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

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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 hemin transfer from hemin-containing donors (holo-protein) to different hemin-free acceptors (apo-protein) were examined, and the rates of the transfer reactions were compared with that of indirect loss of hemin from the relevant donor to H64Y/V68F apomyoglobin. The efficiencies, spectral changes, and kinetics of the transfer reactions demonstrate that: 1) metHb directly transfers hemin to apo-IsdB, but not to apo-IsdA, apo-IsdC, and apo-IsdE; 2) holo-IsdB directly transfers hemin to apo-IsdA and apo-IsdC, but not to apo-IsdE; 3) apo-IsdE directly acquires hemin from holo-IsdC, but not from holo-IsdB and holo-IsdA; and 4) IsdB and IsdC enhance hemin 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 hemin to apo-IsdC, these results provide direct experimental evidence for a model in which IsdB acquires hemin 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)[2] 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 hemin from methemoglobin (15). IsdA and IsdC are also important for hemin 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 hemin 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 hemin-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

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[2] The abbreviations used are: ABC, ATP-binding cassette; metHb, methemoglobin; Mb, myoglobin.

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′-TACCATGGAAGCAGCAGCTGAAGAAACA-3′/5′-TGGATCCTTAAGTTTGTGGTAATGATTTTGC-3′ and 5′-TACCATGGGTCAATCTTCCAGTTCTCAA-3′/5′-AGGATCCGCAGTTGCGATTAAAATGACT-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 was the mature protein (amino acids 20–292) except that the Cys^{20} residue was changed to Gly to introduce the NcoI site for cloning. The cloned genes were sequenced to rule out spurious mutations. To produce IsdA and IsdC without the His_6 tag, the *isdA* and *isdC* genes in pSIDA and pISDC (30) were subcloned into pET-21d at the NcoI and EcoRI sites.

Protein Purification—The Isd proteins were purified from *Escherichia coli* BL21 containing the individual *isd* genes to >80% (supplemental Fig. S1) as described in the supplemental material. Human hemoglobin was purified as a complex with CO, as described previously (31). Ferric hemoglobin (methemoglobin or metHb) was prepared by oxidizing the CO-hemoglobin complex with excess ferricyanide and passing the sample through a G-25 column (1.5 × 30 cm) to remove excess ferricyanide.

Preparation of Apo- and Holo-Isd 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 NaCl in Tris-HCl to release the bound IsdB, which had ~70% of holo-form (designated holo-$IsdB_{E.\ coli}$) as determined by the pyridine hemochrome and protein concentration assays (see below). IsdC obtained was a mixture of apo-IsdC and the protoporphyrin-IsdC complex. The mixture was loaded onto a DEAE-Sepharose column (1 × 4 cm), and apoIsdC was recovered in flow-through and Tris-HCl wash. Purified IsdE and IsdA were apo-IsdE/protoporphyrin-IsdE complex and apo-/holo-IsdA mixtures, respectively, and homogeneous apo-proteins were prepared by the methyl ethyl ketone method (32).

Besides holo-$IsdB_{E.\ coli}$ directly purified from *E. coli*, holo-IsdB was also prepared by reconstitution from apo-IsdB and hemin (designated holo-$IsdB_R$). Apo-IsdB was incubated with hemin at 1:1.5 apo-IsdB:hemin ratio for 20 min, and the sample was loaded onto a SP Sepharose column (1.0 × 2 cm). The column was extensively washed with 20 mM Tris-HCl to remove free hemin and eluted with 100 mM NaCl to recover holo-IsdB. The IsdB sample obtained contained 95% holo-form according to the measurements of protein and hemin contents. Holo-IsdA, holo-IsdC, and holo-IsdE were obtained by reconstitution from hemin and their apo-proteins. One ml of each protein was incubated with excess hemin for 20 min and loaded onto a Sephadex G-25 column (1 × 30 cm). The column was eluted with 20 mM Tris-HCl, pH 8.0. Holo-proteins without free hemin were collected. More than 95% of IsdA, IsdC, and IsdE obtained were in holo-form based on protein and hemin contents.

Determination of Protein Concentration and Hemin Contents—Protein concentrations were determined using a modified Lowry protein assay kit with bovine serum albumin as a standard. Hemin contents of holo-Isd proteins and metHb were measured with the pyridine hemochrome assay using $\epsilon_{418} = 191.5\ mM^{-1}\ cm^{-1}$ (33).

Rate of Hemin Dissociation from IsdB and IsdE—The rate constants for hemin dissociation from holo-proteins were measured using H64Y/V68F apo-myoglobin as a hemin scavenger (34). Each holo-protein at 3 μM was incubated with 45 μM H64Y/V68F apo-myoglobin (apo-Mb) in 1 ml of 20 mM Tris-HCl, and changes in absorbance at 544 and 600 nm were monitored for up to 6 h. Time courses of $A_{600} - A_{544}$ for each reaction were fit to a single exponential expression to obtain the first order rate constant for hemin dissociation.

Hemin Transfer—Holo-protein (hemin donor) and apo-protein (hemin acceptor) at concentrations specified under "Results" or in the figure legends were incubated in 0.2 ml of 20 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 resin), and the column was washed and eluted, obtaining 0.15-ml fractions. In the separation of Hb from IsdB, IsdA, or IsdE in the reaction mixture, the column was washed with 20 mM Tris-HCl, pH 8, to recover unbound Hb and then eluted with 0.2 M NaCl in Tris-HCl to release bound protein(s). To separate IsdB from IsdA or IsdE, the column was washed 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 M 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 Tris-HCl, 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-IsdA, apo-IsdC, or apo-IsdE and from holo-IsdA, holo-IsdB, or holo-IsdC to apo-IsdE were measured by monitoring the absorbance changes using a conventional spectrophotometer (SPECTRA^{max} 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.

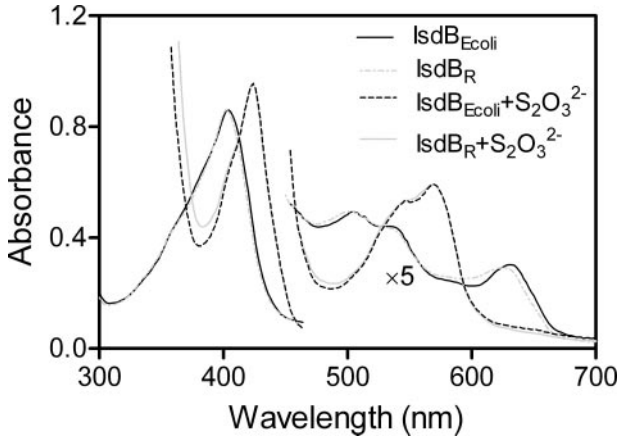

FIGURE 1. **Optical absorption spectra of oxidized and reduced IsdB (8.7 μM).** IsdB$_{E.\ coli}$ and IsdB$_R$ were purified from *E. coli* and reconstituted from apo-IsdB, respectively. The reduced spectra were recorded in the presence of excess dithionite.

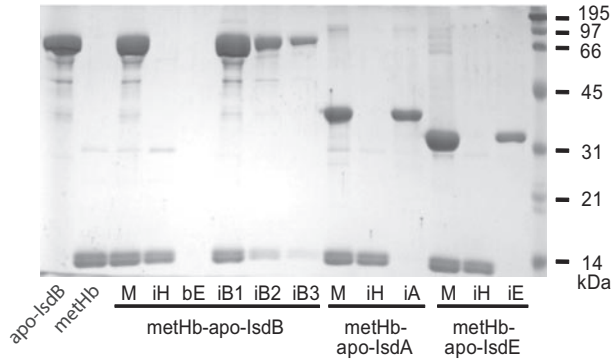

FIGURE 2. **SDS-PAGE evaluation of separation of the proteins in the metHb-apo-IsdB, -apoIsdA, and -apo-IsdE reactions.** The reaction and separation conditions are described in text. *Lane M*, the reaction mixture; *lane iH*, isolated Hb; *lane bE*, fraction right before the IsdB elution; *lanes iB1–iB3*, three fractions of isolated IsdB; *lane iA*, isolated IsdA; *lane iE*, isolated IsdE.

The time courses of the absorbance changes obtained from these reactions were fitted to a single or double phase exponential expression using GraphPad Prism software to obtain the apparent rate constants for the transfer reactions.

RESULTS

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 hemin 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 hemin 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 A_{405}/A_{280} of the IsdB-free Hb sample from the metHb-apo-IsdB reaction was 70% of that of the starting metHb (Fig. 3*A*), 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. 3*B*). 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 A_{405}/A_{280} 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. 3*D*) or IsdE sample (Fig. 3*F*) 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 hemin to apo-IsdB much 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 hemin 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. 4*A*). The spectrum recorded at 9.87 s after mixing almost

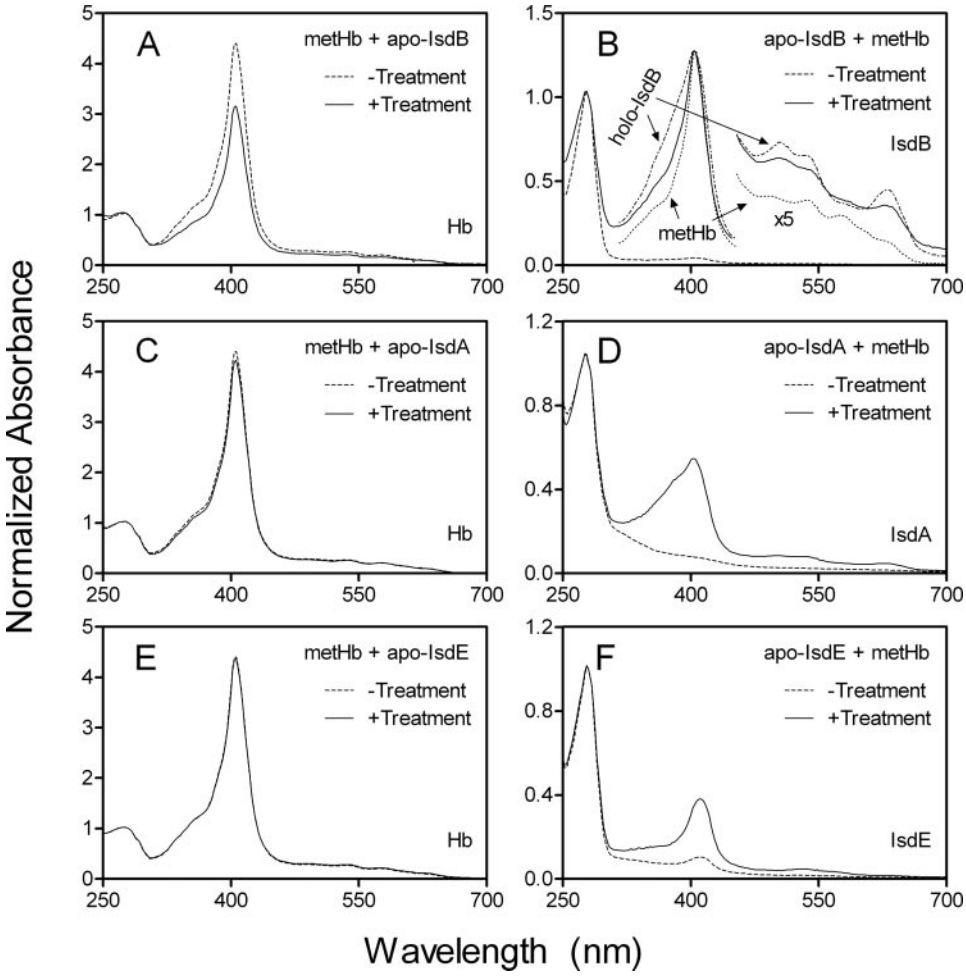

FIGURE 3. **Hemin transfer from metHb to the Isd proteins.** MetHb (20 μM hemin) was incubated with 35 μM apo-IsdB (*A* and *B*), apo-IsdA (*C* and *D*), and apo-IsdE (*E* and *F*) in 0.2 ml of 20 mM Tris-HCl, pH 8.0, for 2 min. The two proteins in each reaction were separated as shown in Fig. 2. Presented are the normalized spectra of Hb and Isd proteins without and with the reaction treatment. The normalized spectra of holo-IsdB and metHb are included in *B* for spectral comparison.

overlays that of holo-IsdB (Fig. 4*B*), 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. 4*C*), 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. 4*C* 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. 4*C*), yielding two observed rate constants of 1.2×10^{-3} and 1.4×10^{-4}, 1.1×10^{-2} and 8.6×10^{-4}, and 4.1×10^{-4} and 6.9×10^{-5} 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-apomyoglobin reaction ($k = 3.5 \times 10^{-3}$ 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-$IsdB_R$ 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 A_{402}/A_{280} ratio of IsdB from the holo-$IsdB_R$/apo-IsdA and holo-$IsdB_R$/apo-IsdE reaction mixtures was ~60 and 90% of that of the starting holo-$IsdB_R$, 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. 5*E*), and IsdB-free IsdC from the same reaction was in holo-form (Fig. 5*F*). Thus, holo-$IsdB_R$ 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-$IsdB_R$ 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-$IsdB_R$ to that for holo-IsdA (Fig. 6*A*) or holo-IsdC (Fig. 6*B*). 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-$IsdB_R$ reactions were 114 and 15 s^{-1}, respectively (Fig. 6*C*). These rate constants are 87,000- and 11,500-fold greater than the rate constant for simple hemin dissociation from holo-$IsdB_R$, which was determined to be 1.3×10^{-3} s^{-1} by the H64Y/V68F apo-Mb assay

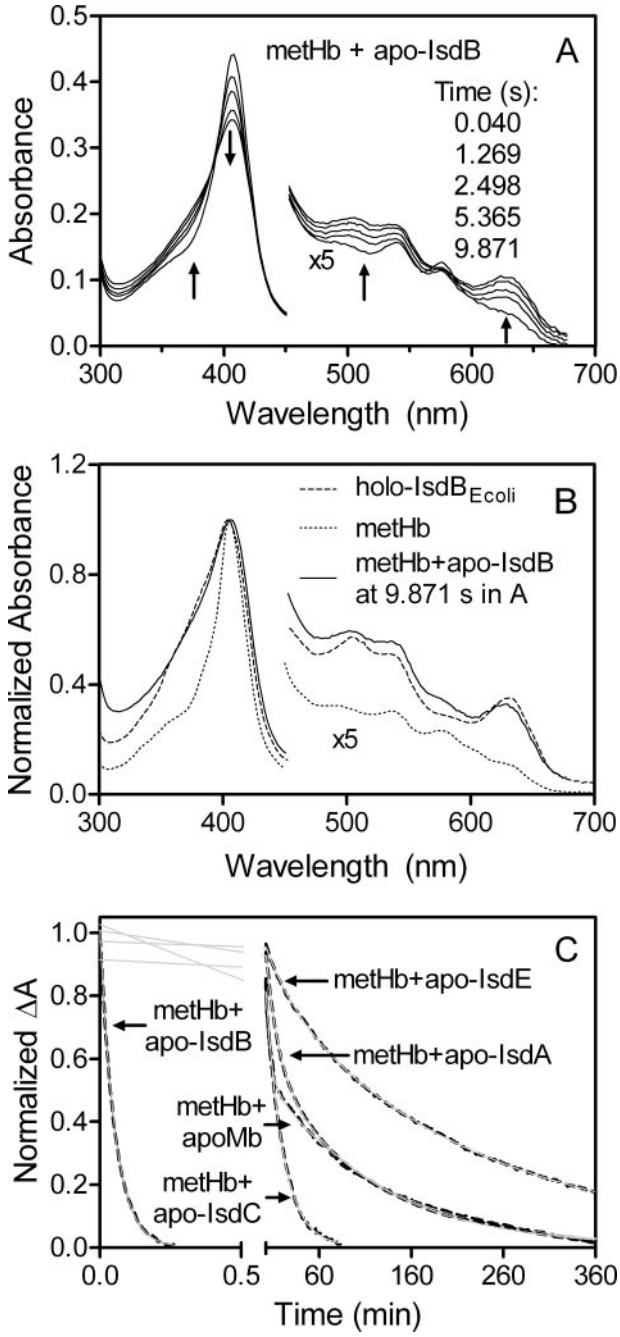

FIGURE 4. **Kinetics of hemin transfer from metHb to the Isd proteins.** *A*, absorption spectra of a mixture of 2.7 μM metHb and 20 μM apo-IsdB as a function of time after mixing. The *arrows* indicate the directions of the spectral change with time. *B*, overlay of the normalized spectra of the metHb/apo-IsdB mixture at 9.871 s in *A*, holo-IsdB$_{E.\ coli}$, and metHb. *C*, time courses of the spectral change in the metHb (2.7 μM) and apo-IsdB (27 μM), metHb (2.7 μM) and apo-IsdA (25 μM), metHb (2.7 μM) and apo-IsdC (25 μM), metHb (2.7 μM) and apo-IsdE (25 μM), and metHb (2.7 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.

(Fig. 6*C*). Clearly, apo-IsdA and apo-IsdC can efficiently and directly acquire hemin from holo-IsdB.

In a reaction of 2.5 μM holo-IsdB$_R$ with 30 μM apo-IsdE, absorption spectrum of the reaction mixture was shifted from

TABLE 1
Apparent rate constants of hemin transfer among methemoglobin, myoglobin, and Isd proteins

Hemin donor	Hemin acceptor	k or k_1[a]	k_2[a]
		s^{-1}	s^{-1}
metHb	apo-IsdA	0.0012	0.00014
	apo-IsdB	0.31	
	apo-IsdC	0.011	0.00086
	apo-IsdE	0.00041	0.000069
	apomyoglobin	0.0035	0.00014
holo-IsdB$_R$	apo-IsdA	114	
	apo-IsdC	15	
	apo-IsdE	0.0016	
	apomyoglobin	0.0013	
holo-IsdB$_{E.\ coli}$	apo-IsdA	87	
	apo-IsdC	10.7	
	apo-IsdE	UD[b]	
	apomyoglobin	UD[b]	
holo-IsdA	apo-IsdC	54.3[c]	
	apo-IsdE	UD[b]	
	apomyoglobin	0.00076	
holo-IsdC	apo-IsdA	UD[b,c]	
	apo-IsdE	0.0062	0.0007
	apomyoglobin	UD[b]	
holo-IsdC^{His}	apo-IsdE	0.0077	0.0029
	apomyoglobin	≤0.00072[d]	

[a] The k and k_1/k_2 values were obtained by fitting data to single and double exponential expressions, respectively.
[b] Unable to determine.
[c] The data are from Ref. 30.
[d] The value was obtained from incomplete transfer reaction and represents an upper estimate of the true hemin dissociation rate constants for IsdC^{His}.

that of holo-IsdB toward but did not completely turn into that of holo-IsdE (supplemental Fig. S5*A*), indicating that only a portion of the hemin in holo-IsdB$_R$ 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-IsdB$_R$. The time course for the holo-IsdB$_R$-apo-IsdE reaction was fit to a single exponential expression (supplemental Fig. S5*B*), 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. S5*B*), but is 71,200- and 9,370-fold slower than those for the holo-IsdB$_R$-apo-IsdA and holo-IsdB$_R$-apo-IsdC reactions, respectively. These measurements indicate that holo-IsdB$_R$ does not directly nor rapidly transfer hemin to apo-IsdE.

Many of the properties of holo-IsdB$_{E.\ coli}$ were similar to those of holo-IsdB$_R$. Holo-IsdB$_{E.\ coli}$ efficiently transfers its hemin to both apo-IsdA and apo-IsdC but not to apo-IsdE (supplemental Fig. S6). The spectra of holo-IsdB$_{E.\ coli}$-apo-IsdA and -apo-IsdC reaction mixtures rapidly shift from that of holo-IsdB$_{E.\ coli}$ to those of the holo-IsdA 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

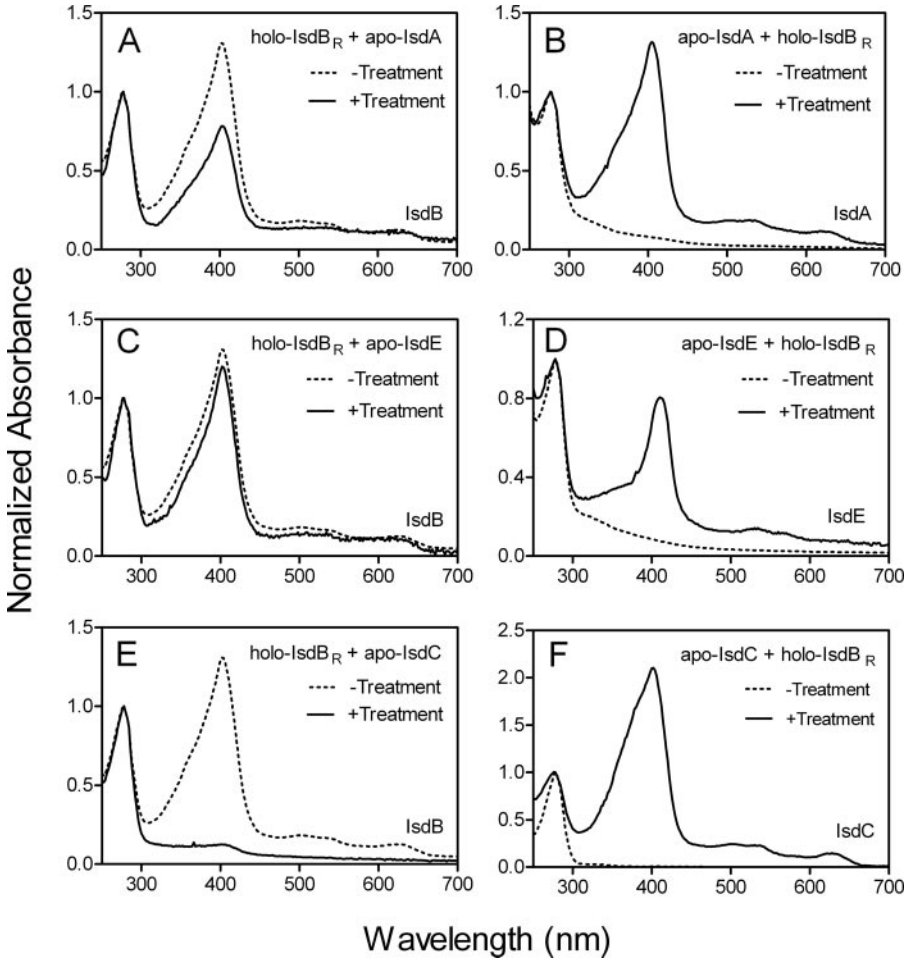

FIGURE 5. **Hemin transfer from holo-IsdB$_R$ to apo-IsdA (*A* and *B*), apo-IsdE (*C* and *D*), and apo-IsdC (*E* and *F*).** 6 μM holo-IsdB$_R$ was incubated with 12 μM apo-IsdA, apo-IsdC, or apo-IsdE for 2 min. The two proteins were separated (supplemental Fig. S4). Presented are the normalized spectra of IsdB and the other Isd proteins with and without the reaction treatment.

(Fig. 7*A*), indicating no significant transfer. In contrast, the Soret maximum of the holo-IsdC-apo-IsdE mixture under the same conditions did shift with time from 402 to 412 nm, and the transfer was apparently incomplete because the spectrum of the reaction mixture does not perfectly overlay that of holo-IsdE, indicating that there is an equilibrium distribution between holo-IsdC and holo-IsdE (Fig. 7*B*). At a 1:1 holo-IsdC: apo-IsdE ratio, the shift was much less dramatic (data not shown), indicating that IsdC has higher affinity for hemin than IsdE and that the equilibrium favors to holo-IsdC. The kinetics of transfer were then examined by reacting 3 μM holo-IsdC with 45 μM apo-IsdE. The time course for $\Delta(A_{418} - A_{378})$ *versus* time was fit to a two exponential expression (Fig. 7*C*), yielding apparent rate constants of 6.3×10^{-3} and 7×10^{-4} s^{-1}. As described below, holo-IsdC does not lose hemin to H64Y/V68F apo-Mb under the similar conditions, and the rate of simple dissociation of IsdC hemin could not be measured to determine whether IsdC-to-IsdE hemin transfer is direct. His$_6$-tagged holo-IsdC (holo-IsdC^{His}) loses ~20% of its hemin in a reaction of 3 μM holo-IsdC^{His} with 48 μM H64Y/V68F apo-Mb (30). The observed rate constant for this partial hemin loss was 7.2×10^{-4} s^{-1} (Fig. 7*C*). The time course of the spectral change in the holo-IsdC^{His}/apo-IsdE reaction is almost identical to that of the holo-IsdC/apo-IsdE reaction (Fig. 7*C*) and had two apparent rate constants of 7.7×10^{-3} and 2.9×10^{-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. 7*A* 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 μM metHb with 22 μM apo-IsdC in the presence or absence of 0.4 μM IsdB. The addition of catalytic amounts of IsdB dramatically enhanced hemin transfer from metHb to apo-IsdC (Fig. 8*A*). A similar catalytic effect of IsdC on hemin transfer from holo-IsdB$_R$ to apo-IsdE was observed (Fig. 8*B*). 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 IsdE 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 ~20:1 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 1×10^{12} M^{-1} at pH 7.0 based on a bimolecular rate constant of 1×10^{8} M^{-1} s^{-1} for

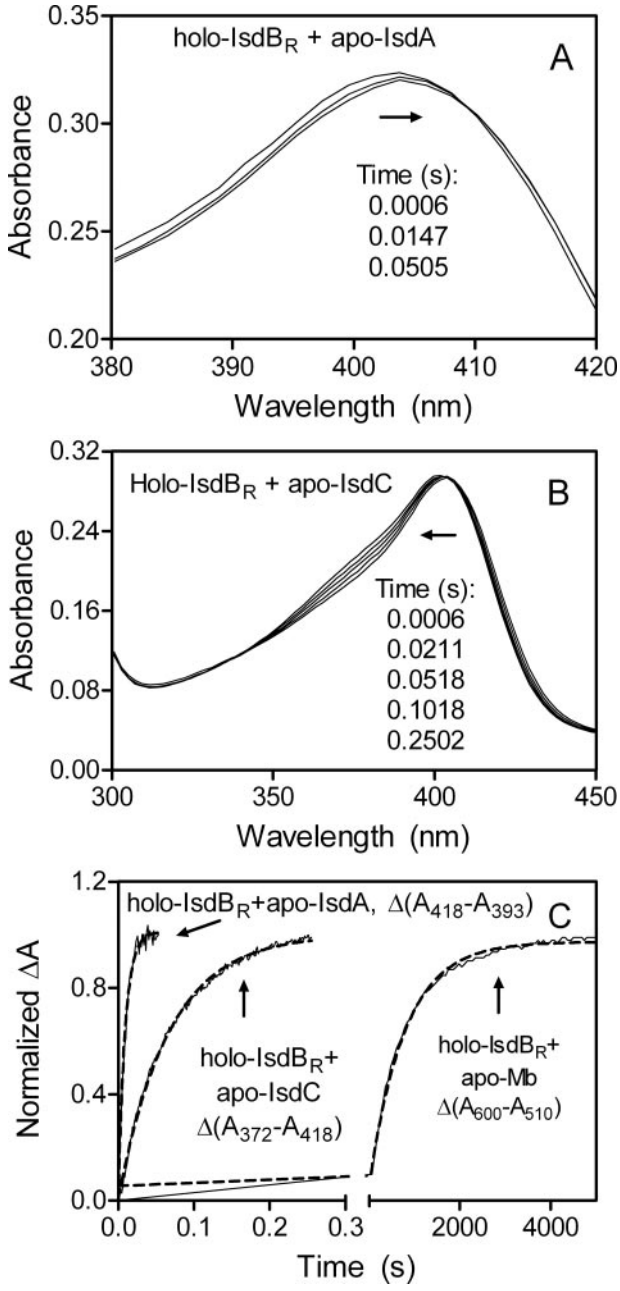

FIGURE 6. **Kinetics of hemin transfer from holo-IsdB$_R$ to apo-IsdA and apo-IsdC.** *A* and *B*, absorption spectra of a mixture of 3 μM holo-IsdB$_R$ with 30 μM apo-IsdA (*A*) or apo-IsdC (*B*) as a function of time after mixing. The *arrows* indicate the direction of the spectral shifts with time. *C*, time courses of normalized spectral changes for the indicated reactions. The *solid* and *dashed curves* represent the observed data and single exponential fitting curves, respectively. The rate constant for the reactions (s^{-1}): IsdA, 114; IsdC, 15; and Mb, 0.0013.

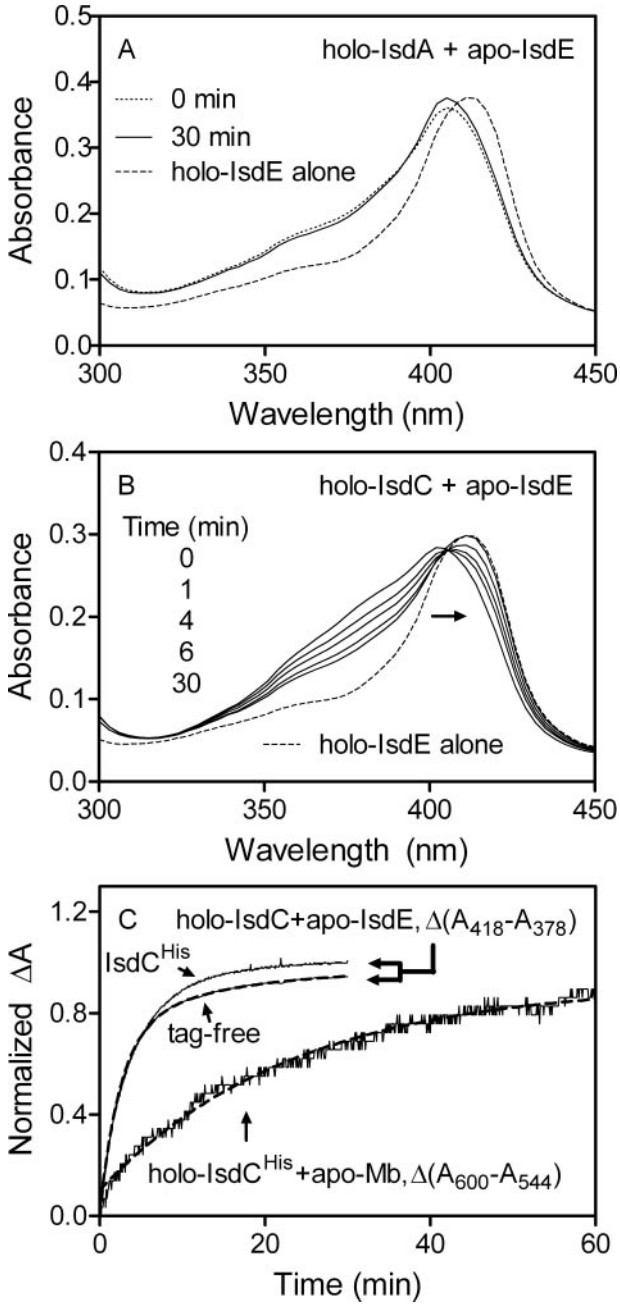

FIGURE 7. **Hemin transfer from holo-IsdC to apo-IsdE.** *A* and *B*, spectra of the mixture of 45 μM apo-IsdE with 3 μM holo-IsdA (*A*) or holo-IsdC (*B*) at the indicated time after mixing. The *arrow* in *B* indicates the direction of the spectral shift with time. *C*, time courses of normalized spectral change in the reactions of 3 μM tag-free or His-tagged (IsdC^{His}) holo-IsdC with apo-IsdE and H64Y/V68F apo-Mb. The *solid* and *dashed curves* are the observed data and theoretical lines obtained by fitting the data to single exponential (apo-Mb reaction) or two-exponential (apo-IsdE reaction) expressions. The rate constant(s) for the reactions (s^{-1}): IsdC/apo-IsdE, 0.0063 and 0.0007; IsdC^{His}/apo-IsdE, 0.0077 and 0.0029; and IsdC^{His}/apo-Mb, 0.00072.

hemin association to H64Y/V68F Mb (4) and a dissociation rate constant of 1.1×10^{-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 > IsdE > IsdB ≈ 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

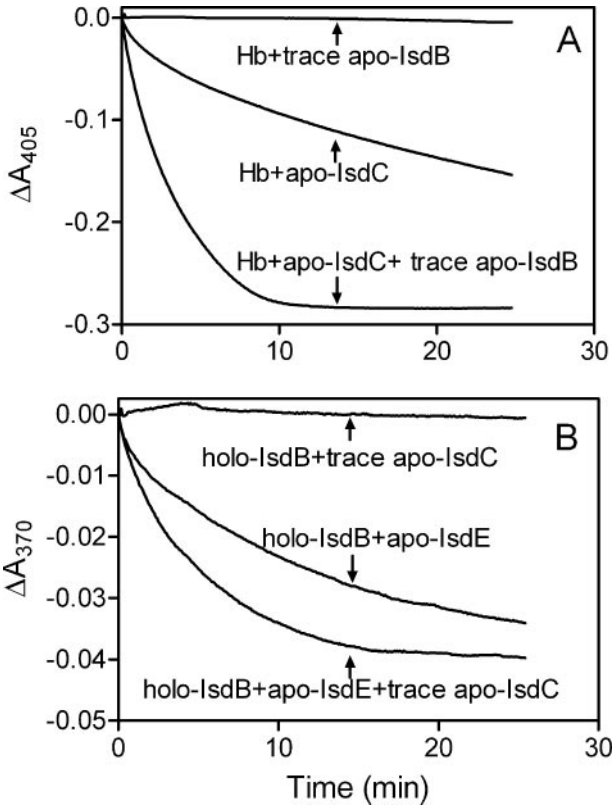

FIGURE 8. **Relay roles of IsdB and IsdC in the hemin acquisition pathway.** Catalytic amounts of IsdB and IsdC speed up hemin transfer from metHb to apo-IsdC and from holo-IsdB$_R$ to apo-IsdE, respectively. Presented are the spectral changes associated with hemin transfer in the reactions of 7 μM metHb with 0.4 μM IsdB or 22 μM apo-IsdC in the presence or absence of 0.4 μM IsdB (*A*) and 4 μM holo-IsdB$_R$ with 30 μM apo-IsdE in the presence or absence of 1 μM apo-IsdC (*B*).

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, ~0.3 s^{-1}, which is 80–2200 times greater than the rates for simple hemin dissociation from the β and α 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 α and β subunits at the same rate, whereas simple thermal dissociation of hemin from the α and β 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 α and β 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. 8*A*).

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 *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. 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-

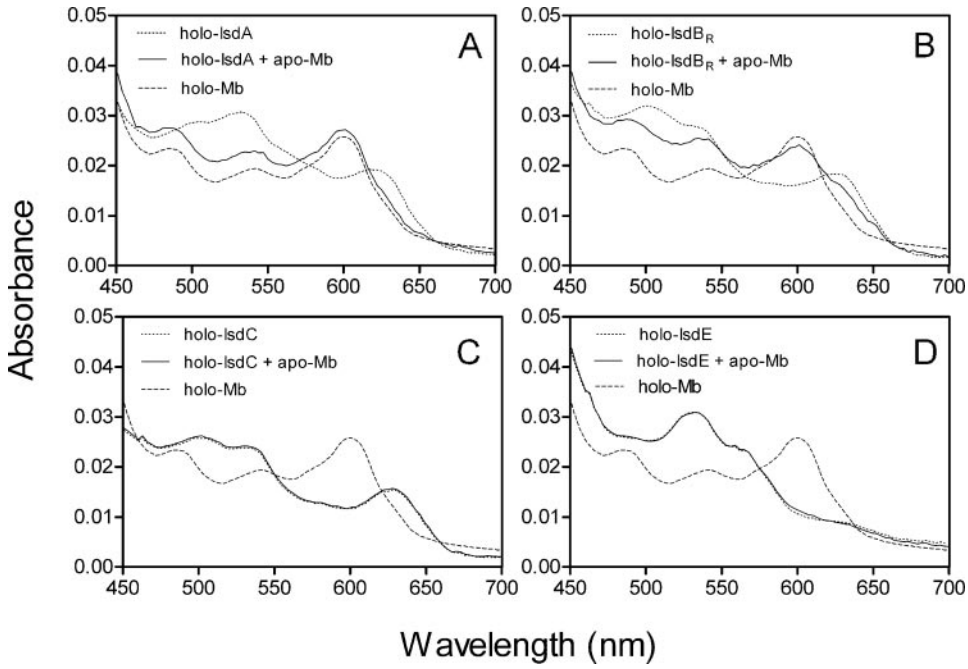

FIGURE 9. **The reactions of H64Y/V68F apo-Mb with the holo-Isd proteins.** Presented are the spectra of the mixtures of 45 μM H64Y/V68F apo-Mb with 2 μM holo-IsdA (*A*), holo-IsdB$_R$ (*B*), holo-IsdC (*C*), and holo-IsdE (*D*) after a 12-h incubation. The spectra of holo-Mb and corresponding holo-Isd protein are included for comparison.

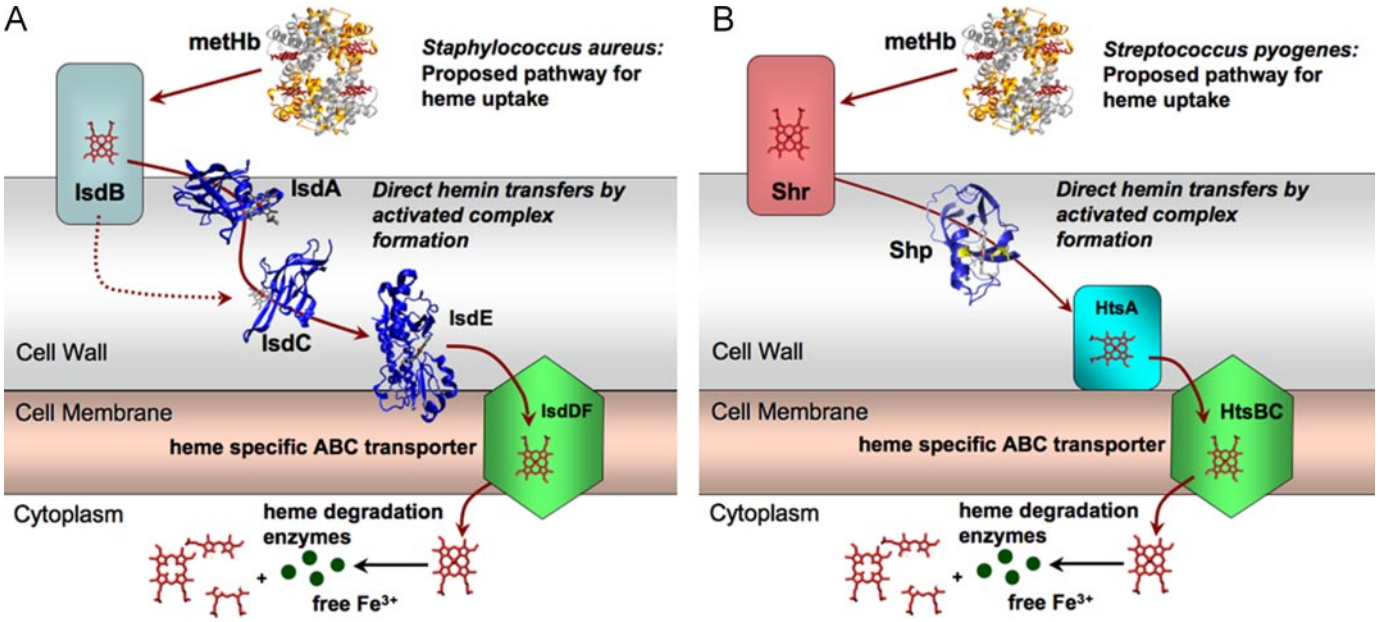

FIGURE 10. **Schematics for the proposed models of hemin acquisition from metHb by the *S. aureus* Isd and *S. pyogenes* Shr/Shp/HtsABC systems.** The *arrows* indicate the direction of direct hemin transfer. The *S. aureus* model (*A*) is derived from the experimental data of this work and Refs. 7, 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 hemin transfer and heme degradation mediated by heme oxygenase have not been experimentally demonstrated in the *S. pyogenes* model. The hemin 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, 2O6P, 2Q7A, and 1HHO.

pates in the extraction of hemin 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 hemin 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 [^{55}Fe]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 hemin from metHb.

Holo-IsdB transfers its hemin to apo-IsdA approximately eight times more rapidly than to apo-IsdC, suggesting that IsdA is the initial acceptor of hemin 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 hemin 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 hemin to apo-IsdE. Holo-IsdB and holo-IsdA, which have lower affinities for hemin than IsdC, inefficiently transfer their hemin to apo-IsdE, whereas holo-IsdC does directly transfer hemin to apo-IsdE under the similar conditions. The rate of hemin transfer from holo-IsdC^{His} to apo-IsdE is faster than simple hemin dissociation from holo-IsdC^{His}. 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 hemin 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 hemin transfer from holo-IsdB to apo-IsdE (Fig. 8*B*).

Although we could not accurately measure the affinities of the Isd proteins for hemin, 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. 4*B*), and, by assuming that 90% of the Hb hemin was transferred to IsdB at the equilibrium, the affinity of IsdB for hemin would be estimated to be ~10-fold higher than that of an α chain in a dimer of Hb, which is $\sim 6 \times 10^{11}\ M^{-1}$ (4). This estimation ($\sim 6 \times 10^{12}\ M^{-1}$) is consistent with the incomplete loss of hemin from 3 μM holo-IsdB to 45 μM H64Y/V68F apo-Mb ($K_{hemin} \approx 1 \times 10^{12}\ M^{-1}$). The affinity of IsdA for hemin is similar to that of IsdB. The hemin 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 hemin. 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 other steps from metHb through the Isd surface proteins to IsdE. These results suggest the model for the heme acquisition that is shown in Fig. 10*A* for *S. aureus*. In this pathway, IsdB binds metHb, extracts hemin from its α and β subunits. The holo-IsdA formed can in turn relay the hemin to IsdC, which then transfers it to IsdE, the lipoprotein component of the IsdDEF transporter. The transported hemin is then degraded inside the cell by IsdG and IsdI to release iron (40). This experimental model is different from the previous model (20, 21) in two respects. First, IsdB is not simply a metHb receptor but actively relays hemin from metHb to IsdA. Second, IsdB can, depending on the physical location of the Isd proteins, directly transfer its hemin to IsdC. It should be pointed out that the role of IsdH in heme acquisition is not addressed in our studies and in this model.

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. 10*B*). 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 at 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.

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.[3] 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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