The *Mycobacterium tuberculosis* Secreted Protein Rv0203 Transfers Heme to Membrane Proteins MmpL3 and MmpL11*

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Background: A novel *Mycobacterium tuberculosis* heme acquisition has recently been discovered.

Results: The membrane protein MmpL11 is required for efficient heme uptake, and secreted Rv0203 may transfer heme to extracellular domains of both MmpL3 and MmpL11.

Conclusion: MmpL3 and MmpL11 are potential heme transporters, whereby heme is transported into the cytosol.

Significance: This work enhances our understanding of *Mycobacterium tuberculosis* heme uptake.

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*The abbreviations used are: MmpL, mycobacterial membrane Large; RND, resistance nodulation cell division; E1, first extracellular domain of MmpL3 or MmpL11; ZnMP, zinc mesoporphyrin; Mb, myoglobin; metHB, methemoglobin; MCD, magnetic circular dichroism.
reported to play roles in siderophore export (25). Finally, MmpL3 has been implicated in both heme uptake (9) and trehalose monomycolate export (26–28).

MmpL3 is encoded by an essential *M. tuberculosis* gene (9, 14) preventing direct investigation of the role of MmpL3 in heme uptake. MmpL3 is the closest homolog to MmpL11 with 24% amino acid sequence identity and is located within close genomic vicinity of MmpL11, hinting at a functional relationship. Gene deletion experiments indicate that MmpL11 is involved in *M. tuberculosis* heme uptake. *M. tuberculosis* mutants deficient in both siderophore production and MmpL11 display a significant growth defect when cultured in the presence of heme, suggesting it plays a role in heme-iron uptake and is required for efficient substrate acquisition (9).

The topologies of MmpL3 and MmpL11 are predicted to be similar, where both contain two predicted extracellular domains E1 and E2, and one intracellular domain C1 (Fig. 1B). Notably, recombinant E1 and E2 domains from MmpL3 and MmpL11 were shown to bind heme (9), suggesting that both MmpL3 and MmpL11 are transmembrane heme transporters. Thus, we hypothesize that the extracellular heme-binding protein Rv0203 can directly transfer heme to MmpL3 and MmpL11 extracellular domains, mirroring the mechanisms found in both Gram-positive and Gram-negative heme uptake systems (7, 29–34).

In this work, because of the challenges of producing full-length transmembrane proteins MmpL3 and MmpL11 (Fig. 1B), we undertook a modular approach to further investigate the heme uptake pathway. Preliminary experiments demonstrated rapid heme transfer from Rv0203 to the E1 domains of both MmpL3 and MmpL11; therefore, we embarked on a detailed characterization of their heme transfer mechanisms. Although both E1 domains bind heme with similar spectroscopic properties, their affinities to heme are quite distinct. Furthermore, heme binding to both E1 domains favors domain oligomerization, which leads to a two-step heme-binding mechanism. Heme transfer experiments demonstrate that holo-Rv0203 rapidly and efficiently transfers heme to either MmpL11-E1 or MmpL3-E1 domain, suggesting an interaction-driven mechanism, providing further evidence that MmpL3 and MmpL11 play a role in heme transport across the mycobacterial membrane. Additionally, we use the nonpathogenic and fast-growing model organism *Mycobacterium smegmatis* to demonstrate that MmpL11 is required for the efficient uptake of a heme analog.

**EXPERIMENTAL PROCEDURES**

**MmpL3-E1 and MmpL11-E1 Cloning, Expression, and Purification**—DNA sequences of residues 32–187 and 41–187 from MmpL3 and MmpL11, respectively, were cloned into pET28a (Novagen) encoding a fusion protein of the respective E1 domain with a cleavable His6 tag using NdeI and HindIII and BamHI and XhoI restriction enzyme sites (Fermentas Scientific) for MmpL3-E1 and MmpL11-E1, respectively. The respective E1 domain was transformed into BL21-Gold (DE3) cells and grown at 37 °C in LB medium containing 30 μg/ml kanamycin. Protein expression was induced when cells reached an A_{600} of 0.8 by the addition of 1 mM isopropyl 1-thio-D-galactopyranoside and cells harvested after 4 h by centrifugation at 5100 rpm for 20 min, followed by resuspension in 50 mM Tris, pH 7.4, and 350 mM NaCl. Cells were then lysed by sonication after addition of egg hen lysozyme (5 mg, Sigma) with phenylmethylsulfonyl fluoride (40 μM, Sigma), and the cell lysate was centrifuged at 14,000 rpm for 20 min. After addition of 400 μl of Proteoblock protease inhibitor mixture (Fermentas), the supernatant was loaded onto a Ni^{2+}-charged HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (between 100 and 250 mM imidazole). Fractions containing E1 were identified by SDS-PAGE, pooled, and concentrated using a Centricon centrifugal concentrator (Millipore). Further purification of E1 was achieved by running the protein over an S75 gel filtration column (GE Healthcare) eluted with a linear imidazole gradient (between 100 and 250 mM imidazole). Fractions containing E1 were identified by SDS-PAGE, pooled, and concentrated using a Centricon centrifugal concentrator (Millipore). Further purification of E1 was achieved by running the protein over an S75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris, pH 7.4, 150 mM NaCl, which yielded nearly 100% homogeneous protein. Cleavage of the His_{6} tag was conducted in cleavage buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM CaCl_{2}) by adding 1 ml of thrombin-agarose suspension (Sigma) to the protein. After an overnight incubation at 4 °C, the thrombin-agarose was removed on a glass frit. Each E1 domain was then run over an S75 gel filtration column equilibrated with 50 mM Tris, pH 7.4, 150 mM NaCl to separate E1 from the His_{6} tag.

 FIGURE 1. A, proposed heme uptake mechanism in *M. tuberculosis* (9–11). B, topology of MmpL3, predicted with TMHMM (12), consists of 11 transmembrane helices and three soluble domains (E1, C1, and E2). A similar topology is also predicted for MmpL11.
Reconstitution of the Apoproteins with Heme—Approximately 4 mg of heme (hemin, Sigma) were dissolved in 0.4 ml of ice-cold 0.1 M NaOH and vortexed periodically. After 30 min, 0.4 ml of 1 M Tris, pH 7.4, was added to the solution. The solution was subsequently centrifuged for 10 min at 4 °C at 13,000 rpm. The heme solution was then diluted with chilled 50 mM Tris, pH 7.4, 150 mM NaCl and centrifuged again at 5100 rpm to remove any heme aggregates. Final concentrations were determined using ε_{385} = 58.44 mM⁻¹cm⁻¹ (35). Heme solutions were used within 12 h.

For spectroscopy and kinetics purposes, apoprotein was fully reconstituted by slowly adding heme to the respective E1 domains in small increments to a 1.5-fold molar excess. After an overnight incubation at 4 °C, excess heme was removed using an S200 gel filtration column (GE Healthcare) or a desalting column (GE Healthcare), and the protein collected in 1-ml fractions. Protein concentrations were measured using the modified Lowry assay (Pierce). The extinction coefficient for the Soret peak for both E1 domains was determined to be equal to 60 mM⁻¹cm⁻¹ by the pyridine hemochromogen assay (36) and used for hemoprotein concentration determination.

To determine the oligomerization state of the respective E1 domains, heme was incrementally added to the protein to the molar ratio indicated in the text and incubated overnight at 4 °C. The heme-loaded E1 domains were run on an S200 gel filtration column (GE Healthcare), and their respective molecular weights were calculated using molecular weight standards (Bio-Rad).

Spectroscopic Measurements—All absorption spectra were recorded at room temperature on a Beckman DU800 spectrophotometer using cells with a 1-cm pathlength, except for heme titration measurements, which were recorded on a Varian Cary 3E dual beam spectrophotometer.

Electron paramagnetic resonance spectra were taken in-house on a Bruker EMX500 spectrometer fitted with a Bruker ER041x microwave bridge and an Oxford Instruments continuous flow liquid helium cryostat. The temperature was maintained at 4.5 K and monitored using an Oxford iTC503S temperature control unit. The magnetic field frequency was calibrated using a 2,2-diphenyl-1-picrylhydrazyl standard (g = 2.0036).

Far-UV circular dichroism (CD) spectra were recorded at 4 °C on a Jasco J-715 spectropolarimeter using 0.1-cm pathlength cells. The bandwidth was set to 2 nm; the response time was equal to 8 s; scanning speed was 10 nm/min, and data pitch was 0.5 nm.

Magnetic circular dichroism (MCD) samples were prepared by exchanging fully reconstituted holoprotein samples into 50 mM potassium phosphate buffer, pH 7.4, with 150 mM NaCl. The solutions were concentrated in Amicon stirred cells (Millipore) and then combined in a 60:40 (v/v) mixture of glycerol and protein solution. The samples were loaded into custom-built sample cells and flash-frozen. MCD spectra were recorded on a home-built MCD setup consisting of a Jasco J-815 CD spectrometer with an Oxford SM4000-8T Spectromag, controlled by a Mercury iTC temperature controller and a Mercury iPS power supply. Data were collected between 900 and 300 nm with a bandwidth of 1 nm, an integration time of 0.25 s, a data pitch of 0.5 nm, and a scan speed of 200 nm/min.

On- and Off-rate Measurements—The on-rates from MmpL3-E1 and MmpL11-E1 were measured anaerobically using ferrous-CO heme (2 μM for MmpL3-E1 and 1 μM for MmpL11-E1) binding to the respective E1 domain using previously described methods (13, 37). The off-rate was measured using the apo-H64Y/V68F-myoglobin (Mb) assay described previously (13, 38).

Ferric Heme Binding Experiments—To measure ferric heme binding, varying molar excess amounts of apo-E1 were mixed with ferric heme (5 μM) in an SX.18MV stopped-flow spectrophotometer (Applied Photophysics) by rapid, equal volume mixing at room temperature. Spectra were recorded between 260 and 700 nm, and the resulting time courses at 410 and 372 nm were fitted to single exponential functions using Graphpad Prism, as described under “Results.”

Pulldown Assay—All procedures described in this section were done in 20 mM PBS, pH 7.4, 150 mM NaCl. Rv0203 was biotinylated in a 1:1 protein/biotin ratio using the Pierce biotinylation kit according to the manufacturer’s directions. Heme was subsequently added to biotinylated apo-Rv0203 as described above. The absorption spectrum of biotinylated holo-Rv0203 was identical to that of unbiotinylated holo-Rv0203, which suggests that the biotin group does not interfere with heme binding.

Holo-Rv0203 (5 μM) was mixed with a 10-fold molar excess apo-E1 in a 500-μl reaction volume. After an incubation period, the length of which is described under “Results,” 120 μl of streptavidin-agarose beads (Novagen) were added to the reaction mixture. The beads were incubated for at least 10 min before the reaction mixture was placed in a spin filter and spun at 1000 rpm for 5 min. The flow through was collected, and the beads were subjected to two wash steps with 250 μl of buffer. All fractions were run on an SDS-polyacrylamide gel, and absorption scans of each fraction were recorded. When holo-Rv0203 was incubated with streptavidin-agarose beads only, the flow through contained minimal amounts of heme indicating heme loss from holo-Rv0203 was not due to nonspecific interactions with the agarose beads.

Heme Transfer between holo-Rv0203 and E1 Domains—In an SX.18MV stopped-flow spectrophotometer (Applied Photophysics), 5 μM Rv0203 in an ~1:1 complex with heme was mixed with the respective E1 domain at various concentrations, as indicated under “Results,” and spectra were recorded between 260 and 700 nm. Reverse transfer experiments with holo-MmpL11-E1 and apo-Rv0203 were conducted in a stopped-flow device in the same manner as the forward reaction at the protein concentrations indicated in the text. Heme transfer between holo-MmpL3-E1 and apo-Rv0203 was initially monitored by stopped flow, but only a fraction of the heme was transferred within the instrument time range. Therefore, reverse transfer between holo-MmpL3-E1 and Rv0203 was recorded in a Beckman DU-800 spectrophotometer. Time courses were fitted to single or double exponential functions using Graphpad Prism as indicated in the text.

Construction of M. smegmatis Knock-out—The M. smegmatis mutant was constructed as described previously (39). To gen-
**TABLE 1**

Primer used to construct the suicide vectors for deletion strains

| Strain          | Primers               | Primer sequence                       | Genes deleted     |
|-----------------|-----------------------|---------------------------------------|-------------------|
| MsmegA0241      | 0241for1              | CGCTGACAGGTCTGGCTAAGCGGTCAAATGCA      | MSMEG_A0241       |
|                 | 0241rev1              | CCACTTCACACGATGGCGAGAGGTCAAATGCA      |                   |
|                 | 0241for2              | CCACTTCACACGATGGCGAGAGGTCAAATGCA      |                   |
|                 | 0241rev2              | CCACTTCACACGATGGCGAGAGGTCAAATGCA      |                   |

**RESULTS**

**Iron Depletion and Testing of Mutant Growth in Various Iron Supplements**—Strains were first grown in LB 0.05% tyloxapol liquid media to late log phase. They were then diluted to 0.1 OD̄/750 in iron-free 7H9 0.05% tyloxapol/NoFe (7H9 without ferric ammonium citrate) and grown again to late log phase. The strains were then passed again as described previously (7) to deplete intracellular iron and then inoculated at A₃₀₀ nm of 0.1 into 7H9 0.05% tyloxapol/NoFe media with 1 μM FeCl₃, 1 μM heme, or no iron. Cell density was measured over 62 h. Knock-out mutant strain was complemented with its respective M. tuberculosis gene, p-NBV1-mmpL11.

**Zinc Mesoporphyrin Uptake Assay**—Zinc mesoporphyrin (ZnMP, Frontier Scientific) was dissolved into 100% DMSO to 1 mM. The resulting solution was filter-sterilized before use in cultures.

Strains were grown in LB 0.05% tyloxapol and passed once into 7H9 0.05% tyloxapol/NoFe as described previously. Each strain was then inoculated into fresh 7H9 0.05% tyloxapol/NoFe and grown to A₃₀₀ nm of 0.4. At this point, 1 μM ZnMP was added to the cultures, and they were further incubated for 1 h. After incubation, cells were harvested by centrifugation at 3500 rpm for 15 min. The resulting pellets were washed in PBS with 1% BSA. BSA was included to remove any membrane-associated ZnMP that could otherwise contaminate cell lysate measurements. The cells were then pelleted again and washed four additional times with PBS without BSA to be sure that the cells were washed clear of any contaminating ZnMP. The resulting pellet was resuspended in 200 μl of PBS and sonicated for 45 s. The lysed cells were then centrifuged at 13,000 rpm at 4 °C. The cytosolic fraction (supernatant) was separated, and pelleted cell debris was discarded. The cytosolic fraction was analyzed by fluorimetry using an excitation wavelength of 416 nm, using a slit width of 2.5 nm.

**Expression and Purification of MmpL3-E1 and MmpL11-E1**—MmpL3-E1 and MmpL11-E1 domains were heterologously expressed and purified from *Escherichia coli*. The respective E1 domain boundaries were chosen based on the in silico domain and transmembrane helix predictions (40). Several E1 constructs for both MmpL3 and MmpL11 were generated with varying N and C termini and tested for solubility, stability, and secondary structural elements. The final MmpL3-E1 and MmpL11-E1 constructs consisted of residues 32–187 and 41–187, respectively. A sequence alignment and secondary structural prediction of MmpL3-E1 and MmpL11-E1 are shown in Fig. 2A. Fig. 2B shows an SDS-PAGE of both E1 domains purified to near homogeneity.

**Heme Reconstitution and Absorption Spectra of Ferric MmpL3-E1 and MmpL11-E1**—Titration experiments suggest that MmpL3-E1 and MmpL11-E1 bind heme in a 1:1 stoichiometry (Fig. 3, A and B). These results were confirmed by reconstitution experiments for both MmpL3-E1 and MmpL11-E1. The E1 domains were reconstituted by adding 1.5-fold molar excess heme to the respective proteins. Removal of
unbound heme over a desalting column resulted in the formation of a 1:1 heme to protein complex as determined by the pyridine hemochromogen (41) and Lowry assays, which measure the heme and protein concentrations, respectively.

The Soret peak maximum of MmpL3-E1 (Fig. 4A) is located at 388 nm, and the Soret peak maximum of MmpL11-E1 is located at 379 nm (Fig. 4A). Both domains feature a charge transfer band near 620 nm (Table 2), which is characteristic of a high spin heme molecule and shifted compared with that of aqueous heme, which has a charge transfer band at 611 nm. The Soret peaks of MmpL3-E1 and MmpL11-E1 are broad, which may indicate the presence of multiple heme species.

**Electron Paramagnetic Resonance (EPR) Spectra of MmpL3-E1 and MmpL11-E1**—The EPR spectra of MmpL3-E1 and MmpL11-E1 are identical (Fig. 5), revealing mixed spin states with a high spin population characterized by $g_{\perp} = 5.99$ and $g_{\parallel} = 1.96$ and at least two low spin populations characterized by $g = 2.92$, $g = 2.26$, and $g = 2.04$. The high and low spin mixture seen in the E1 domains resemble those of ChaN (42), which also exhibits both high and low spin heme populations, although with different low spin $g$ values. Furthermore, the EPR spectra of MmpL3-E1 and MmpL11-E1 also resemble that of *Rhizobium leguminosarum* Irr (RI-Irr), an iron response protein, with heme bound to its low affinity site (43), which has nearly identical high and low spin $g$ values as the E1 domains.

**MCD Spectra of MmpL3-E1 and MmpL11-E1**—The MCD spectra of MmpL3-E1 and MmpL11-E1 at 5 K display very similar spectral features to one another (Fig. 4B), in agreement with the absorption spectra of the two E1 domains (Fig. 4A). The most intense feature in the MCD spectrum of both species is a derivative-shaped feature centered at 410 nm, which corresponds to a shoulder observed in the absorption spectrum of MmpL3-E1. The wavelength and intensity of this feature are consistent with the Soret band of a low spin heme species (44, 45). Absorption spectra were acquired for both MmpL3-E1 and MmpL11-E1 at 20 K (data not shown) using the same samples and instrumental setup as described for MCD spectroscopy, and the Soret peak maxima were between 380 and 390 nm, consistent with the presence of a high spin heme species. Thus, taken together, the absorption (Fig. 4A), EPR (Fig. 5), and MCD (Fig. 4B) data all demonstrate that a mixture of low spin and high spin heme is present in both MmpL3-E1 and MmpL11-E1.

**Heme Promotes Protein Oligomerization**—The oligomeric state of MmpL3-E1 and MmpL11-E1 was investigated using analytical gel filtration chromatography (Fig. 6). Both apo-MmpL11-E1 and apo-MmpL3-E1 are monomeric; addition of increasing molar amounts of heme to both MmpL3-E1 (Fig. 6A) and MmpL11-E1 (Fig. 6B) results in the incremental formation of larger oligomers, with the predicted molecular weight corresponding to either tetramers or pentamers. Of note, after gel
filtration heme is not bound to the monomer but exclusively to the oligomeric species for both E1 domains. Moreover, MmpL3-E1 has higher oligomer to monomer ratios compared with MmpL11-E1, at the heme to protein ratios tested (Fig. 6C), suggesting that heme binding to MmpL3-E1 is tighter than that to MmpL11-E1, which is further supported by the determination of heme off-rates described below.

**Circular Dichroism (CD) Spectroscopy on MmpL3-E1 and MmpL11-E1**—To ensure heme-induced oligomerization of both E1 domains is not a result of major structural changes, we determined the secondary structural composition for each E1 domain in the absence and presence of heme. The CD spectrum of apo-MmpL3-E1 (Fig. 7A) reveals the $\alpha$-helix and $\beta$-strand content based on the program K2D3 (46) to be 38 and 12%, respectively, which is in good agreement with the structural prediction (Fig. 2A). Addition of heme to MmpL3-E1 causes both a slight decrease and a shift in the absolute spectral minimum from 207.5 to 205.5 nm, resulting in a predicted slight decrease of $\alpha$-helix and an increase of $\beta$-strand content (34 and 15%, respectively) (see Fig. 7A). In contrast to MmpL3-E1, the CD spectra of apo- and holo-MmpL11-E1 are nearly identical consisting of a mixture of $\alpha$-helices (34%) and $\beta$-sheets (13%) (Fig. 7B), also in good agreement with secondary structural prediction. These results demonstrate that heme binding does not induce drastic structural rearrangement of either E1 domain.

**Heme Off-rates for MmpL3-E1 and MmpL11-E1**—To measure the respective heme binding affinities, the off- and on-rates, $k_{off}$ and $k_{on}$, of MmpL3-E1 and MmpL11-E1 were determined. The off-rate is highly variable between heme-binding proteins, and typically determines the protein’s heme binding affinity. It is measured using the apo-H64Y/V68F-Mb assay...
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**TABLE 3**

| Protein (Ref.) | On-rate $k_{on}$ $\mu M^{-1} s^{-1}$ | Off-rate $k_{off}$ $s^{-1}$ | $K_a$ $M^{-1}$ |
|---------------|---------------------------------|---------------------------|---------------|
| MmpL3-E1 (this work) | $29 \pm 11$ | $k_{oE1} = 0.0036 \pm 0.0007$ (44%) | $8.1 \times 10^{8a}$ |
| MmpL11-E1 (this work) | $53 \pm 18$ | $0.34 \pm 0.02$ | $1.6 \times 10^{8a}$ |
| Rv0203 (13) | 133 | 0.08 | $1.6 \times 10^{8}$ |
| metHb ($\alpha$ subunit) (47) | 100 | $0.00016$ | $6.0 \times 10^{9a}$ |
| metHb ($\beta$ subunit) (47) | 100 | $0.0042$ | $2.4 \times 10^{10a}$ |
| IsdA (48) | 100 | $0.00026$ | $3.8 \times 10^{11}$ |
| PhuS (49) | 0.18 | 0.04 (calculated from $k_{off} = k_{on}/K_a$) | $5.0 \times 10^{6}$ |
| Shp (50) | 1.6 | 0.0003 | $5.3 \times 10^{5}$ |
| HmuT (51) | | | $3.4 \times 10^{5}$ (1st heme molecule)$^a$ |

$^a$ $K_a$ values are calculated using ferrous-CO heme binding on-rates and ferric heme binding off-rates.

$^b$ $K_a$ values are calculated using ferric heme binding on- and off-rates.

$^c$ $K_a$ values are calculated by direct titration of ferric heme into apoprotein.

$^d$ $K_a$ values are calculated isothermal titration calorimetry with ferric heme.

(38). Neither E1 domain interacts with Mb, and when apo-H64Y/V668F-Mb is present in excess, the rate of heme transfer is dependent exclusively on the rate of heme release, $k_{off}$.

The experimental data for the Mb assay of MmpL3-E1 follows biphasic kinetics with the fast phase $k_1$ equal to 0.0036 ± 0.0007 s$^{-1}$ and the slow phase $k_2$ equal to 0.0004 ± 0.0001 s$^{-1}$ (Table 3). These values are similar to heme dissociation from human methemoglobin (metHb) and Mb, respectively (Table 3) (47). MmpL11-E1 features significantly faster monomeric heme dissociation as compared with MmpL3-E1 with $k_{off}$ equal to 0.34 ± 0.02 s$^{-1}$ (Table 3). These values suggest that MmpL3-E1 binds heme more tightly than MmpL11-E1.

Heme On-rates for MmpL3-E1 and MmpL11-E1—Heme binding can be described by the two-step mechanism shown in Scheme 1.

$$
E1 + \text{heme} \rightarrow E1-\text{heme} \rightarrow \text{holo-E1}
$$

**SCHEME 1**

The respective E1 domain forms an initial protein-heme complex. The final holoprotein is formed after subsequent structural changes within the heme-binding pocket governed by the rate $k_{coord}$. The on-rate was measured by adding increasing amounts of MmpL3-E1 or MmpL11-E1 to ferrous-CO heme. Because ferric heme tends to be dimeric, we used ferrous-CO heme as it is monomeric (52). It has been demonstrated that monomeric heme binding to proteins is usually not affected by the oxidation state of the heme molecule because the on-rates for monomeric ferric CN-heme (53) and monomeric ferrous-CO heme (52) binding to Mb are within experimental error of each other.

The experimental data for ferrous-CO heme binding were fitted to single exponential functions, which yield the experimental $k_{obs}$ at each individual protein concentration. Under conditions where apo-MmpL3-E1 and apo-MmpL11-E1 are in low molar excess compared with the heme concentration, the rate $k_{obs}$ depends linearly on the protein concentration, and $k_{on}$ is equal to the slope. The on-rate for MmpL3-E1 is $29 \pm 11 \mu M^{-1} s^{-1}$ and that for MmpL11-E1 is $53 \pm 18 \mu M^{-1} s^{-1}$. These values are comparable with the on-rates of many heme proteins (Table 3).

**Ferric Heme Binding to E1 Domains**—Although determination of $k_{on}$ necessitates using ferrous-CO, MmpL3-E1 and MmpL11-E1 are unlikely to encounter this heme adduct under physiological conditions, where CO concentrations are low. We therefore attempted to determine ferric heme binding to both MmpL3-E1 and MmpL11-E1.

Ferric heme binding to MmpL3-E1 and MmpL11-E1 is a two-step process. The first, governed by the rate $k_1$, results in an initial fast increase in absorption in the 400–410 nm range, which for MmpL3-E1 is coupled with a drop at 372 nm (Fig. 8, A and C, for MmpL3-E1, and B and D for MmpL11-E1). This species does not resemble the holo-MmpL3-E1 or holo-MmpL11-E1 spectra shown in Fig. 4A. Therefore, we propose that this species represents a heme binding intermediate. In the second step, $k_2$, we observe an increase in absorption at 372 nm (Fig. 8, E and F) over several minutes, although the Soret peak blue shifts to 388 nm for MmpL3-E1 and shifts slightly to 379 nm for MmpL11-E1. The final spectra for ferric heme binding to MmpL3-E1 (Fig. 8A) and MmpL11-E1 (Fig. 8B) now resemble the holo-E1 spectra shown in Fig. 4A.

When the rate of ferric heme binding to E1 domains is measured at two different protein concentrations, the rates $k_1$ and $k_2$ display inverse concentration dependences and are slower when the E1 concentration is increased (Fig. 8 and Table 4). The inverse rate dependence could potentially be caused by concentration-dependent oligomerization of the respective apo-E1 domains. To test this possibility, the experiment was conducted at a constant E1 concentration and two different concentrations of heme (data not shown). This resulted in rates $k_1$ and $k_2$ for both E1 domains that display the same inverse rate dependence as described above. Thus, the inverse concentration dependence of $k_1$ and $k_2$ is not due to oligomerization of apo-E1 domains. Instead, we propose that the inverse rate dependences of $k_1$ and $k_2$ for both MmpL3-E1 and MmpL11-E1 are due to changes in the oligomerization behavior of E1 domains due to heme binding, which is likely a multistep process, and the proposed mechanism of which will be outlined under the “Discussion.”

Rv0203 Transfers Heme to MmpL3-E1 and MmpL11-E1—To investigate whether heme was transferred from the hemotransport protein Rv0203 to MmpL3-E1 and/or MmpL11-E1, holo-Rv0203 was mixed with either apo-E1 domain, and the reaction was followed spectrophotometrically. Mixing holo-
Rv0203 and apo-E1 domains in a conventional spectrophotometer resulted in a rapid spectral shift from that of holo-Rv0203 to that of the corresponding E1 domain. The observation that heme is transferred from Rv0203 to E1 domains raised the question of whether a stable Rv0203-E1 complex can be formed. To investigate this possibility, holo-Rv0203 was biotinylated and incubated with a 10-fold molar excess of apo-MmpL3-E1 or apo-MmpL11-E1 for 60 min. Streptavidin-agarose beads were added to the protein mixture, and the mixture was run over a spin filter. SDS-PAGE subsequently detected proteins bound to streptavidin-agarose and those located in the flow-through. Inspection of the gel revealed that the streptavidin-agarose beads contained only Rv0203, whereas MmpL3-E1 and MmpL11-E1 were entirely located in the flow-through (Fig. 9A). These results suggest Rv0203 and the E1 domains do not form high affinity protein complexes.

**FIGURE 8.** Time-resolved absorption spectra of MmpL3-E1 (A) and MmpL11-E1 (B) binding to ferric heme (5 μM) are shown. The spectra at 0 s represents free heme; 1 s represents the formation of an intermediate species, and 100 s represents the final holo-E1 species observed in Fig. 4A. Formation of the intermediate species, monitored at the change of absorbance at 410 nm, was fit to single exponentials between 0 and 1 s and for MmpL3-E1 (C) and MmpL11-E1 (D) for ferric heme binding at different apo-E1 concentrations. Formation of the holo-E1 species was followed at the change of absorbance at 372 nm for MmpL3-E1 (E) and MmpL11-E1 (F) and was fit to single exponentials, whereby the fitting window was 1–60 s.

**TABLE 4**

| Apo-protein, fold molar excess versus 5 μM heme                      | \( k_1 \) \( \times 10^{-3} \) | \( k_2 \) \( \times 10^{-3} \) |
|---------------------------------------------------------------|----------------|----------------|
| MmpL3-E1, 10X                                                 | 15.81 ± 1.26   | 0.12 ± 0.05   |
| MmpL3-E1, 50X                                                 | 6.87 ± 0.33    | 0.05 ± 0.03   |
| MmpL11-E1, 10X                                               | 14.64 ± 1.52   | 0.64 ± 0.25   |
| MmpL11-E1, 50X                                               | 5.0 ± 0.4      | 0.16 ± 0.05   |

\(^a\) The rate \( k_1 \) represents the formation of the E1-heme intermediate characterized by an amplitude change at 410 nm in the time interval 0–1 s.

\(^b\) The rate \( k_2 \) is obtained from the change in absorption at 372 nm in the 1–60 s interval and the 0–60 s interval for MmpL3-E1 and MmpL11-E1, respectively.

The observation that heme is transferred from Rv0203 to E1 domains raised the question of whether a stable Rv0203-E1 complex can be formed. To investigate this possibility, holo-Rv0203 was biotinylated and incubated with a 10-fold molar excess of apo-MmpL3-E1 or apo-MmpL11-E1 for 60 min. Streptavidin-agarose beads were added to the protein mixture, and the mixture was run over a spin filter. SDS-PAGE subsequently detected proteins bound to streptavidin-agarose and those located in the flow-through. Inspection of the gel revealed that the streptavidin-agarose beads contained only Rv0203, whereas MmpL3-E1 and MmpL11-E1 were entirely located in the flow-through (Fig. 9A). These results suggest Rv0203 and the E1 domains do not form high affinity protein complexes.
Absorption spectra of the flow-through fractions in both experiments are characteristic of holo-E1, suggesting that heme is transferred from Rv0203 to E1 domains (Fig. 9, B and C). Measurement of the total heme content within the flow-through and wash fractions demonstrates that for both MmpL3-E1 and MmpL11-E1/H11011 80% of total heme initially bound to Rv0203 was transferred, suggesting that heme transfer goes to near completion (Fig. 9 D).

The efficient transfer of heme from Rv0203 to E1 domains was initially conducted with a 10-fold molar excess of apo-E1, and the reaction was allowed to proceed for 60 min. To test whether transfer would occur with reduced molar excess of E1 domains or after a shorter incubation period, the incubation time and E1 domain concentration were varied. In the first experiment, a 2.5-fold molar excess of both E1 domains was used. In the second experiment, the reaction mixtures were incubated for only 5 min. In both cases, ~80% of the heme was transferred suggesting low molar excess of each E1 domain was sufficient to drive the reaction to completion and that the reaction was rapid (data not shown).

**Heme Transfer Kinetics**—Because heme transfer to MmpL3-E1 and MmpL11-E1 is rapid, stopped-flow techniques were used to investigate the mechanism of heme transfer. The overall kinetics of heme transfer between holo-Rv0203 and the respective apo-E1 domains are similar to that of ferric heme binding to E1 domains, where an initial increase of the Soret peak at 400–410 nm is observed ($k_1$), followed by a slower increase at 372 nm ($k_2$) (Fig. 10, A and B). Importantly, the rate constants $k_1$ of heme transfer between holo-Rv0203 and both MmpL3-E1 and MmpL11-E1 domains are significantly faster than passive heme dissociation from holo-Rv0203, which has an off-rate, $k_{off}$, equal to 0.08 s$^{-1}$ (Table 5) (13).

For both MmpL3-E1 and MmpL11-E1, the rate $k_1$ of heme transfer does not change significantly as the concentration of E1 increases (Fig. 10, C and D, and Table 5). Furthermore, similar to ferric heme binding to MmpL3-E1, the rate $k_2$ (Fig. 8E and Table 5) decreases slightly with increasing apo-MmpL3-E1 concentrations, and the values of $k_2$ are also similar to $k_2$ from the ferric heme binding experiments (Tables 4 and 5). Similarly, for heme transfer from holo-Rv0203 to MmpL11-E1, the rate $k_1$ was determined using either the pyridine hemochromogen assay (36) or the respective protein’s extinction coefficient at the Soret peak.
M. tuberculosis Rv0203 Transfers Heme to MmpL3 and MmpL11

Directionality of Heme Transfer—To determine whether the reaction is unidirectional, the reverse reaction from the respective holo-E1 domains to Rv0203 was measured. For both holo-MmpL3-E1 and holo-MmpL11-E1, transfer proceeded at a rate similar to passive heme dissociation from holo-Rv0203 but different from ferric heme binding to MmpL3-E1 or MmpL11-E1. Subsequently, the observed spectral changes upon heme transfer from holo-Rv0203 to holo-MmpL3-E1 and MmpL11-E1 are similar to those observed during ferric heme binding to the respective E1 domain.

Zinc-Mesoporphyrin Uptake Requires MmpL11—The subcellular localization of heme that is acquired by mycobacteria is unknown; heme may be incorporated into the mycobacterial cell wall and membrane-bound proteins or be imported into the mycobacterial cytosol to be broken down to release iron by the heme-degrading protein MhuD (10, 11). To determine subcellular localization of acquired heme, ZnMP, a fluorescent heme analog previously used in assessing heme uptake in other examples including Mycobacterium smegmatis (59) from Mycobacterium tuberculosis (60). However, IsdX1 and Shp exhibit different spectroscopic features compared with ChaN and HmuT, sug-

k_{\text{off}} (Fig. 10F) is slower than k_{\text{on}} and slower at high apo-MmpL11-E1 concentration (Table 5), which is the same trend observed for ferric heme binding to MmpL11-E1. Taken together, the experimental evidence suggests that heme is first rapidly transferred to the respective E1 domain at a rate faster than that permitted by passive heme dissociation from holo-Rv0203 but different from ferric heme binding to MmpL3-E1 or MmpL11-E1. Subsequently, the observed spectral changes upon heme transfer from holo-Rv0203 to holo-MmpL3-E1 and MmpL11-E1 are similar to those observed during ferric heme binding to the respective E1 domain.

M. tuberculosis MmpL3 is encoded by an essential gene, preventing direct investigation of its role in mycobacterial heme uptake (9, 14). ZnMP uptake in M. smegmatis ΔmmpL11 was nearly abolished (Fig. 11), whereas complementing M. smegmatis with M. tuberculosis ΔmmpL11 displays an emission signal at 580 nm (Fig. 11). These results suggest that MSMEG_0241 is crucial for ZnMP uptake, and M. tuberculosis mmpL11 restores ZnMP uptake.

Table 5

| Reaction                        | k_{\text{on}} | k_{\text{off}} |
|--------------------------------|--------------|---------------|
| Holo-Rv0203 to 10× apo-MmpL3-E1 | 4.22 ± 0.64  | 0.08 ± 0.01   |
| Holo-Rv0203 to 50× apo-MmpL3-E1 | 3.35 ± 0.47  | 0.03 ± 0.03   |
| Holo-Rv0203 to 10× apo-MmpL11-E1 | 4.16 ± 1.37  | 0.13 ± 0.01   |
| Holo-Rv0203 to 50× apo-MmpL11-E1 | 5.37 ± 1.61  | 0.09 ± 0.04   |
| Holo-MmpL3-E1 to 5× apo-Rv0203 | 0.003 ± 0.001 | ND            |
| Holo-MmpL11-E1 to 5× apo-Rv0203 | 0.20 ± 0.08   | ND            |

* Experiments were conducted with holoprotein concentrations of 5 μM.
* Experiments were conducted with holoprotein concentrations of 10 μM.
* ND means not determined.
M. tuberculosis Rv0203 Transfers Heme to MmpL3 and MmpL11

**FIGURE 12. Proposed two-phase mechanism for heme transfer between holo-Rv0203 and apo-E1 domain (from either MmpL3 or MmpL11).**

suggesting that spectral features of MmpL3-E1 and MmpL11-E1 are not necessarily indicative of cofacially stacked heme molecules.

Instead, the absorption data may be indicative of a five-coordinate, Tyr-ligated heme irrespective of whether the heme is monomeric or cofacially stacked. This possibility is supported by the similarity of the MmpL3-E1 and MmpL11-E1 absorption spectra to that of H25Y human heme oxygenase (61) and H93Y Mb (Table 2) (62). Both of these proteins bind a single five-coordinate, Tyr-ligated heme.

Both the EPR and MCD spectral data indicate that a mixture of high and low spin heme states is present in both E1 domains. A closer analysis of the MCD spectra of MmpL3-E1 and MmpL11-E1 suggests that three heme species are present (Fig. 4B) as follows: high spin, nitrogen-ligated (His or Lys) heme; high spin, oxygen-ligated (Tyr or Ser) heme; and low spin, six-coordinate nitrogen/oxygen-ligated heme. The lowest energy feature in the MCD spectra of MmpL3-E1 and MmpL11-E1 is a negative band at 638 nm. This feature is similar to the ligand-to-metal charge transfer bands observed in ferric Mb at pH 6.8 and cyclohexylamine-ligated H93G Mb, suggesting that one of the high spin species present in MmpL3-E1 and MmpL11-E1 is a His- or Lys-ligated heme (44, 45). The wavelength and sign of the broad positive feature centered at 594 nm is most similar to a His- or Lys-ligated heme (44, 45). The wavelength and sign of the broad positive feature centered at 594 nm is most similar to a His- or Lys-ligated heme (44, 45). The wavelength and sign of the broad positive feature centered at 594 nm is most similar to a His- or Lys-ligated heme (44, 45). The wavelength and sign of the broad positive feature centered at 594 nm is most similar to a His- or Lys-ligated heme (44, 45).

Interestingly, ferrous-CO and ferric heme display very different binding kinetics. Ferric-CO heme binding is characterized by heme binding rates, $k_{obs}$, which increase linearly with the apoprotein concentration. In contrast, ferric heme binding to both E1 domains is multiphasic, described by $k_1$ and $k_2$. The inverse rate dependence of $k_1$ and $k_2$ for both MmpL3-E1 and MmpL11-E1 may be an effect of the oligomerization behavior of the respective holo-E1 domains. Based on the observed two-step binding process, we propose a mechanism in which E1 initially forms an E1-heme intermediate characterized by a red shift in the Soret peak. The E1-heme intermediate undergoes oligomerization with apo-E1 forming weakly associated, unsaturated E1 oligomers. In a second step, the final product oligomeric holo-E1 is formed, which is characterized by a Soret peak increase at 372 nm. Oligomeric holo-E1 is the only heme-bound E1 species detected after gel filtration experiments. Therefore, we believe its formation is essentially irreversible. The inverse rate dependence may be explained by the fact that when the apo-E1 concentration is high, formation of the holo-E1 product is relatively slow because E1-heme first undergoes oligomerization reactions with excess molar apo-E1 before the irreversible holo-E1 product forms (Fig. 12).

**MmpL3-E1 and MmpL11-E1 Heme Binding Kinetics**—The heme binding kinetics of MmpL3-E1 and MmpL11-E1 were studied by measuring on- and off-rates of the respective domains. The heme binding affinity of MmpL3-E1 is $8.1 \times 10^9$ M$^{-1}$ and that of MmpL11-E1 is $1.6 \times 10^8$ M$^{-1}$. The $K_m$ for MmpL3-E1 is similar to that of Hb (47), whereas MmpL11-E1 binds with approximately one-fifth the affinity as compared with Rv0203 (13). These values indicate that MmpL3-E1 binds heme more tightly than MmpL11-E1.

ChaN (42), where the apoprotein is monomeric and the holo-protein is dimeric. Furthermore, fully heme-bound RI-Irr is a hexamer, whereas apo-RI-Irr is a mixture of dimer and hexamer (65), which the authors suggest is heme promoted oligomerization of RI-Irr.

We note, however, that unlike RI-Irr, ChaN, HmuT, and PhuT, both E1 domains are part of a large membrane protein. Therefore, the oligomerization state described for holo-MmpL3-E1 and holo-MmpL11-E1 domains in vitro may not reflect their behavior under physiological conditions; nevertheless, understanding E1 domain oligomerization is important for the interpretation of the heme binding and transfer experiments in this study.

$\text{MmpL3-E1 and MmpL11-E1}$

$\text{Heme Binding Kinetics}$—The heme binding kinetics of MmpL3-E1 and MmpL11-E1 were studied by measuring on- and off-rates of the respective domains. The heme binding affinity of MmpL3-E1 is $8.1 \times 10^9$ M$^{-1}$ and that of MmpL11-E1 is $1.6 \times 10^8$ M$^{-1}$. The $K_m$ for MmpL3-E1 is similar to that of Hb (47), whereas MmpL11-E1 binds with approximately one-fifth the affinity as compared with Rv0203 (13). These values indicate that MmpL3-E1 binds heme more tightly than MmpL11-E1.

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Because of the lack of positive rate dependence of \( k_I \) for ferric heme binding to E1, it is not possible to determine the on-rate for ferric heme, and the \( K_a \) was instead determined using the on-rate from ferrous-CO heme experiments. However, given the lower values for \( k_I \) compared with \( k_{abs} \), it is very likely that the on-rate for ferric heme is lower than that for ferrous-CO heme. This in turn suggests that under physiological conditions, where MmpL3-E1 and MmpL11-E1 will encounter ferric heme (7), the proteins’ respective heme binding affinities may not be as high as those determined in vitro using ferrous-CO heme.

**Heme Transfer from Rv0203 to the E1 Domains Occurs via a Transient Protein-Protein Interaction**—Heme uptake in bacteria is typically accomplished by the transfer of heme between a series of proteins from outside to inside (7, 31, 66). Previously, we have determined the heme binding properties of Rv0203 and suggested that it acts as a heme transport protein (9, 13).

Additionally, the two homologous transmembrane proteins MmpL3 and MmpL11 have been suggested to function as transmembrane heme transporters (9). In this work, we have demonstrated that the soluble domains, MmpL3-E1 and MmpL11-E1, bind heme, and heme is transferred from Rv0203 to MmpL3-E1 and MmpL11-E1.

Pulldown experiments between holo-Rv0203 and MmpL3-E1 and MmpL11-E1 have shown that heme is efficiently transferred to the E1 domains (Fig. 9). Furthermore, comparing the rates of heme transfer between holo-Rv0203 and the E1 domains with the rate of passive heme transfer between holo-Rv0203 and apo-H64Y/V68F-Mb revealed that the heme transfer reaction to either E1 domain is significantly faster than passive heme dissociation from holo-Rv0203, which suggests that transfer is perhaps driven by a transient protein-protein interaction (Fig. 10). The kinetic features of the heme transfer between holo-Rv0203 and the respective E1 domains resemble the biphasic kinetics of ferric heme binding. However, the rates \( k_f \) for transfer are slower compared with ferric heme binding and do not display concentration dependence suggesting that heme transfer is a slower process than ferric heme binding to the respective E1 domain. The fastest observed rate of heme transfer, \( k_p \), is a zero-order reaction as it is independent of the apo-E1 concentration. This suggests that the rate-limiting step is the rate at which heme is transferred within the Rv0203-E1 protein-protein complex. The concentration independence of \( k_f \) further suggests that the initial formation of the holo-Rv0203-E1 complex (Fig. 12), which is a bimolecular process, involves the rapid formation of a reversible, weak complex governed by rates of formation and dissociation \( k_f \) and \( k_r \), respectively. The formation of the holo-Rv0203-E1 complex can be considered sufficiently rapid to be in equilibrium throughout the heme transfer process. The spectrophotometric changes at 372 nm after heme transfer, \( k_{ps} \), are similar to those seen during ferric heme binding to MmpL3-E1 and MmpL11-E1. Therefore, \( k_f \) is likely to be independent of the heme transfer reaction itself and arises from the mechanism with which the respective E1 domain binds heme post-transfer. A proposed mechanism for heme transfer is outlined in Fig. 12.

Heme transfer via protein-protein interactions between holo-Rv0203 and apo-E1 resembles the mode of transfer observed in other heme uptake systems such as the HasA-HasR interaction (33) in *Serratia marcescens* and the IsdX1-IsdC interaction in *B. anthracis* (67). The binding affinity of the protein-protein interaction between Rv0203 and E1 is likely to be very weak as the Rv0203-E1 complex was not captured during pulldown experiments. The protein-protein interactions encountered in heme uptake pathways are often transient (29, 48). Heme transfer in *S. aureus* has been extensively studied. IsdA is a heme transport protein that transfers heme to IsdC. Van’t Hoff analysis estimates the \( K_a \) value between IsdA and IsdC to be equal to 17 \( \mu M \) (48), whereas NMR experiments suggest it to be in the millimolar range (34). A complication when studying heme transfer arises from the fact that the heme recipient may have different affinities for the holo-heme donor (Rv0203) compared with its apo-form. Support for this hypothesis is offered by experiments between the *B. anthracis* hemeophore IsdX1 and its heme receptor IsdC (67). By surface plasmon resonance analysis, holo-IsdX1 was shown to bind IsdC with a \( K_d \) of 5 \( \mu M \). In contrast, no interaction between apo-IsdX1 and IsdC was recorded.

**Downstream Heme Transfer**—This work demonstrated that MmpL3 and MmpL11 receive heme from Rv0203 via their respective E1 domains. The subsequent steps to transfer heme across the membrane are not yet known. In the Gram-negative organism *S. marcescens*, the HasA receptor HasR is part of a TonB-dependent transport complex (6, 30). In Gram-positive bacteria such as *S. aureus*, the membrane-bound heme receptors are part of ABC transporters, such as IsdE within the ABC transporter IsdDEF (31, 32). MmpL3 and MmpL11 contain low sequence homology to nonmycobacterial proteins and are part of a different protein family than either HasR and IsdDEF. In addition, heme import mediated by MmpL3 and MmpL11 is likely to occur via a mechanism that is distinct from other RND transporters, which are typically efflux pumps that remove substrates from the cell using protonmotive force (17, 68). The predicted topology of MmpL3 and MmpL11 (Fig. 1B) is different from that of the four RND protein structures in the Protein Data Bank, CusA (16), MexB (69), SecDF (70), and AcrB (71), which may reflect a difference in their substrate transport mechanism.

Recent research efforts have discovered that MmpL3 is the target of a variety of compounds that have antimycobacterial activities, including adamantyl urea-based compounds (72), SQ109 (73), and BM212 (27). The investigation of the mode of action of these compounds revealed that they inhibited mycobacterial growth by disrupting trehalose monomycolate transport across the membrane (27), preventing proper assembly of the mycobacterial cell wall. This suggests MmpL3 may be a bifunctional transporter. Such bifunctionality has been observed among other heme uptake transport proteins. For example, the *S. aureus* transmembrane protein HtsA displays dual roles as both a heme and siderophore receptor (74, 75). Because MmpL3 is involved in both heme and trehalose monomycolate transport but not in siderophore transport, it is likely that the evolutionary origins of MmpL3 heme transport are distinct from that of HtsA. Recently, MmpL4 was shown to mediate siderophore export (25). The evolutionary origins of heme and siderophore transporters are often linked (76), and
thus the MmpL family of proteins may harbor members of a mycobacterium-specific group of iron and heme uptake proteins. The observation that MmpL3 is a necessary protein and that the MmpL3 heme uptake mechanism may be unrelated to that of other bacteria suggests MmpL3 represents an excellent anti-tuberculosis drug target.

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