MetHb to N2 by ND-N1-MD

Since ND-N1-MD-N2 is the minimal region of IsdB for the rapid and efficient heme acquisition, the ND-N1-MD region must play roles in the rapid and efficient heme transfer reaction. To further examine the role of this region in the rapid and efficient heme transfer reaction, we determined whether the ND-N1-MD fragment can restore the rate and efficiency of the metHb/apo-N2 to those of the metHb/IsdB reaction. A rapid shift from the spectrum of metHb to that of holo-N2 was observed within 16 s after mixing metHb with apoN2 and ND-N1-MD (Figure 4B) but not after mixing metHb with apoN2 (Figure 4A). The spectral change of the metHb/apo-N2/ND-N1-MD reaction had a single kinetic phase (Figure 4C) with an observed rate constant of 0.21 s-1, and the spectrum of the reaction almost overlapped with that of holo-N2 (Figure 4D), indicating that ND-N1-MD enhanced the efficiency of the heme transfer reaction. These results indicate that ND-N1-MD can rescue in trans the metHb/apoN2 in kinetics and equilibrium.

Linkage between ND-N1 and MD is Required for the Kinetic Rescue of the MetHb/Apo-N2 by the ND-N1-MD

We further examined the features of the ND-N1-MD region that are required in the kinetics and equilibrium rescue of the metHb/apo-N2 reaction. As summarized in Table 2, either MD and ND-N1 or their combination could not rescue the kinetics of the metHb/apoN2 reaction. In addition, N1 could not rescue the kinetics of the metHb/MD-N2 reaction. Therefore, both MD and ND-N1and the linkage between MD and ND-N1 are required to rescue the kinetics of the metHb/apo-N2 reaction.

Table 2. Apparent Rate Constants and Heme Transfer Efficiency in Various Reactions

heme donor	heme acceptor	k or k_1 (s^{-1})[a]	k_2 (s^{-1})	*transferred heme%*
metHb	IsdB[b]	0.31		72
	ND-N1-MD-N2	0.28		70
	N1-MD-N2	0.025	0.00191	64
	MD-N2	0.0031	0.00023	67
	N2	0.0030	0.00019	10
	N2/ND-N1-MD	0.21		78
	N2/ND-N1	0.0039	0.00047	15
	N2/ND-N1/MD	0.0034	0.00033	69
	N2/N1	0.0031		13
	N2/MD	0.0025	0.00024	68
	MD-N2/ND-N1	0.0046	0.00033	65
	H64Y/V68F Mb[c]	0.0034	0.00020	
	H64Y/V68F Mb/ND-N1-MD	0.0037	0.00040	
N2	H64Y/V68F Mb[c]	0.0034		
	H64Y/V68F Mb/ND-N1-MD[c,d]	0.00014		

[a]The data were obtained using 3 mM heme donor and 20 mM each component in heme acceptor mixtures unless specified otherwise.
[b]Data were from ref 42.
[c]40 μM H64Y/V68F Mb was used. [d]8 μM ND-N1-MD was used.

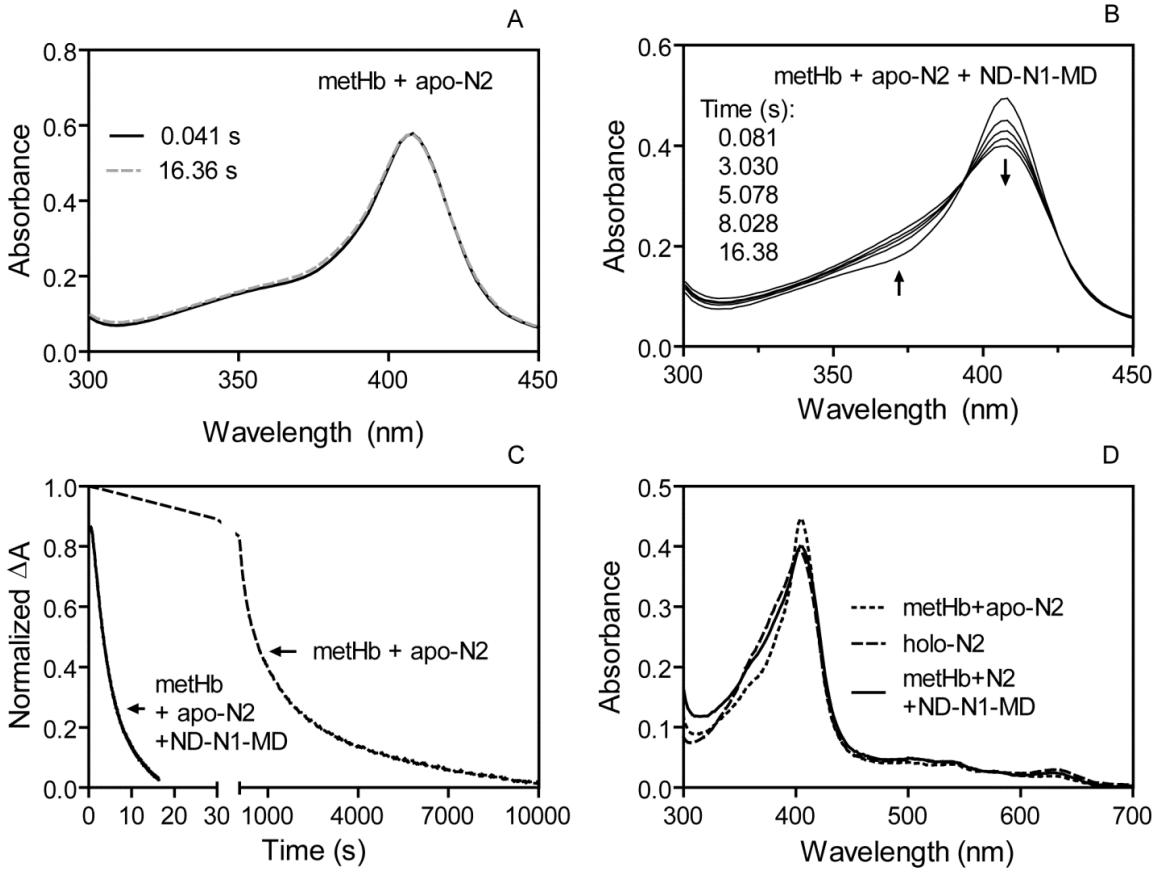


Figure 4: ND-N1-MD enhances the rate and efficiency of heme transfer from metHb to N2. (A) No shift in the spectrum of a mixture of 3.0 μM metHb heme and 30 μM N2 at the indicated times after mixing in a stopped-flow spectrophotometer. (B) Shift of absorption spectra after mixing 3 μM metHb heme with 30 μM apo-N2 and 30 μM ND-N1-MD. The arrows indicate the directions of the spectral shift during the reaction. (C) Time course of normalized spectral changes in the 3.0 μM metHb heme/30 μM apo-N2 reactions in the absence and presence of 30 μM ND-N1-MD. (D) Overlay of the absorption spectra of metHb/apo-N2 after 12-h incubation, holo-N2, and metHb/apo-N2/ND-N1-MD after 30-min incubation.

In Trans Rescue of Efficient Heme Transfer from MetHb to N2 by MD

The earlier data indicate that MD-N2 is the minimal region for efficient heme transfer from metHb to N2, suggesting that MD is able to change the equilibrium of the metHb/N2 reaction. I hypothesize that MD interacts with N2 to drive the heme transfer from metHb to N2. If the hypothesis is correct, MD can influence the equilibrium of the

metHb/N2 reaction in trans. To examine in trans rescue of the efficient heme transfer from metHb to N2 by MD, the metHb/N2 transfer reaction was performed in the presence and absence of MD, ND-N1-MD, or ND-N1, and the in trans rescue reactions were also compared with the metHb/MD-N2 reaction. The metHb/apo-N2/ND-N1-MD and metHb/apo-N2/MD reactions exhibited similar final spectral profiles (Figure 5A), and 78% and 68% of metHb heme was transferred to N2 tin the presence of ND-N1-MD and MD, respectively. The metHb/apo-N2/ND-N1 reaction transferred only 15% of metHb heme to N2, which was similar to the efficiency of heme transfer in the metHb/apo-N2 reaction (10%). These data indicate that MD, but not ND-N1, is responsible for the high efficiency of the metHb-to-IsdB heme transfer reaction. The metHb/apo-N2/MD and metHb/apo-MD-N2 reactions had similar final absorption spectra (Figure 5B), indicating similar transfer efficiencies in the two reactions. Therefore, the covalent linkage of MD to N2 is not required for the efficient reactions, indicating that MD can in trans restore the efficiency of the metHb/apo-N2 to that of the metHb/IsdB reaction.

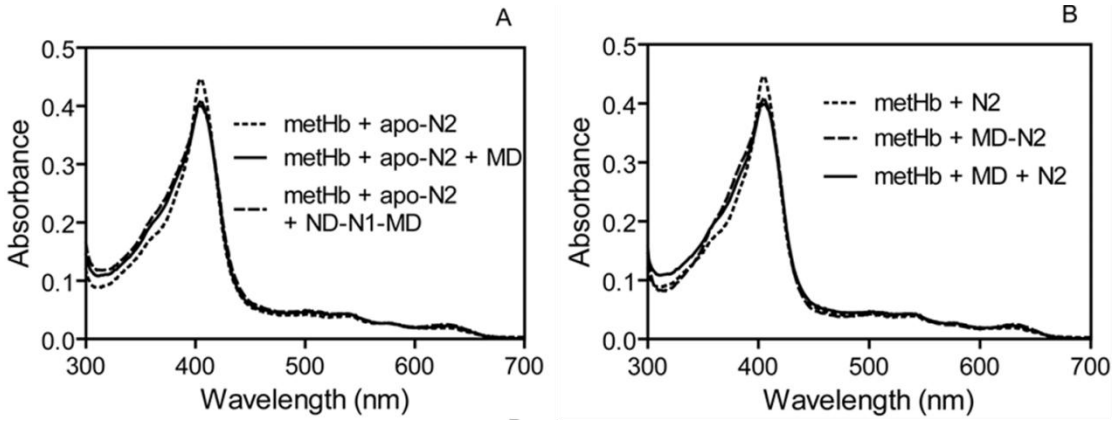


Figure 5: Effect of MD on the efficiency of the metHb/apo-N2 reaction. (A) Overlay of the spectra of the metHb/apo-N2, metHb/apo-N2/MD, and metHb/apo-N2/ND-N1-MD. (B) Overlay of the spectra of the metHb/apo-N2, metHb/apo-MD-N2, and metHb/apo-N2/MD.

MD Slows Down the Passive Dissociation of Heme from Holo-N2

There are two possible reasons for the effect of ND-N1-MD and MD on the equilibrium of the metHb/apo-N2 reaction. First, ND-N1-MD and MD could increase the affinity of N2 for heme. Second, ND-N1-MD and MD could reduce the affinity of metHb for heme. To investigate these possibilities, we examined the effect of ND-N1-MD and MD on the passive heme dissociation from holo-N2 and metHb using apo-H64Y/V68F myoglobin as a heme scavenger. We recorded the time courses of heme dissociation from holo-N2 in the presence of ND-N1-MD at different concentrations (Figure 6A). The dissociation of heme from holo-N2 was completed within 2500 s in the absence of ND-N1-MD. Addition of ND-N1-MD slowed down the heme dissociation from holo-N2, and this slowdown depended on the ND-N1-MD concentration (Figure 6A). In contrast, ND-N1-MD had no effect on the heme dissociation from metHb (Figure 6B). These results indicate that ND-N1-MD affects the affinity of N2 for heme but not the affinity of metHb for heme. The effect of MD on the heme association from holo-N2 was similar to that of ND-N1-MD (Figure 6C), and the effect of MD was specific because ND-N1 did affect the heme dissociation from holo-N2 (Figure 6D). Thus, MD drives the heme transfer from metHb to N2 by increasing the affinity of N2 for heme by slowing down heme dissociation from N2.

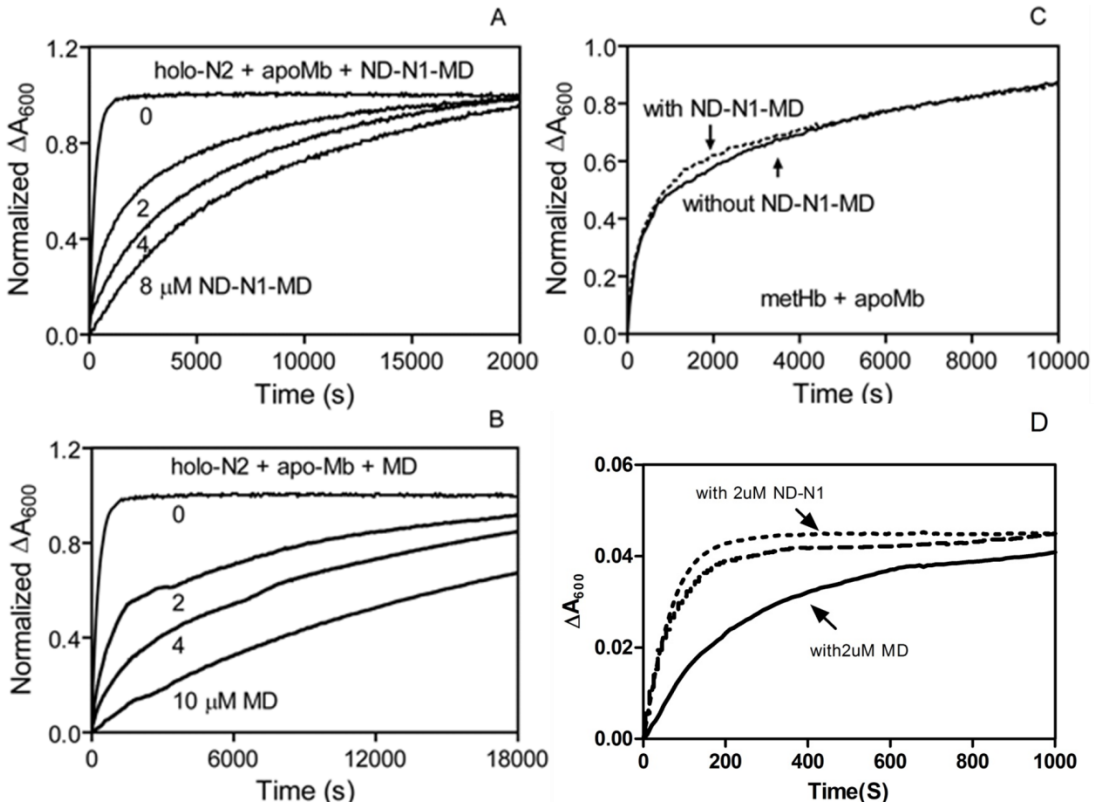


Figure 6: Effects of ND-N1-MD and MD on heme dissociation from holo-N2. (A and B) Time courses of normalized ΔA_{600} measuring heme association from holo-N2 using 40 μM H64V/H68F apo-myoglobin as a heme scavenger in the presence of various ND-N1-MD (A) or MD (B) concentrations. (C) Heme dissociation from metHb in the absence and presence of 30 μM ND-N1-MD as measured using H64V/H68F apo-myoglobin as heme scavenger. (D) Heme dissociation from holo-N2 in the presence of 2 μM ND-N1 or in the presence of 2 μM MD.

Analysis of MD/N2 Interaction by Size Exclusion Chromatography

The earlier data on the in trans rescue of the metHb/apo-N2 reaction in kinetics and equilibrium by ND-N1-MD and MD implies that MD can interact with N2. We examined the MD/N2 interaction using size exclusion chromatography. The elution profile of the MD/apo-N2 mixture displayed two elution peaks that overlapped perfectly

with the elution peaks of MD and apo-N2 alone, indicating that there was no detectable stable interaction between MD and apo-N2 (Figure 7A). In contrast, a mixture of MD and holo-N2 had a shifted holo-N2 peak and reduced holo-N2 peak compared with the elution profiles of MD and holo-N2 alone (Figure 7C). However, there is not a new peak for a stable MD-holo-N2 complex. In controls, N1 had no effect on the elution profile of apo-N2 or holo-N2 (Figure 7B and D). These results suggest that an interaction between MD and holo-N2 was detectable but did not lead to a tight MD/holo-N2 complex.

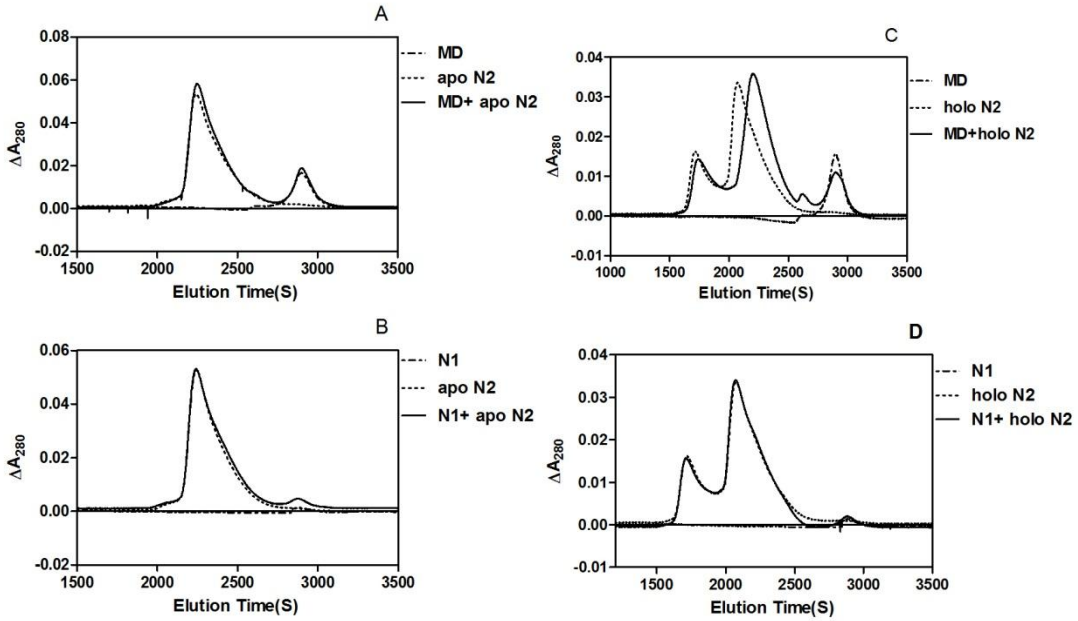


Figure 7: Direct interactions between MD and holo-N2. (A and B) Chromatogram overlay of 30μM MD, 46.8μM apo-N2, and mixture of MD/apo-N2 (A), and 30μM N1, 46.8μM apo-N2, mixture of N1/apo-N2 (B), (C and D) Chromatogram overlay of 30μM MD, 46.8μM holo-N2, and mixture of MD/holo-N2 (C), and 30μM N1, 46.8μM holo-N2, mixture of N1/holo-N2 (D). The concentrations of fragments in each mixture, are equal to the concentrations of the runs in their alone.

DISCUSSION

Iron is an essential nutrient for the growth of *S. aureus*. Previous studies have already demonstrated that *S. aureus* assimilates heme from hemoglobin as an iron source through the Isd system and that IsdB functions as a metHb receptor and directly extract heme from metHb (42). However, the mechanism of heme transfer from metHb to IsdB remains unknown. In this project, we investigated the roles of different IsdB domains in the kinetics and equilibrium of the metHb/IsdB heme transfer reaction.

We found that ND-N1-MD-N2 is the minimal region of IsdB for the rapid heme assimilation from metHb and promises high heme-transfer efficiency. This finding indicates the CD domain is not necessary in the rapid and efficient heme transfer from metHb to IsdB. IsdB, IsdA, and IsdC are covalently linked to the bacterial cell wall at the C-terminus (43, 44). IsdA and IsdC also have a CD domain, and the length of the CD domain in these proteins is in the order of CDIsdB > CDIsdA > CDIsdC. These would arrange the heme-binding domains across the cell wall envelope to allow relay through the cell wall (Figure 1). Thus, we propose that the function of CD domain of IsdB is to place the other domains of IsdB at right position to be able to extract heme from metHb and then donate its heme to IsdA and then to IsdC.

In accordance with a previous finding (45), N2 alone is unable to efficiently acquire heme from metHb. MD alone or linked to N2 restores the efficiency of heme transfer from metHb to N2 to levels that are similar to those in the reaction of metHb with intact IsdB, illustrating an important role of MD in driving the reaction to the formation of the heme transfer products. Moreover, MD slows down the heme release

from holo-N2. These observations suggest that MD drives the equilibrium of the metHb/IsdB reaction to the formation of holo-IsdB by enhancing the affinity of IsdB for heme by slowing down heme dissociation from N2.

The other major finding in this study is that the ND-N1 and its linkage to MD is critical for the rapid kinetics of the metHb/IsdB reaction. ND-N1-MD is able to dramatically enhance the rate of heme transfer from metHb to N2 but has no effect on heme dissociation from metHb, suggesting that IsdB directly extracts heme from metHb, and ND-N1 plays a critical role in the direct extraction process. IsdH, another metHb receptor in *S.aureus's* Isd system, is also capable of acquiring heme from metHb rapidly (46). As Clubb et al. suggested, there is a release and capture mechanism in the metHb/IsdH reaction (46). IsdH rapidly assimilates heme from metHb, through a NEAT 2-NEAT 3 segment. NEAT 2 of IsdH interacts with metHb and facilitates its heme release, and the released heme is then picked up by the NEAT 3. The authors suggested that this mechanism also may be followed in the metHb/IsdB reaction (46). However, a recent study by Gell et al. solved the structure of a complex of metHb with NEAT2-NEAT3 fragment of IsdH, revealing the metHb/IsdH complex structure, implies that NEAT2 of IsdH binds to metHb to position NEAT 3 to facilitate the direct assimilation of NEAT 3 from metHb (47). Thus, this mechanism of direct heme assimilation may have a wide range of applications, and the metHb/IsdB, and metHb/IsdH reactions use a similar mechanism to extract heme from metHb. The interaction of streptococcal surface protein Shp and HtsA is another analogy of heme transfer to the metHb/IsdB reaction. In the Shp/HtsA reaction, the heme axial residues in heme acceptor HtsA specifically displace

the axial residues in the heme donor Shp (48-51). Together with our finding, we conclude that heme is transferred directly through direct protein-protein interaction but not by a release and capture mechanism.

Our data also suggest that inter- and intra-protein interactions are very important for rapid and efficient heme transfer. First, the interaction between MD and N2 dramatically enhances the affinity of IsdB for heme. The depletion of the linkage between MD-and N2 compromising neither the rapid nor the efficient heme transfer process may suggest the possibility, that MD has the ability to interact with N2. Our analysis from size-exclusive chromatography supports this possibility and further reveals that MD interacts stronger with holo-N2 than with apo-N2. The ND-N1 fragment is unable to change the rate of the metHb/N2 reaction, suggesting there is not a strong interaction between ND-N1 and N2, which is supported by our results using the size-exclusive chromatography. Since ND-N1-MD can in trans rescue the rapid and efficient heme transfer of the metHb/N2 reaction, ND-N1 must directly interact with metHb to enhance the heme transfer rate. However, we cannot detect stable interaction between metHb and ND-N1-MD, ND-N1-MD-N2, or intact IsdB in solution. The inability to detect this stable interaction implies that the metHb and IsdB interaction may be transient and weak, which is similar to the interaction between IsdA and IsdC (52).

CONCLUSION

This project was aimed at elucidating the role of the various domains or regions of the hemoglobin receptor IsdB of *S. aureus* in the rapid and efficient heme transfer from metHb to IsdB. Heme binding tests demostrated that the N2 domian is the heme-binding site for IsdB. N2 alone cannot rapidly acquire heme from metHb but indirectly and inefficiently scavenges heme dissociated from metHb. By examining the effects of a series deletion of various domain(s) on the kinetics and efficiency of heme transfer from metHb to IsdB, we found that ND-N1-MD-N2 is the minimal structure retaining the ability to rapidly and efficiently acquire heme from metHb and that MD-N2 is the minimal region of IsdB to efficiently but passively scavenge heme dissociated from metHb. These conclusions also imply that the CD domain of IsdB is not directly involved in the heme extraction reaction from metHb by IsdB. IsdA and IsdC also possess a CD domain next to their heme-binding NEAT domain. The CD domains of IsdB, IsdA, and IsdC are comprised of 155, 132, and 42 amino acids, respectively, and all these proteins are covalently linked to the bacterial cell wall at the C-terminal end of their CD domain. Our data suggest that the CD domain of Isd proteins functions as a spacer to position these proteins or their heme-accepting domain appropriately across the cell wall envelope to enable the sequential relay of heme across the cell wall from metHb → IsdB → IsdA → IsdC.

The MD domain can in trans restore the efficiency of the metHb/N2 heme transfer reaction to levels that were similar to those of the metHb/IsdB reaction, indicating that MD plays a critical role in driving equilibrium of the metHb/IsdB reaction toward the

formation of holo-IsdB. Interestingly, the MD domain slows down the dissociation of heme from holo-N2. These data support the notion that the affinity of the N2 domain for heme is not sufficiently strong for efficient heme abstraction and that the MD domain drives the equilibrium of the reaction toward the formation of holo-IsdB by increasing the affinity of the heme-binding domain of IsdB for heme via the lowering of heme dissociation from the heme-binding domain. Our findings suggest that the MD of the IsdB domain functions as a heme binding affinity switch. In this proposed model of affinity switching (Figure. 8), the MD domain of IsdB would interact with N2, increasing its affinity for heme to allow efficient capture and transfer of heme from metHb to IsdB, and subsequently, interactions between IsdB and IsdA could disrupt interactions between MD and N2, returning N2 to a lower the heme binding affinity state such that efficient heme transfer from IsdB to IsdA can occur.

Both the ND and N1 domains of IsdB are required for the rapid kinetics of the metHb-to-IsdB heme transfer. The ND-N1 fragment does not promote rapid dissociation of heme from metHb. Our data are consistent with a model that ND-N1 interacts with metHb to more efficiently bring N2 to the heme pockets in metHb for rapid, direct heme extraction by IsdB.

Our data also provide insight into inter- and intra-protein interactions that are critical determinants of the kinetics and equilibrium of the metHb/IsdB heme transfer reaction. One critical intra-protein interaction within IsdB is the interaction between the MD and N2 domains. This interaction is apparently sufficiently strong to alter the affinity of N2 for heme and mediate the effect of ND-N1 on the kinetics of the metHb/N2

reaction. The ND-N1 fragment could not enhance the rate of heme transfer from metHb to MD-N2, indicating that there exists no strong interaction between ND-N1 and MD-N2. Therefore, ND-N1 must interact directly with metHb to enhance the heme transfer rate. The ability of ND-N1 to enhance the rate of heme transfer from metHb to N2 may enhance specific metHb/N2 interactions.

Our findings support a functional model for the role of the different IsdB domains in the metHb/IsdB heme transfer reaction. In this model (Figure. 9), the CD domain of IsdB acts as spacer to position IsdB in the right location for relaying heme from metHb to the other Isd protein(s). The MD domain interacts with and enhances the affinity of N2 to thermodynamically drive heme transfer from metHb to IsdB, and ND-N1 enhances specific metHb/N2 interactions to facilitate direct extraction of heme by N2 from the heme pocket in metHb.

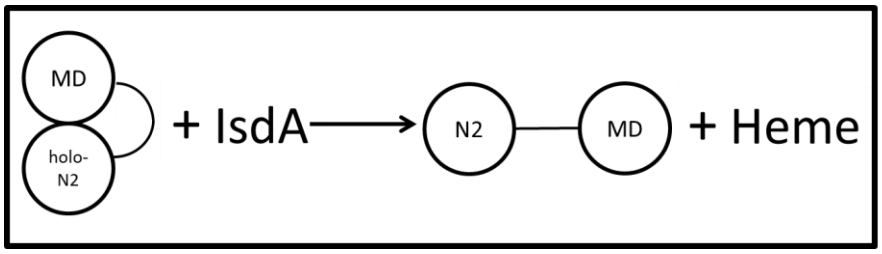


Figure 8: A model for MD to switch N2's affinity to heme. In this model, MD interacts with heme, increases its affinity to heme, after the presence of IsdA, the interaction is interrupted, returns N2's affinity to heme to a low affinity, and facilitates heme transfer from IsdB to IsdA.

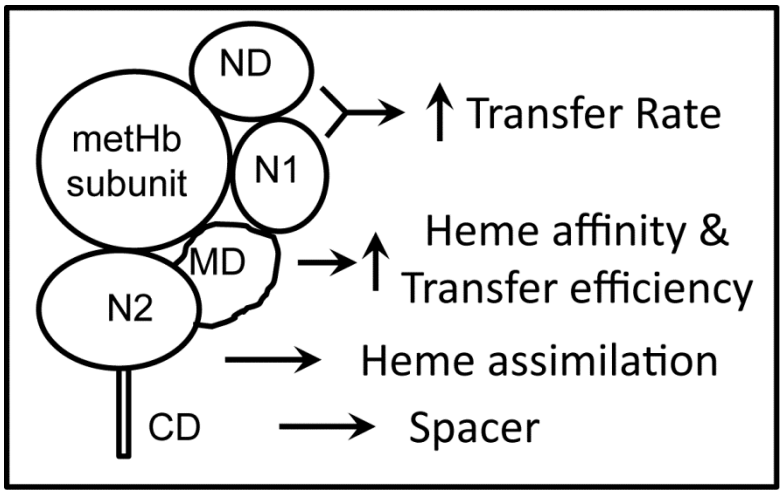


Figure 9: A schematic for a model for the functions of individual domains of IsdB for the kinetics and equilibrium of the metHb/IsdB heme transfer reaction. In this model, ND-N1 specifically enhances direct heme assimilation from α and β subunits of metHb by N2; MD enhances the affinity of N2 for heme and drives the equilibrium of the reaction toward the formation of holo-IsdB; and CD functions as a spacer to place IsdB at an appropriate position in the cell wall envelope of *S. aureus* for heme relay by the Isd heme acquisition system.

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