The *Staphylococcus aureus* Protein IsdH Inhibits Host Hemoglobin Scavenging to Promote Heme Acquisition by the Pathogen*

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Hemolysis is a complication in septic infections with *Staphylococcus aureus*, which utilizes the released Hb as an iron source. *S. aureus* can acquire heme *in vitro* from hemoglobin (Hb) by a heme-sequestering mechanism that involves proteins from the *S. aureus* iron-regulated surface determinant (Isd) system. However, the host has its own mechanism to recapture the free Hb via haptoglobin (Hp) binding and uptake of Hb-Hp by the CD163 receptor in macrophages. It has so far remained unclear how the Isd system competes with this host iron recycling system *in situ* to obtain the important nutrient. By binding and uptake studies, we now show that the IsdH protein, which serves as an Hb receptor in the Isd system, directly interferes with the CD163-mediated clearance by binding the Hb-Hp complex and inhibiting CD163 recognition. Analysis of truncated IsdH variants indicating one or more of three near iron transporter domains, IsdH<sup>N1</sup>, IsdH<sup>N2</sup>, and IsdH<sup>N3</sup>, revealed that Hb binding of IsdH<sup>N1</sup> and IsdH<sup>N2</sup> accounted for the high affinity for Hb-Hp complexes. The third near iron transporter domain, IsdH<sup>N3</sup>, exhibited redox-dependent heme extraction, when Hb in the Hb-Hp complex was in the oxidized form but not in the reduced form. IsdB, the other *S. aureus* Hb receptor, failed to extract heme from Hb-Hp, and it was a poor competitor for Hb-Hp binding to CD163. This indicates that Hb recognition by IsdH, but not by IsdB, sterically inhibits the receptor recognition of Hb-Hp. This function of IsdH may have an overall stimulatory effect on Hb-Hp heme acquisition and growth.

*Staphylococcus aureus* is a Gram-positive bacterium that colonizes approximately one-third of the human population (1). It can be invasive and cause an array of diseases including hemolysis and septic shock. Successful host invasion involves compromising the efficacy of the immune system and efficient acquisition of essential nutrients including iron. Like a number of other pathogenic bacteria (*e.g.* strains of *Escherichia coli*, *Pseudomonas*, and *Streptococci*) (2–4), *S. aureus* secretes an α-hemolysin that integrates in red blood cell membranes and induces osmotic hemolysis. Liberation of Hb into plasma facilitates *S. aureus* acquisition of iron by means of an iron-sequestering pathway designated the iron-regulated surface determinant (Isd)<sup>3</sup> system (5, 6). *S. aureus* expresses several different Isd proteins (IsdA, IsdB, IsdC, IsdE, IsdF, IsdG, and IsdH) that orchestrate the acquisition of host Hb heme iron. The functions of most of these proteins have been elucidated: extraction of heme is achieved by the two bacterial surface-exposed Hb-receptors, IsdB and IsdH; transport of heme across the bacterial cell wall and plasma membrane is performed by IsdA and IsdC together with the membrane protein IsdEF complex; and the heme oxygenase enzymes IsdG and IsdH, located in the cytoplasm, finally cleave the porphyrin ring (reviewed in Ref. 7). Although the role of Isd proteins in the sequestering of iron from free Hb is well understood, this may not apply to the situation in the blood where extracellular Hb is found in complex with Hp. The heme-binding function of *S. aureus* Isd proteins—IsdA, IsdB, IsdC, and IsdH—is conferred by the presence of a near iron transporter (NEAT) domain with a conserved heme-binding pocket (8). Importantly, the heme-binding domain alone is unable to extract heme from Hb, and IsdB and IsdH contain additional NEAT domains to achieve this function (9). Thus, IsdH contains three NEAT domains of which the first and second NEAT domain (IsdH<sup>N1</sup> and IsdH<sup>N2</sup>) bind to Hb but lack heme binding activity, whereas the third, C-terminal, NEAT domain (IsdH<sup>N3</sup>) carries the single heme-binding site of IsdH. IsdH<sup>N2</sup> and IsdH<sup>N3</sup> are connected by an α-helical linker domain and the IsdH<sup>N2</sup>-linker-IsdH<sup>N3</sup> region is the minimal fragment of the IsdH receptor that retains native ability to capture heme from Hb (9–11). IsdB has a two-NEAT domain

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§ This abbreviation used is: Isd, iron-regulated surface determinant; NEAT, near iron transporter; Hb, hemoglobin; Hp, haptoglobin; SEC, size exclusion chromatography; RALS, in-line right angle light scattering; CHO CD163, CD163-expressing CHO cells; SRCR, scavenger receptor cysteine-rich; MFI, mean fluorescence intensity; SPR, surface plasmon resonance.
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Binding of IsdH to Hb-Hp Complexes—IsdH truncation variants IsdH\textsuperscript{N1}, IsdH\textsuperscript{N2N3}, IsdH\textsuperscript{N1N2N3}, and IsdB\textsuperscript{N1N2} with a His\textsubscript{6} tag were expressed recombinantly in E. coli with N-terminal His\textsubscript{6} tags, purified, and analyzed by SDS-PAGE (Fig. 1). Binding of the IsdH variants to Hb-Hp complexes was evaluated by SPR analysis (Fig. 2, A and B). The apparent equilibrium dissociation constants for binding of IsdH\textsuperscript{N1} (one Hb binding site), IsdH\textsuperscript{N2N3} (one Hb binding site), or IsdH\textsuperscript{N1N2N3} (two Hb binding sites) to Hb-Hp\textsubscript{1-1} or Hp\textsubscript{2-2} were 70, 120, and 30 nM, respectively (Fig. 2). The binding curves of IsdH\textsuperscript{N1} and IsdH\textsuperscript{N1N2N3} were different in the sense that IsdH\textsuperscript{N1} and IsdH\textsuperscript{N2N3} displayed faster off rates, compared with IsdH\textsuperscript{N1N2N3}. These results suggest that the two Hb-binding domains in IsdH\textsuperscript{N1} and IsdH\textsuperscript{N2}, together increase the overall functional affinity of IsdH\textsuperscript{N1N2N3} for Hb-Hp. No significant binding of any of the IsdH constructs was seen to Hp\textsubscript{1-1} and Hp\textsubscript{2-2} (Fig. 2, C and D).

In the present study, we show that IsdH only binds Hb-Hp via a direct Hb interaction without direct contact to the Hp subunit, in contrast to previous reporting (12, 13, 23) of a direct low affinity interaction between IsdH and Hp. Furthermore, this study describes how the interaction with Hb-Hp leads to heme extraction and obstruction of CD163-mediated clearance of Hb-Hp complexes in macrophages.

Results

Binding of IsdH to Hb-Hp Complexes—IsdH truncation variants IsdH\textsuperscript{N1}, IsdH\textsuperscript{N2N3}, IsdH\textsuperscript{N1N2N3}, and IsdB\textsuperscript{N1N2} with a His\textsubscript{6} tag were expressed recombinantly in E. coli with N-terminal His\textsubscript{6} tags, purified, and analyzed by SDS-PAGE (Fig. 1). Binding of the IsdH variants to Hb-Hp complexes was evaluated by SPR analysis (Fig. 2, A and B). The apparent equilibrium dissociation constants for binding of IsdH\textsuperscript{N1} (one Hb binding site), IsdH\textsuperscript{N2N3} (one Hb binding site), or IsdH\textsuperscript{N1N2N3} (two Hb binding sites) to Hb-Hp\textsubscript{1-1} or Hp\textsubscript{2-2} were 70, 120, and 30 nM, respectively (Fig. 2). The binding curves of IsdH\textsuperscript{N1} and IsdH\textsuperscript{N1N2N3} were different in the sense that IsdH\textsuperscript{N1} and IsdH\textsuperscript{N1N2N3} displayed faster off rates, compared with IsdH\textsuperscript{N1N2N3}. These results suggest that the two Hb-binding domains in IsdH\textsuperscript{N1} and IsdH\textsuperscript{N2}, together increase the overall functional affinity of IsdH\textsuperscript{N1N2N3} for Hb-Hp. No significant binding of any of the IsdH constructs was seen to Hp\textsubscript{1-1} and Hp\textsubscript{2-2} (Fig. 2, C and D).

To further characterize the interaction between IsdH\textsuperscript{N1} and Hb or Hb-Hp complexes in solution, we employed size exclusion chromatography (SEC) with in-line right angle light scattering (RLAS). The molecular mass of IsdH\textsuperscript{N1} determined by SEC-RLAS in solution (18.6 kDa) was close to the monomer molecular mass based on sequence and determined by mass spectrometry (18,907 Da) (Fig. 3). The major component of the Hp\textsubscript{1-1} preparation was a 93-kDa species, consistent with the presence of the Hp\textsubscript{1-1} dimer, including 20% mass contribution because of glycosylation; a minor peak at the exclusion volume of the SEC column was assumed to be an aggregate. Hb eluted as a single asymmetric peak, indicative of the well characterized dimer-tetramer equilibrium (24); the measured molecular mass of 57 kDa suggested that the protein was predominantly in the tetrameric state under the experimental conditions. Analysis of a 1:1 (by mass) mixture of IsdH\textsuperscript{N1} with Hp showed no significant change in the peak elution times or molecular masses as determined by RLAS (Fig. 3); thus no interaction between IsdH\textsuperscript{N1} and Hp could be detected. In contrast,
Hp and Hb in an 1:1 (by mass) mixture formed a complex with a molecular mass of 152 kDa, consistent with a stoichiometry of one Hp1-1 dimer bound to two Hb/Hb/Hb/Hb-dimers; the absence of any detectable free Hb is consistent with the known high affinity binding between Hp and Hb. The elution of this complex shifted to an earlier time point, and the molecular mass of the complex increased upon addition of IsdHN1 (Fig. 3), indicating formation of a ternary Hb-Hp-IsdHN1 complex. Taken together, the SPR and SEC-RALS data show that Hb is necessary for interaction between Hb-Hp and IsdH, most likely via a direct interaction between IsdH and Hb.

IsdH Acquisition of Heme from Hb-Hp—To determine whether IsdHN3 is capable of extracting heme from Hb-Hp, heme transfer from Hb-Hp to IsdHN2N3 was monitored using UV-visible spectroscopy, taking advantage of the different spectra generated when heme is bound to the globin or bound to the IsdH protein. Previous studies have confirmed rapid heme transfer to IsdHN2N3 from metHb (9, 13). Similar results were obtained when metHb was incubated in the presence of added IsdHN2N3 (Fig. 4A). Significant absorption changes were also evident when metHb-Hp1-1 was incubated with IsdH2N3 (Fig. 4C), indicating that IsdH is capable of extracting heme from Hb in complex with Hp. When IsdHN2N3 was mixed with ferrous oxyHb or oxyHb-Hp1-1, no spectral changes were detected (Fig. 4, E and F). Thus, ferrous oxyHb has to be oxidized to metHb for IsdH to be able to extract heme.

IsdH Interference with CD163 Binding of Hb-Hp—Surface plasmon resonance (SPR) analysis was used to determine whether the high affinity binding of IsdH to Hb-Hp interferes with the binding of the complex to purified CD163. A robust SPR response was obtained when Hb-Hp1-1 was injected over CD163 immobilized on the SPR chip surface (Fig. 5, A, Buffer), indicating a complex forming between Hb-Hp1-1 and CD163. In contrast, when Hb-Hp1-1 was injected together with an equimolar concentration of IsdHN1, the SPR response was strongly attenuated (Fig. 5, A), suggesting that IsdHN1 interferes with the interaction of Hb-Hp1-1 with CD163. Similarly, the IsdHN3 and IsdHN1N2N3 proteins also blocked the Hb-Hp1-1 interactions with CD163 (Fig. 5, A and C). Similar results were obtained for interactions of Hb-Hp2-2 with CD163 (Fig. 5, B and D). All IsdH constructs exhibited dose-dependent inhibition of Hb-Hp binding to CD163 with IsdHN1N2N3 showing the highest potency. The IsdH variants were slightly more potent in
their inhibition of Hb-Hp2-2 (Fig. 5, B and D) binding compared with Hb-Hp1-1 (Fig. 5, A and C).

To investigate how these interactions may impact on clearance of Hb-Hp complexes by cellular CD163, we analyzed the endocytosis of fluorophore-labeled Hb-Hp2-2 by transfected CD163-expressing CHO cells (CHO CD163) in the presence of IsdHN1N2N3, IsdHN2N3, or IsdHN1. As expected, confocal microscopy showed that fluorophore-labeled Hb-Hp (Fig. 6, green) only was endocytosed when CHO cells were expressing CD163 (Fig. 6A, red). A substantial inhibition of CD163-mediated vesicular Hb-Hp uptake was observed in the presence of IsdH^{N1N2N3}, IsdH^{N2N3}, or IsdH^{N1} compared with the positive control (Fig. 6A). The inhibitory effect by IsdH was quantified by analyzing the cellular uptake of fluorescent Hb-Hp2-2 by flow cytometry (Fig. 6B). All IsdH constructs inhibited Hb-Hp uptake in a dose-dependent manner, with IsdH^{N1N2N3} being the most efficient showing a 6-fold reduction in uptake using an equimolar concentration of the Hb-Hp units. Complete inhibition, corresponding to the background level in the mock transfected CHO cells, was seen at 5 molar excess of IsdH^{N1N2N3} and IsdH^{N1}. The inhibitory effect by IsdH was also evident in flow cytometric analysis of monocytes/macrophages, the cell type responsible for the \textit{in vivo} clearance of Hb-Hp (Fig. 7). The flow cytometric analysis shows that an equimolar concentration of IsdH^{N1N2N3} reduced the mean fluorescence intensity (MFI) signal of Hb-Hp_{11011} by 4-fold. Similar inhibition was seen with IsdH^{N1} and IsdH^{N2N3}, although 5-fold higher concentrations of these IsdH truncation mutants were needed to obtain the same level of inhibition.

\textbf{Comparative Analyses of IsdB—Next, because IsdH and IsdB share a high degree of structural homology and both are reported to function as Hb receptors, interactions of IsdB with the Hb-Hp complex and its removal by CD163 were investigated. IsdB^{N1N2} was analyzed by UV-visible spectroscopy for its ability to transfer heme (Fig. 8, A and B). Although IsdB^{N1N2} rapidly transfers heme from metHb, we could not detect any heme transfer from metHb-Hp. In contrast to IsdH, SPR analysis showed no or very weak inhibition of Hb-Hp binding to...}
CD163 by IsdBN1N2 (Fig. 8C), although IsdBN1N2 was able to bind to Hb-Hp (Fig. 8D). Finally, IsdBN1N2 did not inhibit uptake of fluorophore-labeled Hb-Hp2-2 in CHO CD163 (Fig. 8E).

Discussion

This study shows that S. aureus IsdH blocks the CD163-mediated uptake of Hb-Hp complexes, which instantly form when Hb is released during intravascular hemolysis. The blockage is biologically meaningful because it inhibits the normal mechanism for Hb degradation in the host and therefore secures a pool of Hb-iron for the pathogen.

The present heme transfer kinetic studies shows that IsdH is able to extract heme from metHb-Hp but not from oxyHb-Hp. This is in line with previous studies on free Hb showing that the third NEAT domain, IsdHN3, binds ferric heme tighter than ferrous heme (25) and studies with the other S. aureus receptor, IsdB, which does not extract heme from oxyHb (26). It is tempting to speculate that the IsdH blocking of the CD163-mediated pathway for Hb-Hp uptake may benefit S. aureus by providing time for the spontaneous oxidation of Hb from its ferrous form to ferric metHb, from which IsdH then can extract the heme.

Our data indicate that both the IsdHN1 and IsdHN2 domains contribute to the binding of the Hb-Hp complexes. This is in line with previous data showing that IsdHN1 only binds the Hb α-subunit, whereas IsdHN2 binds either the Hb α- or the Hb β-subunit (27). Given that Hb-Hp complexes contain Hb αβ dimers, the higher affinity of IsdHN1N2N3 suggests that IsdHN1 and IsdHN2 are bound simultaneously to the Hb α- and β-subunits, thereby stabilizing the interaction. By presenting the known Hb-Hp-IsdHN1 crystal structure in a view together with a model of two CD163 scavenger receptor cysteine-rich (SRCR) domains, previously shown to bind Hp in the Hb-Hp complex, Fig. 9 illustrates how IsdHN1 blocks the CD163 binding site on Hb-Hp (28, 29). Fig. 9 also illustrates how two positively charged amino acids, Lys317 and Arg307, in Hp interact with acidic calcium-coordinating residues in CD163. The structural model suggests that this interaction (28) will be obstructed when IsdH binds to adjacent Hb subunit. However, x-ray crystal structures are needed to confirm the domain interactions of IsdHN1N2N3 with Hp-bound Hb.

IsdHN1 has, in contrast to our findings, been reported to bind Hp with a $K_D$ in the micromolar (12) or nanomolar range (13).
In our study, we could not detect Hp binding by any of the IsdH constructs using either the sensitive SPR analyses or SEC-RALS in line with size exclusion chromatography. Furthermore, a recently published structure of IsdHN1 binding to Hb-Hp shows that IsdHN1 molecules only bind to Hb α-subunits (22) with no contact to Hp. Given that Hb-Hp is one of the strongest known non-covalent interactions (30), it is possible that trace contamination of Hp preparations with Hb gave rise to false positive interactions with IsdH observed in previous studies.

IsdH shares a high degree of homology with IsdB (11) and can extract heme from metHb (26). However, our data revealed that IsdB cannot extract heme from metHb-Hp, and neither is IsdB a strong inhibitor of the Hb-Hp-CD163 interaction. This clearly differentiates the role of IsdH and IsdB. Because Hb is complexed instantly with Hp in the human circulation as long as Hp exceeds the Hb concentration in plasma, the ability to sequester this pool of iron through the IsdH receptor may have provided a selective advantage during S. aureus evolution, explaining the presence of two different Hb receptors in this organism.

From a clinical point of view, IsdH blocking of Hb-Hp uptake by CD163 may worsen hemolytic sepsis by stimulating bacterial growth. Furthermore, it may enhance the inflammatory response, not only because the iron supplementation stimulates bacterial growth but also because of inhibition of Hb uptake in plasma, which is a prerequisite for conversion of heme to anti-inflammatory heme metabolites (CO and bilirubin) (31). The inhibitory effects of the S. aureus heme extraction system on the CD163-mediated Hb removal may also affect the validity of plasma Hp as a biomarker of hemolysis during S. aureus sepsis, because the well known negative correlation between Hp and hemolysis relies on Hb-Hp scavenging (32). Direct inhibition by IsdH and thereby inefficient Hp scavenging may skew this correlation and underestimate hemolysis. Finally, it is tempting to speculate that other hemolytic bacteria that extract heme from Hb might also have mechanisms to block clearance of Hb-Hp complexes.

**Experimental Procedures**

**Protein Production**—The DNA sequence encoding IsdH(N1) (residues 86–229) with an added N-terminal His6 tag and a thrombin cleavage site was cloned into the NdeI and BamHI sites (Genscript) of the pET-22b(+) (Novagen) vector as previously described (22). The sequence encoding IsdH(N2N3) (residues 321–655) was cloned in the XhoI and BamHI sites of pET-15b (Novagen) in line with the vector encoded His6 tag and purified over nickel affinity, anion exchange chromatography as previously described (10). IsdH(N1N2N3) (residues 82–655) and IsdB(N1N2) (residues 120–459) were expressed and purified according to the same method as used for IsdH(N2N3) purification. The protein purity was assessed by SDS gel electrophoresis.

**Cell Culture**—Human CD163-expressing Chinese hamster ovary cells (CHO CD163) were generated and cultured as previously described (33). Human mononuclear leukocytes were purified from blood by Ficoll-Paque centrifugation and seeded in 24-well plates (Nunc, Roskilde, Denmark) at a density of 5 × 10⁵ monocytes/well. Monocytes were isolated by plastic adherence, as described (34) washing away non-adherent cells after 3 h of incubation at 37°C and 5% CO₂ in RPMI. The monocytes were cultured for 3 days in RPMI supplemented with 2% AB serum (Biowest, Nuaille, France), 20
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ng/ml M-CSF (Gibco, Life Technologies), and 2.5 $\times$ 10^{-7} M dexamethasone (Sigma-Aldrich) to increase CD163 expression as previously reported (34).

Fluorophore Labeling of Hp—Human Hp2-2 (Sigma-Aldrich) was fluorescently labeled as described previously (35). Briefly, Hp2-2 was labeled with Atto-488 succinimidyl ester (Sigma-Aldrich) by adding 65 $\mu$g of dye/mg of protein to yield a degree of labeling of 3 as confirmed by spectroscopy.

Confocal Microscopy—Confocal imaging was done as described earlier (36). Briefly, CHO CD163 or mock transfected CHO cells were seeded 3 $\times$ 10^4 cells/well on poly-D-lysine-coated 8-well chambered coverslips (Nunc) and cultured in serum-free CHO medium (CCM5; HyClone, Logan, UT) until the next day. Hb-Hp complexes were formed by incubating equimolar amounts of ferrous stabilized HbA0 (Sigma-Aldrich) with Atto-488-labeled Hp2-2 for 1 h. The cells were incubated for 1 h with labeled Hb-Hp2-2 (46 $\mu$g/ml) in the presence of 5-fold molar excess of IsdH^N1, IsdH^N2N3, or IsdH^N1N2N3 (a 1:1 stoichiometry of IsdH binding to a Hb-Hp monomer unit was assumed). The cells were washed in PBS and fixed in 4% formaldehyde for 10 min at room temperature. Immunostaining of CD163 was performed by incubating the cells with 10 $\mu$g/ml rabbit anti-human CD163 IgG (Dako, Glostrup, Denmark) in 1% BSA in PBS for 45 min, followed by washing and 45 min of incubation with donkey anti-rabbit-conjugated Alexa Fluor 647 (Molecular Probes, Eugene, OR) in 1% BSA in PBS. The nuclei were stained with 300 ng/ml DAPI (Molecular Probes). The cells were visualized using an Olympus FV10i confocal microscope.

Flow Cytometry—CHO cells were seeded in 24-well plates at a density of 2 $\times$ 10^5 cells/well. The following day the cells were

FIGURE 8. IsdB heme transfer kinetics, SPR analysis, and cellular uptake of Hb-Hp2-2. A and B, spectral changes over time after mixing of 13 $\mu$M stdB^N1N2 with 1.5 $\mu$M metHb (tetramer) (A) or 1.5 $\mu$M metHb-Hp1-1 (Hp dimer; two Hb dimers) (B). For comparison, 1.5 $\mu$M metHb or 1.5 $\mu$M metHb-Hp1-1 mixed 1:1 with PBS are included. By estimating from the absorbance changes, 30.2 $\pm$ 0.5% ($n = 3$) of heme was transferred from metHb to IsdB in 300 s, whereas no measurable transfer to metHb-Hp1-1 was seen. C, relative plateau response of immobilized CD163 binding of Hb-Hp1-1 (C) and (D) Hb-Hp2-2 plotted against the indicated molar ratios of IsdB^N1N2. D, sensorgrams of 100 nM Hp-Hp1-1, Hb-Hp2-2, Hb, Hb-Hp1-1, or Hb-Hp2-2 binding to immobilized IsdB^N1N2. E, confocal microscopy images of endocytosis of Hb-Hp by CD163-expressing cells. CHO CD163 cells were incubated with 46 $\mu$g/ml Atto-488-labeled Hb-Hp2-2 and IsdB^N1N2 in 5-fold molar excess over Hb-Hp. Atto-488-labeled Hb-Hp2-2 is shown in green, DAPI staining of nuclei is shown in blue, and CD163 surface immunostaining (Alexa 647) is shown in red. Mock transfected CHO cells (right panel) were included as a negative control.
incubated with Atto-488-labeled Hb-Hp2-2 (23 \mu g/ml) mixed with increasing ratios of IsdHN1, IsdHN2N3, or IsdHN1N2N3 in CCM5 medium for 1 h at 37 °C 5% CO2. The cells were detached by trypsinization and washed twice in PBS 1% BSA. Similar experiments were performed on 72-h cultured monocytes with modifications. Briefly, uptake was performed in RPMI 1% BSA, and monocytes were detached using macrophage detachment solution (Promocell, Heidelberg, Germany) according to the manufacturer’s instructions. Polyclonal rabbit anti-human CD163 IgG (Dako), earlier shown to specifically inhibit Hb-Hp uptake by CD163 (37), and irrelevant polyclonal rabbit anti-human IgG (Dako), earlier shown to specifically inhibit Hb-Hp uptake by CD163 (37), and irrelevant polyclonal rabbit anti-human IgG (Dako) were included as inhibition controls, both at a concentration of 50 \mu g/ml. Cellular uptake of fluorescent Hb-Hp2-2 and CD163 expression was analyzed by flow cytometry using a LSR II flow cytometer and FACSDiva (BD Biosciences, San Jose, CA) using the 488- and 633-nm lasers. Geometric MFI of a population of 10,000 cells was calculated with FlowLogic software (Inivai Technologies) and used to quantify Hb-Hp uptake.

**Surface Plasmon Resonance**—CD163 binding to Hb-Hp with or without IsdH and IsdBN1N2 was studied by SPR on a Biacore 3000 instrument (Biacore, Uppsala, Sweden) with immobilized CD163 on a CM5 chip as previously described (29). Hb-Hp2-2 and Hb-Hp1-1 complexes were formed as described above using unlabeled Hb and diluted to 12.5 \mu g/ml in running buffer (10 mM HEPES, 150 mM NaCl, 2 mM CaCl2, and 0.005% Surfactant P-20, pH 7.4). The Hb-Hp complexes were mixed with increasing ratios of IsdHN1, IsdHN2N3, IsdHN1N2N3, or IsdBN1N2. Each sample (40 \mu l) was injected with a flow rate of 5 \mu l/min.

To confirm the affinity of IsdBN1N2 for Hb, Hb-Hp1-1, and Hb-Hp2-2, IsdBN1N2 was immobilized on a CM5 chip by injecting 10 \mu g/ml IsdBN1N2 in 10 mM sodium acetate, pH 4.0, to a surface density of \sim 0.075 pmol/mm² and capping with 1 M ethanolamine, pH 8.5. Samples (40 \mu l) of Hb, Hb-Hp, or Hb-Hp complexes in running buffer were injected over the chip at a flow rate of 5 \mu l/min in concentrations ranging from 50 to 500 nM.

For the affinity measurements of IsdH, Hb1-1 and Hb2-2 were immobilized on a CM5 chip in different flow cells in a 10 mM sodium acetate pH 4.0 buffer. Hb-Hp complexes were formed by injecting 40 \mu l of 100 \mu g/ml Hb in running buffer over the chip in each cycle followed by injection of 40 \mu l of 25, 50, 100, 200, 300, 400, or 500 nM IsdHN1, IsdHN2N3, or IsdHN1N2N3 at a flow rate of 5 \mu l/min. Regeneration was obtained by injecting two cycles of 10 \mu l of regeneration buffer (10 mM glycine, 20 mM EDTA, 500 mM NaCl, and 0.005% Surfactant P-20, pH 3.0). In parallel, endoglycosidase H, expressed with a His6 tag, was used as a negative control to exclude the possibility of the His tag binding to Hb-Hp or Hb. The data were analyzed using BLAevaluation software 4.0.1, and the apparent dissociation constants were obtained by fitting the binding response of IsdH constructs to Hb-Hp1-1 and Hb-Hp2-2 flow cells just prior to the end of injection (Rmax) to the previously described expression (38).

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\frac{1}{R_m} = \frac{1}{R_{max}} + \frac{K_d}{R_{max}[\text{IsdH}]} \quad (\text{Eq. 1})
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**Heme Transfer Kinetics**—The transfer rate of heme from free Hb and from Hb1-1-bound Hb to IsdH was measured using a Cary 60 (Agilent Technologies, Glostrup, Denmark) UV-visible spectrophotometer at 20 °C. Pure ferrous adult human HbA0 (Sigma-Aldrich) in the oxygenated form (oxyHb) was oxidized to the ferric (met) form (metHb) by ferricyanide, as described (39). Briefly, the lyophilized Hb was dissolved in milliQ water at a concentration of 4–6 mM heme and incubated in the dark at room temperature for 1 h with a 1.2 molar excess of potassium ferricyanide K3Fe(CN)6 over heme. MetHb was desalted on a PD-10 column (GE Healthcare) equilibrated with 100 mM HEPES, pH 7.5, to remove ferricyanide and dialyzed against milliQ water before use.

Concentrations of metHb and oxyHb were determined using extinction coefficients 179 mM⁻¹ cm⁻¹ at 405 nm and 14.6 mM⁻¹ cm⁻¹ at 577 nm, respectively (39). Hb-Hp complexes were made by mixing 1.5 \mu M (tetrameric) metHb or oxyHb with a 1.5 molar excess of Hb1-1 (Sigma-Aldrich) in PBS (pH 7.4), assuming that one Hb1-1 dimer binds two Hb dimers. IsdHN2N3, IsdHN1, or IsdBN1N2 (all 13 \mu M in PBS) was mixed in a 1-cm quartz cuvette 1:1 with either metHb or oxyHb (both 1.5
µm in PBS) alone or bound to Hp1-1. Absorbance spectra were recorded every 5 s in the range 350 – 450 nm for 300 s.

Size Exclusion Chromatography with In-line Right Angle Light Scattering—SEC was performed on a Viscotek P2500 column (Malvern, Worcestershire, UK). RALS and refractive index were measured on a Viscotek 305 Triple Detector Array instrument (Malvern). The detectors and column were maintained at 30 °C. The light scattering cell was illuminated by a laser diode (670 nm), and light scattered at an angle of 90° was measured by a photodiode detector. The refractive index detector was a dual cell design. Calibration of the detectors and calculation of sample weight-average molecular mass was performed using the Omnisec software (Malvern). The instrument was calibrated using multiple protein and polyethylene oxide standards. Sample molecular mass was calculated using a specific refractive index increment with respect to sample concentration (dn/dc) of 0.185 g/ml, neglecting possible deviations because of the presence of co-factors or glycosylation.

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References


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