Pathway for Heme Uptake from Human Methemoglobin by the Iron-regulated Surface Determinants System of *Staphylococcus aureus* 

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The iron-regulated surface proteins IsdA, IsdB, and IsdC and transporter IsdDEF of *Staphylococcus aureus* are involved in heme acquisition. To establish an experimental model of heme acquisition by this system, we have investigated hemin transfer between the various couples of human methemoglobin (metHb), IsdA, IsdB, IsdC, and IsdE by spectroscopic and kinetic analyses. The efficiencies of heme transfer from hemin-containing donors (holo-protein) to different heme-free acceptors (apo-protein) were examined, and the rates of the transfer reactions were compared with that of indirect loss of heme from the relevant donor to H64Y/V668F apomyoglobin. The efficiencies, spectral changes, and kinetics of the transfer reactions demonstrate that: 1) metHb directly transfers heme to apo-IsdB, but not to apo-IsdA, apo-IsdC, and apo-IsdE; 2) holo-IsdB directly transfers heme to apo-IsdA and apo-IsdC, but not to apo-IsdE; 3) apo-IsdE directly acquires heme from holo-IsdC, but not from holo-IsdB and holo-IsdA; and 4) IsdB and IsdC enhance heme transfer from metHb to apo-IsdC and from holo-IsdB to apo-IsdE, respectively. Taken together with our recent finding that holo-IsdA directly transfers its heme to apo-IsdC, these results provide direct experimental evidence for a model in which IsdB acquires heme from metHb and transfers it directly or through IsdA to IsdC. Hemin is then relayed to IsdE, the lipoprotein component of the IsdDEF transporter.

Iron is an essential metal for the growth and survival of most bacterial pathogens. Because of its extremely low solubility under physiological conditions, iron exists in complexes with proteins and other compounds. The most abundant iron complex in mammals is the iron-protoporphyrin complex or heme, which is a cofactor of hemoglobin and has been shown to be preferred as an iron source *in vitro* for some bacterial pathogens such as Gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes* (1–3). Because of its functions and toxicity in its free form, heme is bound to hemoglobin and other host proteins with extremely high affinity (4, 5). To acquire heme from host hemoproteins, bacterial pathogens have evolved sophisticated heme acquisition machineries. Gram-positive bacteria produce surface proteins to extract heme from hemoglobin and relay it through the cell wall to a specific ATP-binding cassette (ABC) transporter (6–10), which transports heme across the cytoplasmic membrane (3, 11, 12). Gram-negative pathogens utilize an outer membrane protein or hemophore/outer membrane receptor to relay heme from hemoglobin to an inner membrane, heme-specific ABC transporter (13, 14).

The heme acquisition machinery in *S. aureus* consists of the iron-regulated surface determinants (Isd), including the surface proteins IsdA, IsdB, and IsdC and the ABC transporter IsdDEF (7). IsdB is a hemoglobin receptor and is required for uptake of heme from methemoglobin (15). IsdA and IsdC are also important for heme uptake (16, 17). Another surface protein, IsdH/HarA, binds haptoglobin-hemoglobin (18) and hemoglobin (19) and thus is also proposed to be part of the *S. aureus* heme uptake machinery (20, 21). However, the *isdH* gene is not located at the locus of the other *isd* genes, nor is it critical for using methemoglobin heme as an iron source *in vitro* (15, 18). IsdB, IsdA, IsdC, and IsdE, the lipoprotein component of the IsdDEF transporter, bind heme (7, 22–26). Structural studies show that IsdA and IsdC bind hemin in a pentacoordinate complex with a tyrosine residue as the only axial ligand (17, 28), whereas the heme iron in IsdE is hexacoordinate with axial coordination to histidine and methionine side chains (29).

It has been hypothesized that heme is transferred from the IsdB-caught hemoglobin to IsdA, then to IsdC, and finally to the ABC transporter IsdDEF (20, 21). We have experimentally demonstrated that heme-containing IsdA (holo-IsdA) rapidly transfers its hemin to hemin-free IsdC (apo-IsdC) through an activated holo-IsdA-apo-IsdC complex (30). In this report, we examined hemin transfer between the other couples of the human methemoglobin (metHb), IsdA, IsdB, IsdC, and IsdE system. We found that: 1) metHb directly transfers its hemin to...
apo-IsdB, but not to the other apo-Isd proteins; 2) holo-IsdB can directly transfer its hemin to apo-IsdA and apo-IsdC, but not to apo-IsdE; and 3) holo-IsdC does directly transfer its hemin to apo-IsdE, allowing transport into the bacterial cytoplasm. These findings provide the experimental evidence for a model for hemin transport through the Isd system.

**EXPERIMENTAL PROCEDURES**

**Gene Cloning**—The *isdB* and *isdE* genes were cloned from *S. aureus* MW2 with paired primers 5′-TACCATGGAGCAGCAGCAGCTGAAGAAACA-3′/5′-TGGATCCCTAAGTTTGTGGTAATGATTTCGC-3′ and 5′-TACCATGGGCTAATCTTTCCAGTTCTCAA-3′/5′-AGGATCCGCAGTGGCATTAATAAATGACT-3′, respectively. The *isdB* and *isdE* PCR products were digested with NcoI and BamHI and cloned into pET-21d. Recombinant IsdB made from the clone lacked the secretion signal sequence (amino acids 1–39) and transmembrane domain and charged tail at the C terminus (amino acids 614–645). Recombinant IsdE produced from the clone lacked the secretion signal sequence (amino acids 20–292) except that the Cys20 residue was changed to Gly to introduce the NcoI site for cloning.

**Purification of Holo- and Apo-IsdB Proteins**—Purified IsdB containing apo- and holo-IsdB in 10 mM Tris-HCl, pH 8.0, was loaded onto a Q Sepharose column (1 × 4 cm). The column was eluted first with Tris-HCl to recover apo-IsdB and then with 50 mM Tris-HCl at 22 °C for 2 min or 6 h. Each donor-acceptor mixture was loaded onto a SP Sepharose column (0.3 ml of 1 × 4 cm), and apoIsdC was recovered in flow-through and Tris-HCl wash. Purified IsdE and IsdA were apo-IsdB/protoporphyrin-IsdC complex and apo-/holo-IsdA mixtures, respectively, and homogeneous apo-proteins were prepared by the methyl ethyl ketone method (32).

**Rate of Hemin Dissociation from IsdB and IsdE**—The rate constants for hemin dissociation from holo-proteins were measured using Hb (apo-myoglobin as a hemin scavenger) (34). Each holo-protein at 3 μM was incubated with 45 μM ferric hemoglobin (apo-Mb) in 1 ml of 20 mM Tris-HCl, pH 8, to recover unbonded Hb and then eluted with 2.4 ml of 60 mM NaCl in Tris-HCl to release bound protein(s). To separate IsdB from IsdA, the column was eluted with 5 ml of Tris-HCl, eluted with 2.4 ml of 60 mM NaCl to obtain IsdB, washed with 5 ml of 60 mM NaCl, and eluted with 1.5 ml of 0.25 μM NaCl to recover IsdA or IsdE. To separate holo-IsdC from IsdB and apo-IsdC, the column was eluted with 2 ml of Tris-HCl to obtain holo-IsdC, washed with 5 ml of 50 mM NaCl, and eluted with 60 mM NaCl to obtain IsdB and apo-IsdC. Separation of the two proteins in each reaction was checked by SDS-PAGE analysis. The spectra of the separated donor and acceptor were recorded to assess the extent of hemin transfer.

**Kinetics of Hemin Transfer**—The rates of hemin transfers from metHb to apo-IsdB and from holo-IsdB to apo-IsdA or apo-IsdC were measured using a stopped flow spectrophotometer equipped with a photodiode array detector (SX20; Applied Photophysics) as described previously (35). Briefly, holo-protein in one syringe was mixed with apo-protein at >5× holo-protein in another syringe. Entire spectra were recorded over time in each reaction.

The rates of slower hemin transfer from metHb to apo-IsdB, apo-IsdA, and apo-IsdC and from holo-IsdB, holo-IsdA, or holo-IsdC to apo-IsdE 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 >5× holo-protein, and the absorbance changes at the indicated wavelengths were monitored for up to 6 h.
IsdB and IsdE Proteins—Purified recombinant IsdB with ~80% purity was a mixture of apo- and holo-IsdB. Partial resolution of apo- and holo-IsdB proteins with a Q Sepharose column yielded predominantly apo-IsdB (>95% in apo-form) and holo-IsdB (~70% in holo-form) (supplemental Fig. S1). Holo-IsdB proteins directly purified from E. coli and reconstituted from apo-IsdB and hemin have almost identical spectra at both the oxidized and reduced states (Fig. 1). The reduced spectra show two overlapping, unresolved peaks in the region of 500–600 nm, instead of the dominating α band. These absorption features are similar to those of pentacoordinate holo-IsdA and holo-IsdC (22, 30), suggesting that the heme iron in IsdB is pentacoordinate. Reconstituted holo-IsdE has the spectra (supplemental Fig. S2) that are similar to the published spectra of holo-IsdE (25) and S. pyogenes HtsA (35) and are typical of hexacoordinate heme complex with two strong axial ligands.

**Human Methemoglobin Directly Transfers Hemin to Apo-IsdB but Indirectly to Apo-IsdA, Apo-IsdC, or Apo-IsdE**—In transfer reactions, hemin can either be directly channeled from a donor to an acceptor in ternary donor-heme-accepter complex or, alternatively, first dissociate from the donor into solvent and then be scavenged by the acceptor. The indirect transfer is usually slow because of slow heme dissociation into solvent, whereas the direct transfer is usually very rapid. Monitoring transfer efficiency at relatively short reaction times can be used to detect rapid, direct heme transfer reactions. This strategy was used to determine which of the Isd proteins directly acquires hemin from human metHb. MetHb, instead of oxygenated hemoglobin, was used because oxygenated hemoglobin autoxidizes to metHb upon dilution after hemolysis (36). MetHb (20 μM hemin) was incubated with 35 μM apo-IsdA, apo-IsdB, apo-IsdC, or apo-IsdE for 2 min, and separation of the two proteins in each reaction was performed. In the separation of Hb from its reaction with apo-IsdB, ~40% of Hb free of IsdB was recovered in 0.15-ml fractions 2–5 in the flow-through and initial wash with 20 mM Tris-HCl (Fig. 2). After Hb was undetectable in the additional, thorough wash with 10 ml of Tris-HCl, IsdB was recovered with 0.2 M NaCl, and Hb (~60% of total Hb) was also present in each of the fractions containing IsdB (Fig. 2). Because Hb alone (data not shown) or in the IsdA- or IsdE-Hb mixture (Fig. 2) did not bind to the column, the coelution of IsdB and Hb suggests that they are in a complex, consistent with the previous findings of Hb binding to IsdB (7, 15). The ratio of A405/A280 of the IsdB-free Hb sample from the metHb-apo-IsdB reaction was 70% of that of the starting metHb (Fig. 3A), and all recovered IsdB-free Hb contained 23% of the initial Hb hemin. The recovered IsdB/Hb mixture contained 72% of the added Hb hemin and had a spectrum that was a combination of the spectra of holo-IsdB and metHb (Fig. 3B). Although Hb-free IsdB could not be obtained from the reaction mixture to quantify holo-IsdB product, it is obvious that metHb can rapidly lose part of its hemin to apo-IsdB.

Unlike the situation for Hb and IsdB reaction mixture, all of the Hb was recovered in the flow-through and initial Tris-HCl wash in the separation of Hb from IsdA or IsdE in their reactions (Fig. 2) The A405/A280 ratio of Hb from the reaction with apo-IsdA or apo-IsdE did not dramatically change compared with the untreated metHb (Fig. 3, C and E). A majority of the treated IsdA (Fig. 3D) or IsdE sample (Fig. 3F) was still in apo-form after a 2-min incubation with metHb. According to measurements of heme contents, the recovered IsdA and IsdE had heme contents that were 9 and 6% of the added Hb hemin, respectively. Thus, these results suggest that metHb transfers heme to apo-IsdB more efficiently than to apo-IsdA and apo-IsdE. Even though we could not separate IsdC from metHb, it was clear from the observed spectral changes described below that little transfer occurred in 2 min.

We next examined the spectral changes associated with heme transfer from metHb to the apo-Isd proteins and performed kinetic analyses for these reactions. The metHb-apo-IsdB reaction is too fast to use a conventional spectrophotometer. To measure the kinetics of this reaction, 2.7 μM metHb was mixed with 20 μM apo-IsdB in a stopped flow spectrophotometer, and a rapid spectral shift of the mixture was observed (Fig. 4A). The spectrum recorded at 9.87 s after mixing almost
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overlays that of holo-IsdB (Fig. 4B), indicating that metHb almost completely transferred its hemin to apo-IsdB under the conditions used. The time course of spectral change at 405 nm fits a single exponential expression (Fig. 4C), yielding an apparent rate constant of 0.31 s⁻¹ (Table 1). These results are dramatically different from those for simple dissociation of hemin from metHb using H64Y/V68F apo-Mb to scavenge released hemin. The latter reaction is a slow, biphasic process with the observed rate constants of 3.5 × 10⁻³ and 1.4 × 10⁻⁴ s⁻¹ representing dissociation from the β and α subunits, respectively (Fig. 4C and Ref. 5). The differences in kinetic phases and rates between the metHb-apo-IsdB and metHb-apo-Mb reactions indicate that apo-IsdB extracts hemin directly from metHb.

After a 6-h incubation of 3 μM metHb with 25 μM apo-IsdA or apo-IsdE, the spectrum of the reaction mixture shifted from that of metHb toward that of the corresponding holo-Isd protein (supplemental Fig. S3, A–D), and 74 and 65% of Hb hemin was transferred to apo-IsdA and apo-IsdE, respectively, based on heme content measurements of the separated proteins from the reactions. Hemin transfer from metHb to apo-IsdC was complete (supplemental Fig. S3, E and F). The time courses for these reactions each fit a two exponential expression (Fig. 4C), yielding two observed rate constants of 1.2 × 10⁻³ and 1.4 × 10⁻⁴, 1.1 × 10⁻² and 8.6 × 10⁻³, and 4.1 × 10⁻⁴ and 6.9 × 10⁻⁴ s⁻¹ for the fast and slow phases of the apo-IsdA-, apo-IsdC-, and apo-IsdE-metHb reactions, respectively. The rate constants are close to those of the metHb-apomysoglobin reaction (k = 3.5 × 10⁻³ and 1.4 × 10⁻⁴ s⁻¹), indicating that the hemin transfers from metHb to apo-IsdA, apo-IsdC, and apo-IsdE are indirect and involve initial hemin dissociation from metHb and then uptake by the Isd apoproteins.

Holo-IsdB Directly Transfers Hemin to Apo-IsdA and Apo-IsdC but Indirectly to Apo-IsdE—To determine whether holo-IsdB transfers its hemin to apo-IsdA, apo-IsdC, and/or apo-IsdE, 6 μM holo-IsdB was incubated with 12 μM each apoprotein for 2 min, and the two proteins were separated. All of the separated proteins had no detectable levels of their partners except that the IsdB from its apo-IsdC reaction had IsdC (supplemental Fig. S4). The A₄₀₂/A₂₅₀ ratio of IsdB from the holo-IsdB/apo-IsdA and holo-IsdB/apo-IsdE reaction mixtures was ~60 and 90% of that of the starting holo-IsdB, respectively (Fig. 5, A and C). Consistent with these results, isolated IsdA contained much higher levels of holo-protein than isolated IsdE (Fig. 5, B and D). Holo-IsdC does not bind to SP Sepharose, whereas apo-IsdC and IsdB do, which was why the isolated IsdB had IsdC as shown in supplemental Fig. S4. However, the presence of IsdC in the IsdB sample did not affect the data interpretation because the isolated IsdB/IsdC sample from the holo-IsdB/apo-IsdC reaction had little hemin (Fig. 5E), and IsdB-free IsdC from the same reaction was in holo-form (Fig. 5F). Thus, holo-IsdB can transfer its hemin more efficiently to IsdA and IsdC than to IsdE.

Kinetic analyses were then performed to determine whether these transfer reactions are direct. When 3 μM holo-IsdB was mixed with 30 μM apo-IsdA or apo-IsdC in a stopped flow spectrophotometer, the spectra of the mixtures rapidly shifted from that for holo-IsdB to that for holo-IsdA (Fig. 6A) or holo-IsdC (Fig. 6B). Time courses of the absorbance changes were fit to single exponential expressions, and the apparent first order constants for the apo-IsdA-and apo-IsdC-holo-IsdB reactions were 114 and 15 s⁻¹, respectively (Fig. 6C). These rate constants are 87,000- and 11,500-fold greater than the rate constant for simple hemin dissociation from holo-IsdB, which was determined to be 1.3 × 10⁻³ s⁻¹ by the H64Y/V68F apo-Mb assay.
Many of the properties of holo-IsdB were similar to those of holo-IsdBR. Holo-IsdBR apo-IsdB and apo-IsdB reactions mixtures rapidly shift from that of holo-IsdBR to those of the holosA and holo-IsdC products, and the time courses fit a single exponential expression, resulting in a rate constant of 1.6 × 10^{-3} s^{-1}. This rate constant is close to that for simple hemin dissociation from IsdB, 1.3 × 10^{-3} s^{-1} (supplemental Fig. S3A), but is 71,200- and 9,370-fold slower than those for the holo-IsdBR apo-IsdA and holo-IsdBR apo-IsdC reactions, respectively. These measurements indicate that holo-IsdBR does not directly nor rapidly transfer hemin to apo-IsdE.

Many of the properties of holo-IsdB were similar to those of holo-IsdBR. Holo-IsdBR apo-IsdB and apo-IsdC reaction mixtures rapidly shift from that of holo-IsdBR to those of the holosA and holo-IsdC products, and the time courses fit a single exponential expression, resulting in apparent first order rate constants of 87 and 10.7 s^{-1} for the apo-IsdA and apo-IsdC transfer reactions, respectively (supplemental Fig. S7). Thus, both holo-IsdB forms can rapidly and directly transfer hemin to apo-IsdA and apo-IsdC, but neither holo-IsdB form can rapidly or directly transfer hemin to apo-IsdE.

### Holo-IsdC Directly Transfers its Hemin to Apo-IsdE—The Soret peaks of holo-IsdA, holo-IsdC, and holo-IsdE are at 406, 402, and 412 nm, respectively, which allows spectral determination of hemin transfer from holo-IsdA and/or holo-IsdC to apo-IsdE. After a 30-min incubation of 45 μM apo-IsdE with 3 μM holo-IsdA, there was a slight spectral shift in the Soret peak that of holo-IsdB toward but did not completely turn into that of holo-IsdE (supplemental Fig. S5A), indicating that only a portion of the hemin in holo-IsdBR was transferred to apo-IsdE at equilibrium. To determine whether this incomplete transfer is direct, the rate was measured and compared with that of simple hemin dissociation from holo-IsdBR. The time course for the holo-IsdBR apo-IsdE reaction was fit to a single exponential expression (supplemental Fig. S5B), resulting in a rate constant of 1.6 × 10^{-3} s^{-1}. This rate constant is close to that for simple hemin dissociation from IsdB, 1.3 × 10^{-3} s^{-1} (supplemental Fig. S3B), but is 71,200- and 9,370-fold slower than those for the holo-IsdBR apo-IsdA and holo-IsdBR apo-IsdC reactions, respectively. These measurements indicate that holo-IsdBR does not directly nor rapidly transfer hemin to apo-IsdE.

### Table 1

| Hemin donor | Hemin acceptor | k or k'/k'' | k'-k'' |
|-------------|----------------|-------------|--------|
| metHb       | apo-IsdA       | 0.0012      | 0.00014 |
|              | apo-IsdB       | 0.31        |        |
|              | apo-IsdC       | 0.011       | 0.00086 |
|              | apo-IsdE       | 0.00041     | 0.000069|
| apo-myoglobin| apo-IsdA       | 0.0035      | 0.00014 |
| apo-IsdB     | apo-IsdA       | 114         |        |
| apo-IsdB     | apo-IsdC       | 15          |        |
| apo-IsdE     | apo-IsdA       | 0.0016      |        |
| apo-IsdE     | apo-IsdC       | 0.0013      |        |
| apo-IsdE     | apo-myoglobin  | 0.00076     |        |
| holo-IsdA    | apo-IsdA       | 0.0062      | 0.0007 |
| holo-IsdA    | apo-IsdC       | UD          |        |
| holo-IsdA    | apo-myoglobin  | UD          |        |
| holo-IsdC    | apo-IsdA       | 0.0077      | 0.0029 |
| holo-IsdC    | apo-IsdC       | UD          |        |
| holo-IsdC    | apo-myoglobin  | UD          |        |

*The k and k'/k'' values were obtained by fitting data to single and double exponential expressions, respectively.

*Unable to determine.

*The data are from Ref. 30.

*The value was obtained from incomplete transfer reaction and represents an upper estimate of the true hemin dissociation rate constants for IsdCln.

### Figure 4

**Kinetics of hemin transfer from metHb to the Isd proteins.**

**A**. Absorption spectra of a mixture of 2.7 μM metHb and 20 μM apo-IsdB as a function of time after mixing. The arrows indicate the directions of the spectral change with time. **B**. Overlay of the normalized spectra of the metHb/apo-IsdB mixture at 9.87 s in A, holo-IsdBR, and metHb. **C**. Time courses of the spectral change in the metHb (2.7 μM) and apo-IsdB (27 μM), metHb (27 μM) and apo-IsdA (25 μM), metHb (27 μM) and apo-IsdC (25 μM), metHb (27 μM) and apo-IsdE (25 μM), and metHb (27 μM) and H64Y/V68F apo-Mb (45 μM) reactions. The solid and dashed curves are the observed data and single (the apo-IsdB reaction) and double (the other reactions) exponential fitting curves, respectively. The rate constant(s) for the reactions (s^{-1}): IsdB, 0.31; IsdA, 0.0012, 0.00014; IsdC, 0.011, 0.00086; IsdE, 0.00041, 0.000069; and Mb, 0.0035, 0.00014.
holog-IsdC/apo-IsdE reaction (Fig. 7C) and had two apparent rate constants of $7.7 \times 10^{-3}$ and $2.9 \times 10^{-3}$ s$^{-1}$, which are 10- and 4-fold greater than the apparent rate constant of simple hemin dissociation from IsdC$^{His}$. Together these results suggest that holo-IsdC can directly transfer its hemin to apo-IsdE at a rate that is significantly greater than that for simple thermal dissociation of hemin from holo-IsdC. As shown in Fig. 7A and supplemental Figs. S5 and S6, neither holo-IsdB nor holo-IsdA can directly transfer hemin to apo-IsdE. Thus, only holo-IsdC appears to interact directly with apo-IsdE. However, the rates of the IsdC-to-IsdE transfer are much slower than the other direct transfer reactions between metHb and IsdB, IsdB and IsdA, IsdB and IsdC, and IsdA and IsdC.

The Relay Roles of IsdB and IsdC in Hemin Transfer—Because IsdB rapidly acquires hemin from metHb and donates it to apo-IsdC, IsdB should be able to enhance the rate of hemin transfer from metHb to apo-IsdC. To test this idea, time courses measured at 405 nm were monitored for the reaction of 7 mM metHb with 22 mM apo-IsdC in the presence or absence of 0.4 mM IsdB. The addition of catalytic amounts of IsdB dramatically enhanced hemin transfer from metHb to apo-IsdC (Fig. 8A). A similar catalytic effect of IsdC on hemin transfer from holo-IsdB$_R$ to apo-IsdE was observed (Fig. 8B). These results demonstrate that IsdB and IsdC can relay hemin all the way from metHb to IsdE with high efficiency.

Relative Affinities of the Isd Proteins for Hemin—Because the rates of simple hemin dissociation from IsdB and IsdC cannot be accurately determined using H64Y/V68F apo-Mb because of incomplete or no hemin loss, respectively, we could not determine the affinities of the Isd proteins for hemin by measuring the rates of hemin association and dissociation. Thus, we can only estimate the order of the hemin affinity of the Isd proteins based on the transfer reactions. The spectra of $20 \times$ apo-Mb-holo-IsdA, -holo-IsdC, -holo-IsdE, and -holo-IsdB mixtures were recorded after 12 h of incubation. The majority of holo-IsdA and holo-IsdB lost hemin to H64Y/V68F apo-Mb, whereas holo-IsdC and holo-IsdE did not significantly lose hemin (Fig. 9). These results indicate that IsdC and IsdE have much higher affinity for hemin than IsdB and IsdA. These results also suggest that the affinities of these Isd proteins for hemin are more than or equal to that of the H64Y/H68F Mb. $K_{hemin}$ for the Mb reagent is estimated to be $1 \times 10^{12}$ M$^{-1}$ at pH 7.0 based on a bimolecular rate constant of $1 \times 10^{8}$ M$^{-1}$ s$^{-1}$ for
hemin association to H64Y/V68F Mb (4) and a dissociation rate constant of $1.1 \times 10^{-5}$ s$^{-1}$ for this double mutant (34). Another consequence of incomplete transfer in the IsdB reaction is that the observed rate constant is not directly equal to $k_{-\text{hemin}}$, for dissociation from holo-IsdE but instead contains a contribution from the rate of hemin dissociation from the H64Y/V68F Mb reagent (see Equation 3 in Ref. 34). Thus, the $k_{-\text{hemin}}$ value for holo-IsdB is just an upper estimation, and the actual value may be significantly smaller. Little hemin transfer occurs in the mixture of 1:15 holo-IsdA-apo-IsdE or 1:1 holo-IsdC-apo-IsdE.

Together these results suggest that the order of hemin affinity for the Isd proteins is IsdC $> \text{IsdE} > \text{IsdB} \approx \text{IsdA}$.

**DISCUSSION**

This report presents biochemical, spectroscopic, and kinetic evidence for direct hemin transfers from human metHb to IsdB, from IsdB to IsdA and IsdC, and from IsdC to IsdE and for the relay roles of IsdB and IsdC in hemin transfer from metHb to...
apo-IsdE. Taken together with our previous report of direct hemin transfer from IsdA to IsdC (30), these findings establish an experimental pathway for hemin movement from metHb through the Isd surface proteins to the lipoprotein component of the specific ABC transporter IsdDEF.

Four observations indicate that hemin is extracted from metHb by apo-IsdB. First, metHb loses a significant amount of its hemin to apo-IsdB after a brief 2-min reaction with apo-IsdB. Second, the observed rate for hemin transfer from metHb to apo-IsdB is very fast, \( \frac{1}{100} \text{s}^{-1} \), which is 80–220 times greater than the rates for simple hemin dissociation from the \( \alpha \) and \( \beta \) subunits of human metHb, respectively. Third, the metHb-to-apo-IsdB transfer is a single exponential process, suggesting that apo-IsdB acquires hemin from the \( \alpha \) and \( \beta \) subunits at the same rate, whereas simple thermal dissociation of hemin from the \( \alpha \) and \( \beta \) subunits occurs at widely different rates (5). If the metHb-to-apo-IsdB transfer were indirect, the time courses should show two phases, and both observed rates would be slow and close to those for simple hemin dissociation from the \( \alpha \) and \( \beta \) subunits of metHb. The reactions of metHb with apo-IsdA, apo-IsdC, and apo-IsdE display the latter features, showing two phases and very slow transfer rates indicative of indirect hemin transfer. Fourth and most convincing, IsdB can function as a relay to mediate hemin transfer from metHb to apo-IsdC at a rate faster than that of the metHb-apo-IsdC reaction in the absence of IsdB (Fig. 8A).

Serratia marcescens hemophore HasA (37), S. pyogenes heme-binding protein Shp (9), and Shigella dysenteriae outer membrane receptor ShuA (14) have been shown to acquire hemin from metHb \textit{in vitro}. Kinetic analyses indicate that the ShuA-metHb reaction is direct (14), but the Shp-metHb reaction is much slower and indirect (10). Whether the HasA-metHb reaction is direct or indirect is not known. Thus, the rapid reaction of metHb with apo-IsdB is the first experimentally verified example of direct hemin transfer from metHb to a heme acquisition protein in Gram-positive bacteria and the second example for all bacteria.

Holo-IsdB has been shown previously to bind hemoglobin and act as an Hb receptor on the cell wall surface of \( S. \) \textit{aureus} (15). However, the event after metHb binds to IsdB was not known (15, 20, 21). Our findings show unequivocally that IsdB extracts hemin from metHb and that neither IsdA nor IsdC alone can obtain hemin directly from metHb but have to wait for indirect hemin dissociation into solvent in the absence of IsdB. These results demonstrate that IsdB acts not only as a receptor to bring hemoglobin to the cell surface but also actively partici-
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FIGURE 10. Schematics for the proposed models of heme acquisition from metHb by the S. aureus Isd and S. pyogenes Shr/Shp/HtsABC systems. The arrows indicate the direction of direct heme transfer. The S. aureus model (A) is derived from the experimental data of this work and Refs. 7, 15, 30, and 40, whereas the S. pyogenes model (B) is based on the findings in Refs. 9, 10, 35, and 41. The direct metHb-to-Shr heme transfer and heme degradation mediated by heme oxygenase have not been experimentally demonstrated in the S. pyogenes model. The heme transfer from IsdB to IsdC represented by the dotted arrow may be prevented in vivo by their physical locations in the cell wall. The structure models of the proteins were from the Protein Data Bank coordinates 2Q8Q, 2ITF, 2D6P, 2Q7A, and 1HKO.

pates in the extraction of heme from metHb and relays it to apo-IsdA and/or apo-IsdC, but not to apo-IsdE.

According to the hypothesized model (20, 21), one would expect that holo-IsdB would only transfer its heme directly to apo-IsdA. However, as shown in Figs. 5, 6, and 8, rapid and direct transfer from IsdB to apo-IsdC also occurs, suggesting that IsdA may play a redundant role in heme acquisition under some circumstances. This possibility is supported by the finding that isdB is required for S. aureus growth using metHb as the sole iron source (15), whereas inactivation of isdA does not affect S. aureus growth using metHb as an iron source (7, 38). However, inactivation of isdA decreases the amount of [55Fe]hemin uptake (7) and reduces bacterial growth using hemin as a sole iron source (17). Interaction between IsdA and metHb was detected (38) but may not be significant to heme acquisition because IsdA cannot directly acquire heme from metHb.

Holo-IsdB transfers its heme to apo-IsdA approximately eight times more rapidly than to apo-IsdC, suggesting that IsdA is the initial acceptor of heme from the outer IsdB receptor in vivo. In addition, apo-IsdC is almost certainly located deep in the cell wall next to the cytoplasmic membrane and IsdE and thus may not be physically close to IsdB whose heme-binding domain should be located on the cell wall surface. Thus, IsdA may serve to relay heme from IsdB on the surface of the cell wall to the interior portion where IsdC must be located for it to interact with the heme-specific ABC transporter.

Several observations support the conclusion that holo-IsdC directly transfers its heme to apo-IsdE. Holo-IsdB and holo-IsdA, which have lower affinities for heme than IsdC, inefficiently transfer their heme to apo-IsdE, whereas holo-IsdC does directly transfer heme to apo-IsdE under the similar conditions. The rate of heme transfer from holo-IsdC<sup>His</sup> to apo-IsdE is faster than simple hemin dissociation from holo-IsdC<sup>His</sup>. Unexpectedly, the holo-IsdC-to-apo-IsdE transfer is biphasic. A single exponential process would be expected if the transfer were indirect, and the relative rates and biphasic feature of the IsdC-IsdE transfer are similar to those for heme transfer from the S. pyogenes Shp axial mutants (pentacoordinate complexes, like holo-IsdC) to HtsA (a hexacoordinate complex, like holo-IsdE) (39). The biphasic kinetic feature may imply the sequential formation of the two axial bonds of the holo-IsdE product in this pentacoordination-to-hexacoordination conversion. The more convincing evidence is that IsdC catalytically enhances the rate of heme transfer from holo-IsdB to apo-IsdE (Fig. 8B).

Although we could not accurately measure the affinities of the Isd proteins for heme, we can estimate their relative affinity order according to the extent of the transfer reactions. For example, the reaction of 2.7 μM metHb with 20 μM apo-IsdB was almost complete (Fig. 4B), and, by assuming that 90% of the Hb heme was transferred to IsdB at the equilibrium, the affinity of IsdB for heme would be estimated to be ~10-fold higher than that of an α chain in a dimer of Hb, which is ~6 × 10<sup>11</sup> M<sup>−1</sup> (4). This estimation (~6 × 10<sup>12</sup> M<sup>−1</sup>) is consistent with the incomplete loss of heme from 3 μM holo-IsdB to 45 μM H64Y/V68F apo-Mb (K<sub><em>hemin</em></sub> ≈ 1 × 10<sup>12</sup> M<sup>−1</sup>). The affinity of IsdA for heme is similar to that of IsdB. The heme affinity of IsdE is greater than those of IsdA and IsdB but lower than that of IsdC, suggesting that IsdC may function as a sink for heme. In some transfer reactions, we used high ratios of [donor] over [acceptor]. In most cases, a high concentration of apoprotein acceptor was used to achieve pseudo-first order conditions for kinetic analyses. However, significant transfer from holo-IsdC to apo-
IsdE could be observed only at an initial [apo-IsdE]:[holo-IsdC] ratio of >5. Thus, the IsdC-to-IsdE transfer is thermodynamically unfavorable. However, this situation will not impede the net uptake because in this case the transfer is driven forward by the following step, which uses the free energy of ATP hydrolysis to bring hemin across the membrane by the ABC transporter.

The Isd system has been extensively characterized genetically and structurally (3, 7, 15, 16–19, 22–29, 38). However, until this work a kinetically determined pathway has been missing. The previously proposed transfer order was: IsdH-haptoglobin-hemoglobin/IsdB-hemoglobin → IsdA → IsdC → IsdDEF (20, 21). IsdB has been shown to be the receptor for Hb (15), supporting this model, but no other direct experimental evidence had been reported to verify this model until we recently reported that IsdA directly transfers its hemin to IsdC (30). We have now confirmed direct hemin transfer for the recently reported that IsdA directly transfers its hemin to IsdC (15), supporting this model, but no other direct experimental evidence had been reported to verify this model until we recently reported that IsdA directly transfers its hemin to IsdC (30).

However, the S. pyogenes system involves two surface proteins, whereas the S. aureus system utilizes at least three surface proteins (four proteins if IsdH is involved). All of the genes of the S. pyogenes system are in the same operon (41), whereas isdA, isdB, and isdCDEF are transcribed separately (7), suggesting that S. aureus might have picked up some of the isd genes after divergence from S. pyogenes if they had shared a same ancestor. Furthermore, S. pyogenes Shp can directly and rapidly transfer its hemin to the HtsA homologue of Streptococcus equi (8) but not to IsdE. Thus, the two systems have apparently diverged enough to be considered as two distinct systems even they might have evolved from a common ancestor.

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**Heme Acquisition in S. aureus**

The S. aureus Isd system represents one of two different sets of heme acquisition genes in Gram-positive bacteria that are known to use surface proteins for heme acquisition. The other set of genes is represented by the system in S. pyogenes, which consists of the genes encoding the surface proteins, Shr and Shp, and the membrane transporter HtsABC (6, 10, 11, 41). S. pyogenes HtsA and IsdE share a high level of sequence homology (29). The structure of the Shp heme-binding domain is similar to those of the NEAT domains of IsdA, IsdC, and IsdH (17, 24, 28, 42), and Shr has two NEAT domains (27). Shr has been proposed to bind Hb (41), and it does bind heme and efficiently transfer it to apo-Shp but not to HtsA (10). Shp transfers its hemin to HtsA (9, 33, 35, 39). Based on these findings, we propose a model in which Shr acquires hemin from metHb and transfers it to HtsA through Shp (Fig. 10B). Transported hemin is presumably degraded by heme oxygenase, which has not been identified. It should be pointed out that direct hemin transfer from metHb to apo-Shr has not been documented. There are parallel functions of the components in the S. aureus and S. pyogenes models. IsdB, IsdA/C, and IsdE have functions similar to those of Shr, Shp, and HtsA, respectively, in relaying the hemin from metHb to the cell surface into the bacterial cytoplasm. In addition, the Shp/HtsA and IsdA/IsdC reactions follow the same mechanism of activated heme transfer (30, 35). These similarities suggest that these systems may share a common ancestor and have similar biochemical mechanisms for heme acquisition.

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