The Mechanism of Direct Heme Transfer from the Streptococcal Cell Surface Protein Shp to HtsA of the HtsABC Transporter

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The heme-binding proteins Shp and HtsA are part of the heme acquisition machinery found in Streptococcus pyogenes. The hexacoordinate heme (Fe(II)-protoporphyrin IX) or hemochrome form of holoHtsA (hemoShp) is stable in air in Tris-HCl buffer, pH 8.0, binds to apoHtsA with a $K_d$ of 120 ± 18 μM, and transfers its heme to apoHtsA with a rate constant of $28 ± 6$ s$^{-1}$ at 25 °C, pH 8.0. The hemoHtsA product then autoxidizes to the hexacoordinate heme (Fe(III)-protoporphyrin IX) or hemichrome form (hemiHtsA) with an apparent rate constant of 0.017 ± 0.002 s$^{-1}$. HemiShp also rapidly transfers heme to apoHtsA through a hemiShp-apoHtsA complex ($K_d$ = 48 ± 7 μM) at a rate ~40,000 times greater than the rate of simple heme dissociation from hemoShp into solvent ($k_{transfer} = 43 ± 3$ s$^{-1}$ versus $k_{hemin} = 0.0003 ± 0.00006$ s$^{-1}$). The rate constants for heme binding to and dissociation from HtsA ($k_{hemin} = 80$ μM$^{-1}$ s$^{-1}$, $k_{hemin} = 0.0026 ± 0.0002$ s$^{-1}$) are 50- and 10-fold greater than the corresponding rate constants for Shp ($k_{hemin} = 1.6$ μM$^{-1}$ s$^{-1}$, $k_{hemin} = 0.0003$ s$^{-1}$), which implies that HtsA has a more accessible active site. However, the affinity of apoHtsA for hemin ($K_{hemin} = 31,000$ μM$^{-1}$) is roughly 5-fold greater than that of apoShp ($K_{hemin} = 5,300$ μM$^{-1}$), accounting for the net transfer from Shp to HtsA. These results support a direct, rapid, and affinity-driven mechanism of heme and heme transfer from the cell surface receptor Shp to the ATP-binding cassette transporter system.

Heme is a major source of iron for bacterial pathogens (1–3). Gram-negative bacteria contain outer membrane proteins that can sequester heme directly (4) or indirectly through hemoeporphyrins (5) from host hemoproteins. The captured heme is brought into the periplasmic space in a TonB-dependent process (6) and then transported across the cytoplasmic membrane by heme-specific ATP-binding cassette (ABC)2 transporters (7). Similar ABC transporters (3, 8–10) and cell surface proteins (11, 12) are required for heme acquisition in Gram-positive pathogens. The lipoprotein components of these ABC transporters and some of the cell surface proteins involved in heme acquisition in Gram-positive bacteria have been shown to bind heme (10–12). However, the molecular mechanisms by which the cell surface proteins pass the captured heme to the ABC transporter are incompletely understood.

Streptococcus pyogenes is a Gram-positive bacterium that causes a variety of human diseases (13). This organism is capable of utilizing heme derived from human hemoproteins as a source of iron (14, 15). It expresses two heme-binding proteins, HtsA (10) and Shp (11). HtsA is the lipoprotein component of the heme-specific ABC transporter called HtsABC, whereas Shp is a cell surface protein. The genes encoding Shp and HtsABC are present at the same locus as an operon in the S. pyogenes chromosome (11).

We have chosen Shp and HtsA as a model system to investigate how heme (Fe(II)-protoporphyrin IX) is transferred from a cell surface protein to a heme-specific ABC transporter in Gram-positive bacteria. In this system, heme-free Shp (apoShp) scavenges heme (Fe(III)-protoporphyrin IX) that has dissociated from methemoglobin. Then holoShp rapidly transfers the iron porphyrin to apoHtsA (16). In this report, the mechanisms of both heme and hemin transfer from holoShp to apoHtsA were examined by measuring the kinetics of these processes and by characterizing each of the observed intermediates. The results reveal the rapid formation of a holoShp-apoHtsA complex and subsequent rapid internal heme or hemin transfer, which is driven by the higher affinity of HtsA for both heme and hemin.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant Shp was expressed in Escherichia coli harboring pSHP and purified by the method of Lei et al. (10)
Heme Transfer from Shp to HtsA

with modifications. After DEAE chromatography, the partially purified Shp was dialyzed against 3 liters of 20 mM Tris-HCl, pH 8.0, loaded onto an SP-Sepharose column (1.5 × 6 cm), washed with 100 ml of Tris-HCl, pH 8.0, and eluted with a 100-ml linear gradient of 0–0.25 m NaCl in Tris-HCl, pH 8.0, yielding holoShp in the reduced form with a purity of >95%, as assessed by SDS-PAGE. Since isolated holoShp contains a tightly bound hexacoordinate Fe(II)-protoporphyrin IX, which is called a hemochrome in the globin literature or a b-type heme in the cytochrome literature. Thus, this form of the protein is designated hemoShp. Alternatively, Shp from the DEAE column was dialyzed against 3 liters of 10 mM sodium acetate buffer, pH 5.5, loaded onto an SP column (2.5 × 3 cm), washed with 100 ml of acetate buffer, eluted with a 100-ml linear gradient of 0–0.3 m NaCl in acetate buffer, yielding a tightly bound hexacoordinate Fe(III)-protoporphyrin IX or hemichrome form of Shp (hemiShp) with a purity of >95%.

HemiShp was also prepared by oxidizing hemoShp with excess ferricyanide and removing any excess ferricyanide by gel filtration on a Sephadex G-25 column (1.5 × 30 cm). As a control, hemoShp was also prepared from hemiShp by reduction with sodium dithionite and subsequent removal of excess dithionite and its products on a Sephadex G-25 column (1.5 × 30 cm). HemiShp or hemoShp samples prepared by these different methods have indistinguishable optical spectra and kinetics of hemin or heme transfer to apoShp. ApoShp was prepared as described previously (16). All proteins were stored in 20 mM Tris-HCl, pH 8.0, at −20 °C prior to experiments.

Recombinant HtsA was isolated from E. coli harboring pLP1795 (10) as a mixture of apoprotein (apoHtsA) and a small amount of hemin-containing holoprotein (10). ApoHtsA was isolated and reconstituted with bovine hemin chloride (Sigma) as previously described (16). Briefly, apoHtsA was incubated with hemin at a 1:2 HtsA/hemin molar ratio for 15 min at room temperature. To remove excess hemin, the sample was loaded onto a Sephadex G-25 column (1.5 × 20 cm), and holoHtsA was eluted with 20 mM Tris-HCl, pH 8.0. The reduced and oxidized forms of HtsA exhibit UV-visible spectra characteristic of hexacoordinate hemochrome and hemichrome complexes and are designated hemoHtsA and hemiHtsA, respectively.

Autoxidation of HtsA—HemiHtsA (500 µl of 7 µM) was reduced to hemoHtsA with ~2 mg of dithionite, and excess dithionite and its oxidation products were removed by a semidry G-25 spin column. To prepare the semidry column, a G-25 column (1.5 × 10 cm) was equilibrated with 20 mM Tris-HCl, pH 8.0, and centrifuged in a swinging rotor at 300 × g for 15 s. The reduced hemoHtsA sample was then loaded onto the semidry column, and the column was spun at 300 × g for 15 s to obtain dithionite-free HtsA. The reduced sample was then immediately placed in a cuvette, and autoxidation of hemoHtsA was monitored by measuring the change in absorbance at 424 nm at room temperature.

EPR Measurement—EPR spectra of hemoShp, hemiShp, and hemiHtsA were recorded with a Varian E-6 spectrometer. The conditions for EPR measurements were as follows: frequency, 9.27 GHz; power, 3 milliwatts; modulation amplitude, 10 G; the modulation frequency, 100 kHz; and temperature, 4.2 K. The high spin signal at g = 6 was quantified by double integration between 800 and 1,700 G and comparison with the signal of a high spin sperm whale metmyoglobin at pH 7 (Mb(Fe(III)-H2O)). Quantification of the low spin signals was based on comparison of the area of the g = 3 absorption-like signal with the analogous low spin signal of metmyoglobin at pH 9.5 (Mb(Fe(III)OH) at known concentration. To assess the oxidation states of the proteins during the heme transfer reaction, ~300 µM hemoShp was reacted with 370 µM apoHtsA, frozen at ~30 s after mixing by quick immersion in a methanol/dry ice bath at ~58 °C, and stored in liquid nitrogen. The EPR spectrum of the sample was recorded as described above. The sample was then warmed to room temperature, incubated for 30 min, and refrozen, and the final EPR spectrum was recorded. A similar experiment was carried out starting with ~300 µM hemoShp and ~370 µM apoHtsA.

Kinetic Experiments—A stopped-flow spectrophotometer (SX18.MV; Applied Photophysics) was used at Montana State University to measure the rate of heme transfer from hemoShp to apoHtsA, the rate of hemin transfer from hemoShp to apoHtsA, and the binding of hemin to apoShp or apoHtsA under pseudo-first-order conditions. In the heme/hemin transfer measurements, one syringe contained 4 µM holoprotein, and the other contained apoprotein at ≥20 µM in 20 mM Tris-HCl, pH 8.0. In the heme binding measurements, initial concentrations of hemin and apoprotein were 2 µM and ≥10 µM, respectively. Changes in absorbance were measured at wavelengths appropriate for each reaction, as indicated under “Results.” Each time course represents the average of 10 consecutive traces for each condition and was analyzed by fitting the observed data to a single exponential expression using Applied Photophysics software to obtain pseudo-first-order rate constants. The dependence of these rate constants on apoprotein concentration was analyzed using the reaction models presented in Schemes I and II to obtain the specific rate parameters described in Equations 1 and 4. Entire spectra were also recorded during hemin and heme transfer using an OLIS RSM 1000 stopped-flow spectrophotometer at Rice University, and the observed time courses were similar to those obtained in the Applied Photophysics apparatus at a single wavelength.

Heme Transfer in the Absence of Oxygen—HemoShp (3.6 µM) was mixed with an equal volume of 15 µM apoHtsA anaerobically in the presence of excess dithionite in 20 mM Tris-HCl, pH 8.0. The spectrum of the mixture was recorded ~5 s after mixing using a SPECTRAX 384 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The time course of this anaerobic reaction was monitored at 424 nm using the Applied Photophysics stopped-flow apparatus.

Rates of Hemin Dissociation from HemiShp and HemiHtsA—The rates of hemin dissociation from hemoShp and hemoHtsA were measured using H64Y/V68F apomyoglobin as a hemin scavenger according to the method of Hargrove et al. (17). The apomyoglobin was prepared using the methyl ethyl ketone method (18). HemoShp or hemoHtsA (5 µM) was incubated with 50 µM apomyoglobin in 1 ml of 20 mM Tris-HCl, pH 8.0, and the change in absorbance at 602 nm was monitored for up to 50 min. The ΔA602 time courses were fit to a single exponential equation to obtain the first-order rate constant.
Other Assays and Measurements—HtsA and Shp protein concentrations were measured using a modified Lowry protein assay kit (Pierce), with bovine serum albumin as a standard, according to the manufacturer’s instructions. Heme or hemin content of Shp and HtsA was determined using a pyridine hemochrome assay (19). Each protein, diluted in 750 µl of 20 mM Tris-HCl was combined with 175 µl of pyridine, 75 µl of 1 N NaOH, and ~2 mg of sodium hydrosulfitie. Absorbance at 418 nm was measured immediately after mixing, and the extinction coefficient, $\epsilon_{418} = 191.5 \text{ mm}^{-1}\text{ cm}^{-1}$, was used to determine heme/hemin concentrations. A SPECTRAmax 384 Plus spectrophotometer was used for absorption measurements, unless otherwise specified.

RESULTS

Oxidation States of HtsA and Shp—The absorption and EPR spectra of holoShp are shown in Fig. 1. Shp expressed and purified from E. coli using DEAE and SP-Sepharose columns at pH 8.0 has a strong Soret absorption peak at 428 nm and two additional peaks at 528 and 560 nm (solid curve in Fig. 1A). This spectrum is only seen for reduced $b$-type cytochromes but not for their oxidized forms (20, 21), indicating that holoShp purified at pH 8.0 is in a hemochrome form. Oxidation of hemoShp was readily achieved by the addition of ferricyanide at pH 8.0 and, after removal of excess ferricyanide, can be seen as a shift of the Soret peak from 428 to 420 nm and replacement of the distinct 528- and 560-nm peaks with a broad band at 530 nm and a weaker one at 570 nm (dotted curve in Fig. 1B).

The spectral properties of hemiShp prepared by ferricyanide treatment are almost identical to those of hemoShp purified at pH 5.5 (Fig. 1B). The addition of dithionite to either of the hemiShp samples instantly results in the hemochrome visible spectrum (Fig. 1A). Shp remains in the hemochrome form in air-saturated Tris-HCl, pH 8.0, after removal of excess dithionite (Fig. 1A), consistent with the observation that purified hemoShp is stable in Tris-HCl, pH 8.0, for weeks in the cold. These results indicate that hemoShp is not sensitive to autoxidation at pH 8.0 but that the conversions between the two oxidation states can be readily achieved by dithionite and ferricyanide.

EPR measurements confirmed the oxidation states of holoShp and their transition. Purified hemoShp (Fig. 1C) and dithionite-derived hemoShp lack any significant EPR signal, whereas ferricyanide-treated holoShp displays strong EPR signals at 4.2 K, which suggest three different populations of the hemin Fe(III) (Fig. 1C), all of which are spectrally distinct from the EPR signal of ferricyanide. These results confirm unambiguously that, when purified at high pH, hemoShp is present initially in a stable reduced state and oxidized readily by ferricyanide.

Approximately 2% of Fe(III) in hemiShp is in a high spin state, characterized by an axial field with $g_x = 5.58$ and $g_y = 2.00$. The dominated population (~80%) exhibits a low spin signal with the rhombic symmetry. However, only $g_z = 3.09$ could be accurately determined for this component, because the other two transitions ($g_x$ and $g_y$) are obscured by overlap with absorption from a third iron species. The third Fe(III) component (15–20%) is also low spin but exhibits more axial symmetry and is characterized by a $g_{||} = 2.1$ transition. Although complex, the EPR spectrum for oxidized holoShp clearly supports the view that bound hemin is coordinated by pairs of two strong axial ligands.

The spectrum of hemiHtsA shows an intense Soret band at 412 nm and strong and weak absorption bands at 530 and 570 nm, respectively (Fig. 2A), which are similar to those of hemiShp, model hemichrome compounds, and oxidized $b$-type cytochromes. Reduction of hemiHtsA with excess dithionite generates a spectrum with peaks at 424, 528, and 558 nm (Fig. 2A), which is characteristic of a hemochrome or a reduced $b$-type cytochrome. However, gel filtration of the hemoHtsA/
Dithionite mixture results in only hemiHtsA (Fig. 2A), indicating that hemoHtsA is unstable and autoxidizes rapidly in air.

The EPR spectrum of hemiHtsA is shown in Fig. 2B. There appear to be two forms of oxidized hemin iron. Approximately 10% of the ferric iron is in a high spin state with \( g = 5.73 \) and \( g = 2.00 \), and about 90% of the iron is in a low spin state with \( g \) factors equal to 2.94, 2.29, and 2.02. Although the derivative signal near \( g = 6 \) appears large (Fig. 2B), its integrated intensity is quite small (10%) compared with a high spin Mb(Fe(III)\text{H}_2\text{O}) control at an equivalent total iron concentration. In contrast, the integrated intensity of the low spin signal is about 90% of that of our low spin Mb(Fe(III)\text{H}_2\text{O}) control at the same total iron concentration (Fig. 2B). The \( g \) values of the low spin signal for hemiHtsA can be used to define the putative axial coordination of the iron. Using the method of Blumberg and Peisach (22), we calculated an axial field of 2.87 and a rhombicity of 0.73 for hemiHtsA. These two parameters define a point in the “truth diagram” (23), and the point fell in the region occupied by \( b \)-type cytochromes and hexacoordinate hemoglobins with two axial N ligands, which are normally but not always bis-His complexes. Unfortunately, in the case of ferric holoShp, only one of the three \( g \) values could be identified from its EPR spectrum, and thus, we cannot identify the axial ligands in holoShp without structural studies.

**Heme Transfer from Shp to HtsA**—When hemoShp is mixed with apoHtsA, there is a rapid shift in the Soret peak from 428 to 424 nm with little change in the visible wavelength region from 500 to 600 nm (Fig. 3A), suggesting the transfer of heme to apoHtsA without any oxidation of the iron atom. The half-time of this heme transfer reaction is on the order of 20–200 ms and depends on [apoHtsA]. This rapid spectral change is followed by a slower process, which involves a further blue shift in the Soret peak from 424 to 414 nm and marked decreases in the 530- and 560-nm peaks, suggesting autoxidation of hemoHtsA (Fig. 3B). To verify this interpretation, hemoShp was mixed with apoHtsA at \( \sim 300 \mu M \) total heme in an EPR tube and frozen.
30 s after mixing. The EPR spectrum of this mixture showed only a small amount of oxidation, indicating that most of the iron atoms were still reduced (Fig. 3C). More importantly, the weak low spin signal is similar to that of hemiHtsA but not that of hemiShp. When the Shp/HtsA reaction mixture was thawed, allowed to stand at room temperature for 30 min, and then refrozen, the strong, primarily low spin signal of hemiHtsA was observed (Fig. 3C). Both the $g \approx 2$ and 5.9 signals were much higher at 30 min than at 30 s, indicating that the slow absorbance changes shown in Fig. 3B correlate with the appearance of the oxidized hemiHtsA EPR signal. These results demonstrate that autoxidation of hemiHtsA is occurring after the heme is transferred from hemoShp to apoHtsA.

Thus, hemoHtsA appears to be formed within seconds after mixing hemoShp and apoHtsA. To test this idea, hemoShp was mixed anaerobically with apoHtsA in the presence of excess dithionite to prevent autoxidation. The spectrum of this mixture was recorded 5 s after mixing using a conventional spectrophotometer and is almost identical to that of hemoHtsA obtained by reducing hemiHtsA but is significantly different from that of hemoShp (Fig. 4A). The time course for hemoHtsA formation was monitored under similar conditions by measuring the change in absorbance at 420 nm using a stopped-flow spectrophotometer. The transfer reaction is complete within 5 s, and no further changes occur (Fig. 4B). The absorbance change corresponds to the rapid spectral shift shown in Fig. 3A, and the slow blue shift shown in Fig. 3B is not observed in the absence of oxygen. These results firmly establish that the rapid and slow phases of spectral changes seen when mixing hemoShp with apoHtsA are the rapid formation and subsequent slow autoxidation of hemoHtsA, respectively.

When hemiShp is reacted with apoHtsA, only a small rapid shift in the Soret peak from 420 to 412 nm is observed (Fig. 5A). To prove that these spectral changes represent hemin transfer, an EPR sample was prepared by mixing hemiShp with apoHtsA, freezing the sample 30 s after mixing, recording its EPR spectrum at 4 K, thawing, refreezing 30 min later, and recording a final EPR spectrum. In this case, the EPR spectra recorded 30 s and 30 min after mixing were similar to those of hemiHtsA but not those of hemiShp (Fig. 5B). Thus, the reaction of hemiShp with apoHtsA involves one spectral transition, representing the transfer of heme without any changes in redox state and suggesting that Shp and HtsA have different ligand(s) at one or both axial positions.

Kinetics of Heme Transfer from HoloShp to ApoHtsA—A minimal model for the heme transfer from hemoShp to apoHtsA is given in Scheme I and based on the optical absorb-
Heme Transfer from Shp to HtsA

Time courses for heme and hemin transfer were measured at single wavelengths, 420 and 414 nm, respectively, in a stopped-flow spectrophotometer by mixing holoShp with varying concentrations of apoHtsA. In the case of the transfer from hemoShp, the absorbance at 420 nm ($A_{420}$) increased rapidly and then slowly decreased over a period of several hundred seconds, as the resultant hemoHtsA autoxidized at 25 °C in air (Fig. 6A). The fast phase was a single exponential process and was complete a few seconds after mixing (Fig. 6B). The pseudo-first-order rate constant ($k_{obs}$) of the fast phase increased hyperbolically with increasing [apoHtsA] (Fig. 6C), suggesting the rapid formation of a holoShp-apoHtsA complex followed by a rate-limiting and first-order transfer of heme to apoHtsA as described in Scheme I. When the initial [apoHtsA] is ≥5 [hemoShp], the heme transfer is a pseudo-first-order process. The expression for the observed rate constant, $k_{obs}$, is given by Equation 1,

$$k_{obs} = \frac{k_{\text{transfer}}[\text{apoHtsA}]}{(k_2 + k_{\text{transfer}})k_1 + [\text{apoHtsA}]} \approx \frac{k_{\text{transfer}}[\text{apoHtsA}]}{K_d + [\text{apoHtsA}]} \quad \text{(Eq. 1)}$$

where $K_d$ is the dissociation constant of the hemoShp-apoHtsA complex, and $k_1$, $k_2$, and $k_{\text{transfer}}$ are the rate constants of the individual reactions proposed in Scheme I. As shown in Fig. 6C, the observed dependence of $k_{obs}$ on [apoHtsA] is described quantitatively by Equation 1, with fitted values of $k_{\text{transfer}}$ and $K_d$ equal to $28 \pm 6$ s$^{-1}$ and $120 \pm 18$ μM, respectively (Table 1).

The slow phase observed after mixing hemoShp with apoHtsA is also a single exponential process, but the observed rate constant is independent of [apoHtsA]. As described in Figs. 2, 3, 4, and 6, this slow process represents autoxidation of hemoHtsA, with $k_{\text{autox}}$ equal to $0.017 \pm 0.002$ s$^{-1}$. This rate of autoxidation was confirmed independently by reducing hemiHtsA with dithionite, removing excess dithionite by centrifugation through a G-25 column, and then measuring autoxidation in air of the newly produced hemoHtsA by monitoring changes in $A_{424}$ in a conventional spectrophotometer. The observed rate of autoxidation of chemically reduced holoHtsA was 0.01 s$^{-1}$ at 22 °C (Fig. 6A, inset), which is almost identical to that observed for hemoHtsA produced by heme transfer from Shp.

Hemin transfer from holoShp to apoHtsA had a kinetic pattern similar to that of heme transfer. The only qualitative difference was the absence of a slow, secondary autoxidation phase for holoHtsA, because the iron was already oxidized (Fig. 6A). Under pseudo-first-order conditions, the time courses at 414 nm can be fit to single exponential expressions. The observed rate constants ($k_{obs}$) for hemin transfer depend hyperbolically on [apoHtsA] (Fig. 6B and C), and this dependence can be described by Equation 1 with $k_{\text{transfer}}$ and $K_d$ equal to $43 \pm 3$ s$^{-1}$ and $48 \pm 7$ μM, respectively.
Heme Transfer from Shp to HtsA

Rate constants and activation parameters for heme and hemin transfer from holoShp to apoHtsA

| Kinetic parameter | Heme | Hemin |
|-------------------|------|-------|
| $k_f/k_i$ or $K_d$ | $120 \pm 18$ $\mu M$ | $48 \pm 7$ $\mu M$ |
| $k_{transfer}$ | $28 \pm 6$ s$^{-1}$ | $43 \pm 3$ s$^{-1}$ |
| $\Delta H_f$ | $-77 \pm 9$ kJ/mol | $-37 \pm 6$ kJ/mol |
| $\Delta S_f$ | $-182 \pm 30$ J/mol K | $-37 \pm 18$ J/mol K |
| $\Delta G_f$ | $-23$ kJ/mol | $-26$ kJ/mol |
| $\Delta S^\circ$ for $k_{transfer}$ | $250 \pm 25$ J/(K/mol) | $35 \pm 4$ J/(K/mol) |
| $\Delta H^0$ for $k_{transfer}$ | $140 \pm 13$ kJ/mol | $75 \pm 9$ kJ/mol |
| $\Delta G^0$ for $k_{transfer}$ | $64.2$ kJ/mol | $64.9$ kJ/mol |
| $k_{auto}$ | $0.3$ kM$^{-1}$ s$^{-1}$ | $0.8$ kM$^{-1}$ s$^{-1}$ |

$K_{auto}$: rate of autooxidation of hemoHtsA$^a$

$0.017 \pm 0.002$ s$^{-1}$

$^a$ The values for $k_f/k_i$ and $k_{transfer}$ at $25^\circ C$ in $20$ mM Tris-HCl at pH 8.0 were obtained from fits of the dependence of the observed rates of transfer on [apoHtsA] to Equation 1.

$^b$ The standard entropy, enthalpy, and free energy for the formation of the holoShp-apoHtsA complexes were obtained by analyzing the dependence of $K_{association}$ (1/$K_d$) on temperature according to the van’t Hoff plot.

$^c$ The activation entropy, enthalpy, and free energy were obtained by analyzing the dependence of $k_{transfer}$ on temperature according to the Eyring equation.

$^d$ The rate of autooxidation of hemoHtsA was obtained from the slow phase following heme transfer.

Thus, both the affinity of apoHtsA for holoShp and the rate of transfer are 2-fold higher when the heme iron is oxidized. At low protein concentrations where $K_d \gg$ [apoHtsA], the transfer process appears second order with an apparent association rate constant equal to $k_{transfer}/K_d$. Under these conditions, the observed bimolecular rate constant for heme transfer is $0.8 \mu M^{-1}$ s$^{-1}$ and roughly 2.5 times larger than that for heme transfer ($0.3 \mu M^{-1}$ s$^{-1}$) at $25^\circ C$.

Activation Parameters for Heme and Hemin Transfers from Shp to ApoHtsA—The activation parameters for heme and hemin transfers in the holoShp-apoHtsA complex were obtained by determining $k_{transfer}$ at temperatures ranging from $15$ to $35^\circ C$. At each temperature, a plot of $k_{obs}$ versus [apoHtsA] was generated experimentally and then fitted to Equation 1. Both the heme and heme transfer rate constants increased with temperature and displayed a linear $\ln(k_{transfer}/T)$ versus $1/T$ plot (Fig. 7A), consistent with the Eyring equation,

$$ k = (k_b/h)T \exp(\Delta S^\circ/RT) \exp(-\Delta H^0/RT) $$

(Eq. 2)

where $k_b$, $h$, and $R$ are Boltzmann’s, Planck’s, and the gas constants, respectively. The activation entropy ($\Delta S^\circ$) and enthalpy ($\Delta H^0$) were calculated from the slope and intercept of the $\ln(k_{transfer}/T)$ versus $1/T$ plots (Fig. 7B) and are $37 \pm 6$ kJ/mol, respectively, and were equal to $-77 \pm 9$ kJ/mol and $-182 \pm 30$ J/(K/mol), respectively, for the formation of hemoShp-apoHtsA and $-37 \pm 6$ kJ/mol.

Thus, the heme transfer rate constants are also approximately twice as high when the heme iron is oxidized. With oxidized heme as well as oxidized iron-porphyrin, the heme transfer rate is $3.1 \times 10^3$ s$^{-1}$, compared to $1.3 \times 10^3$ s$^{-1}$ for heme transfer.

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where $k_b$, $h$, and $R$ are Boltzmann’s, Planck’s, and the gas constants, respectively. The activation entropy ($\Delta S^\circ$) and enthalpy ($\Delta H^0$) were calculated from the slope and intercept of the $\ln(k_{transfer}/T)$ versus $1/T$ plots (Fig. 7B) and are $37 \pm 6$ kJ/mol, respectively, and were equal to $-77 \pm 9$ kJ/mol and $-182 \pm 30$ J/(K/mol), respectively, for the formation of hemoShp-apoHtsA and $-37 \pm 6$ kJ/mol.
Heme Transfer from Shp to HtsA

Kinetics of Hemin Binding to ApoShp and ApoHtsA—The observed rate constants of hemin binding to apoShp and apoHtsA as a function of apoprotein concentration. Hemin (1 mM) reacted with apoShp (open circles) or apoHtsA (solid circles) at the indicated concentrations in 20 mM Tris-HCl, pH 8.0. Each ΔA420 time course was monitored using a stopped-flow spectrophotometer and fit to a single exponential expression to obtain the pseudo-first-order rate constant (k_{obs}). The curves are theoretical lines obtained by fitting the k_{obs} data to Equation 4. B, time courses for hemin dissociation from hemiShp (solid circles) and hemiShp (open circles). H64Y/V68F apomyoglobin (50 μM) reacted with 5 μM hemiShp or hemiHtsA in 20 mM Tris-HCl, pH 8.0. Hemin dissociation was measured by the change in A_{420}, the absorbance at the indicated time minus the absorbance at time 0. The circles and curves represent, respectively, the observed ΔA_{420} values and the theoretical curves obtained by fitting the data to a single exponential expression.

TABLE 2

| Rate and equilibrium constants for hemin binding to apoShp and apoHtsA |
|-----------------|-----------------|-----------------|
| ApoShp          | ApoHtsA         |
| k_{obs}/[apoShp] or k_{obs}/[apoHtsA] (hemin binding)* | 22 ± 2 μM       | 8 ± 0.7 μM      |
| k_{coordination}/[apoShp] or k_{coordination}/[apoHtsA] | 35 ± 4 s⁻¹       | 655 ± 47 s⁻¹    |
| k_{hemin} = k_{coordination}/k_{apo} | 1.6 μM⁻¹ s⁻¹   | 80 μM⁻¹ s⁻¹    |
| k_{apoHtsA} = k_{hemin}/k_{apo} | 0.0003 ± 0.00006 s⁻¹ | 0.0026 ± 0.0002 s⁻¹ |
| k_{apoShp} = k_{hemin}/k_{apo} | 5.300 μM⁻¹       | 31,000 μM⁻¹     |

* The hemin binding reaction at 25 °C in 20 mM Tris-HCl at pH 8.0 appears to occur by a two-step process involving an initial hemin binding step followed by first order iron coordination. In this case, values for k_{apo} and k_{apoHtsA} were obtained from fits of the dependence of the observed rates of transfer on [apoprotein] to Equation 4.

The hemin dissociation rate constants from hemiShp and hemiHtsA were determined by the H64Y/V68F apomyoglobin assay (17).

Apoprotein + hemin ⇌ apoprotein-hemin ⇌ holoprotein

(M) reacts with 5 μM hemiShp or hemiHtsA in 20 mM Tris-HCl, pH 8.0. Hemin dissociation was measured by the change in A_{420}, the absorbance at the indicated time minus the absorbance at time 0. The circles and curves represent, respectively, the observed ΔA_{420} values and the theoretical curves obtained by fitting the data to a single exponential expression.

where k₁ and k₂ are the rate constants for bimolecular formation and unimolecular dissociation of the final apoprotein-hemin complex, respectively, and k_{coordination} and k_{hemin} the internal first order rate constants for iron coordination to and dissociation from the final protein ligands, respectively. A similar two-step mechanism was proposed by Rose and Olson (24) for CO-heme binding to apohemoglobin.

If k₁ and k₂ are much greater than k_{coordination} and k_{hemin} is much smaller than k_{coordination}, the observed pseudo-first-order rate constant (k_{obs}) is described by Equation 4,

k_{obs} = \frac{k_{coordination}[apoHtsA]}{k_{obs} + [apoHtsA]} = \frac{k_{coordination}[apoHtsA]}{K_d + [apoHtsA]} (Eq. 4)

where k₁ is k₂/k₃. Fits of Equation 4 to k_{obs} versus [apoprotein] data for both Shp and HtsA are shown in Fig. 8A. The fitted values for k_{apoShp} and k_{apoHtsA} are 22 ± 2 μM and 35 ± 4 s⁻¹ and 8 ± 0.7 μM and 655 ± 47 s⁻¹ for hemin binding to apoShp and apoHtsA, respectively (Table 2). At low apoprotein concentrations where the reactions appear bimolecular, the apparent second-order rate constants (k_{coordination}/K_d) are 1.6 and 81 μM⁻¹ s⁻¹ for apoShp and apoHtsA, respectively.

The rates of dissociation of hemin from hemiShp and hemiHtsA were measured using excess H64Y/V68F apomyoglobin as a hemin scavenger with a unique absorption peak at 602 nm (17). Low concentrations of hemiShp or hemiHtsA were mixed with apomyoglobin at high concentrations, and the uptake of hemin by apoMb was followed by increases in A_{602} (Fig. 8B). The time courses in Fig. 8B were fitted to single exponential expressions, and the values of k_{hemin} were 0.0003 ± 0.00006 s⁻¹ and 0.0026 ± 0.0002 s⁻¹ for hemiShp and hemiHtsA, respectively. Since the dissociation of hemin is extremely slow, k_{hemin} in Scheme II must be << k₁ or k₂. Therefore, the rate constants obtained from the time courses in Fig. 8 are directly equal to k_{hemin} in Scheme II.

The association equilibrium constants (K_{hemin}) for hemin binding to apoShp or apoHtsA can be estimated by the ratio of
the apparent second order association rate constant \( (k'_{\text{hemin}} = k_{\text{coordination}}/K_q) \) and the hemin dissociation rate constant \( k_{-\text{hemin}} \) (Table 2). The \( K_{\text{hemin}} \) values for hemeShp and hemeHtsA are 5,300 and 31,000 \( \mu M^{-1} \), respectively, indicating that the higher affinity of HtsA for hemin is the driving force for the efficient transfer of hemin from Shp to HtsA. The absolute values of the equilibrium constants for heme binding to apoShp and apoHtsA could not be measured easily by these kinetic methods. However, because hemoShp can efficiently transfer its heme to apoHtsA, HtsA must also have much greater affinity for reduced iron-porphyrin.

**DISCUSSION**

Heme acquisition machineries have been identified in numerous bacteria. However, our understanding of the mechanisms of heme and heme transfer between the proteins in these systems is quite limited. In this work, we have elucidated the kinetic mechanisms for heme and heme transfer from holoShp to apoHtsA, providing the first detailed kinetic characterization of iron-porphyrin transfer between the proteins involved in heme acquisition in bacteria. The efficient transfer of heme and hemin is driven by the relative affinities of the proteins and by direct first-order transfer within holoShp-apoHtsA complexes. The results advance our understanding of how fast heme and hemin can be transferred and why heme binding to cell surface proteins facilitates heme acquisition in Gram-positive bacteria.

Characterization of the oxidation states of holoShp and holoHtsA indicates that hemoShp is stable in air at pH 8.0, whereas hemoHtsA is not. Dithionite and ferricyanide are commonly used to achieve the ferric to ferrous and ferrous to ferric transitions, respectively, in iron-porphyrin-containing proteins (25, 26). HoloShp and holoHtsA treated with dithionite have dominant \( \alpha \)-absorbance bands centered at \( \lambda \approx 560 \) nm. This type of spectrum is seen for reduced \( b \)-type cytochromes but not for their oxidized forms, where the \( \beta \) band at 530–540 nm dominates (20, 21). HemoHtsA is unstable in air and autoxidizes within 5–10 min after reduction. In contrast, hemoShp is stable in aerated Tris-HCl, pH 8.0, for over 24 h at room temperature. When Shp is purified at low pH or in the presence of ammonium sulfate, it is isolated as the oxidized heme chromophore form. However, it can be purified in the hemoShp form using DEAE and SP-Sepharose chromatography at pH 8.0. HemoShp purified at high pH has identical spectral properties to those of hemoShp reduced with dithionite, and both reduced holoShp proteins can rapidly transfer their heme to apoHtsA.

We have firmly established that hemoShp rapidly transfers its heme to apoHtsA and that the resulting hemoHtsA quickly autoxidizes in air. The initial rapid spectral change during heme transfer is due to the formation of hemoHtsA and not to a spin state or ligand switch in holoShp itself when it interacts with apoHtsA. These findings are consistent with our previous report that apoShp and holoHtsA are formed after hemoShp is mixed with apoHtsA (16). A ligand switch mechanism has been proposed to explain the efficient heme transfer from HasA to HasR, which has lower affinity for heme than HasA (27). In contrast, HtsA has higher affinity for hemin and presumably for heme than Shp. Thus, Shp to HtsA heme transfer does not require such a mechanism. Furthermore, the ligand switch mechanism proposed for the HasA/HasR system seems less plausible than a model in which binding to HasR promotes an opening of the heme pocket in HasA and a decrease in affinity (28).

The slower spectral change observed after heme transfer from Shp is due to autoxidation of reduced holoHtsA. Immediately after chemical reduction with dithionite, isolated hemoHtsA underwent autoxidation with a spectral change and rate constant very similar to those observed after heme transfer from holoShp. No slow spectral change occurs when oxygen is absent. The EPR measurements and kinetic analyses shown in Figs. 2–6 support these conclusions.

Cell surface heme-binding proteins are proposed to relay heme through the cell wall to heme-specific ABC transporters in Gram-positive bacteria (12). To have efficient heme or heme transfer between proteins, a donor should have lower affinity for heme and hemin than the acceptor, but neither of these affinities should be extremely high. Shp and HtsA appear to have evolved affinities suitable for their roles in transport. HtsA has a 5-fold higher affinity for hemin than Shp, resulting in directional transfer to HtsA. The affinities of Shp and HtsA for hemin are similar to those of the hemeophore HasA (29), which is involved in heme transport in *Serratia marcescens*, and to serum albumin (30), which is involved in heme transport in the circulatory systems of vertebrates. However, the Shp and HtsA heme affinities are much lower than those of mammalian myoglobin (Mb) (30) and hemoglobin (Hb) (31) (Table 3).

As shown in Table 1, Mb has a very low rate of hemin dissociation, which is critical for its retention of heme in muscle tissue even after oxidative damage. Replacement of the proximal imidazole in Mb with glycine increases the rate of heme dissociation \( \sim 10,000 \)-fold, placing it in the rate range of \( k_{-\text{hemin}} \) values for HtsA and Shp (30) (Table 3). In contrast to Mb, hemiShp and hemeHtsA have low spin \( b \)-coordinated structures, based on their optical and EPR spectra. Normally, these types of hemechromic structures are associated with high hemin affinity and low rates of dissociation. However, both hemiShp and hemeHtsA have the relatively high rates of heme dissociation \( \sim 0.003–0.0003 \) s\(^{-1}\) compared with that for

**TABLE 3**

Comparison of apparent rate and equilibrium constants for heme binding to apoShp, apoHtsA, and other heme protein complexes

| Hemeprotein       | \( K_{\text{hemin}} \) | \( k_{-\text{hemin}} \) | Reference |
|-------------------|------------------------|------------------------|-----------|
| Sperm whale apoMb | \(-70\)                 | 0.000001 ~70,000       | 30        |
| H93G sperm apoMb  | \(-70\)                 | 0.012 ~5               | 30        |
| Bovine serum albumin (hemin) | \(-50\)         | 0.011 ~4              | 30        |
| Hemophore HasA    | 53                     | 29                     |           |
| **ApoShp**        |                        |                        |           |
| Without apoHasA   | 1.6                    | 0.0003                 | 5 This work |
| With apoHasA      | 43                     |                        | 31 This work |
| **ApoHtsA**       | 80                     | 0.0026                 | 31 This work |
| Human apohemoglobin |                       |                        |           |
| \( \alpha \) (tetramers) | \(-100\)           | 0.00008 ~1,200         | 31        |
| \( \alpha \) (dimers)   | \(-100\)            | 0.00016 ~600           | 31        |
| \( \alpha \) (monomers) | \(-100\)            | 0.0033 ~33             | 31        |
| \( \beta \) (tetramers) | \(-100\)            | 0.00041 ~250           | 31        |
| \( \beta \) (dimers)   | \(-100\)            | 0.00042 ~24            | 31        |
| \( \beta \) (monomers) | \(-100\)            | 0.011 ~9              | 31        |
myoglobin ($k_{\text{hemin}} \approx 0.000001 \text{ s}^{-1}$). Thus, although the axial positions of the iron are filled with strong field ligands, the overall binding of hemin to these proteins is relatively weak. The heterogeneity apparent in the EPR spectra of hemeShp and hemiHtsA (Figs. 1C and 2B) suggest that the weaker heme binding is due to less rigid active sites that allow more rapid rates of transfer to facilitate heme uptake across the cell wall and membrane.

Gram-positive bacteria have no outer membrane. So why do Gram-positive bacteria require cell surface heme-binding proteins for heme acquisition? Heme and hemin usually are associated with host proteins that cannot directly interact with heme-specific ABC transporters, and, thus, cell surface heme-binding proteins are needed to relay heme and hemin through the thick cell wall. Another potential reason for the requirement of several cell surface components is that, in combination, these proteins serve as a sink to remove iron porphyrin from the host proteins.

Human hemoglobin is believed to be a major source of heme and hemin for bacterial pathogens. At low concentrations, where dimerization occurs, Hb shows hemin affinities approaching those of the transport proteins (Table 3). By itself, Shp does not possess a high enough affinity to remove hemin from Hb dimers at equilibrium. However, because Shp directly transfers hemin to HtsA, which in turn delivers it to the periplasm of the HtsABC transporter, this set of multiple proteins greatly enhances the affinity of the transport system for heme and facilitates the speed of internalization. Although each individual protein has a relatively low affinity for heme, the complete transport system of several proteins and direct transfer steps have sufficient capacity and affinity to acquire heme and hemin from hemoglobin.

The rates of heme and hemin transfer from holoShp to apoHtsA are $\approx 40,000$ times faster than the rate of simple heme dissociation from either protein. The formation of a holoShp-apoHtsA complex is the major reason for the rapid rate of transfer. The free energy released by the binding of apoHtsA to holoShp is used to weaken hemin and heme coordination in holoShp, which facilitates transfer to apoHtsA. Consequently, the equilibrium association constants for formation of the holoShp-apoHtsA complexes are relatively small (i.e., large $K_d$ values, 50–150 $\mu M$). However, the result of complex formation is direct heme or hemin transfer, with a much lower free energy barrier to movement from one protein to another than complete dissociation of the heme or hemin into solvent.

Another remarkable result is the similarity of the heme and hemin transfer processes. Normally, it is very difficult to remove reduced iron-porphyrin complexes from hemoproteins, and the process often requires partial unfolding of the protein. Although differences do occur, the kinetic parameters for heme and hemin transfer from Shp to HtsA are very similar, and there is no change in mechanism. The biggest difference is that the activation energy for the internal transfer process is much higher for the reduced iron complex. The net result is the greater temperature dependence for heme transfer versus hemin transfer. However, at room temperature, the rates only differ by a factor of 2–3 and are comparable physiologically. As a result, iron-porphyrin can be transferred on millisecond time scales by the Shp/HtsA system, regardless of the redox state of the immediate environment of the bacterium.

Taken together, our kinetic results show that Shp and HtsA of S. pyogenes have evolved to rapidly pass the iron-porphyrin from the cell surface heme-binding protein to the ABC transporter by forming an activated protein complex that is independent of the redox state of the iron and does not require the slow dissociation of bound heme or hemin.

Acknowledgment—We thank Dr. Mark Quinn for critical reading of the manuscript.

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