The Cytoplasmic Heme-binding Protein (PhuS) from the Heme Uptake System of *Pseudomonas aeruginosa* Is an Intracellular Heme-trafficking Protein to the δ-Regioselective Heme Oxygenase*

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The uptake and utilization of heme as an iron source is a receptor-mediated process in bacterial pathogens and involves a number of proteins required for internalization and degradation of heme. In the following report we provide the first in-depth spectroscopic and functional characterization of a cytoplasmic heme-binding protein PhuS from the opportunistic pathogen *Pseudomonas aeruginosa*. Spectroscopic characterization of the heme-PhuS complex at neutral pH indicates that the heme is predominantly six-coordinate low spin. However, the resonance Raman spectra and global fit analysis of the UV-visible spectra show that at all pH values between 6 and 10 three distinct species are present to varying degrees. The distribution of the heme across multiple spin states and coordination number highlights the flexibility of the heme environment. We provide further evidence that the cytoplasmic heme-binding proteins, contrary to previous reports, are not heme oxygenases. The degradation of the heme-PhuS complex in the presence of a reducing agent is a result of $\text{H}_2\text{O}_2$ formed by direct reduction of molecular oxygen and does not yield biliverdin. In contrast, the heme-PhuS complex is an intracellular heme trafficking protein that specifically transfers heme to the previously characterized iron-regulated heme oxygenase *pa-HO*. Surface plasmon resonance experiments confirm that the transfer of heme is driven by a specific protein-protein interaction. This data taken together with the spectroscopic characterization is consistent with a protein that functions to shuttle heme within the cell.

All living organisms require iron for their survival and have developed sophisticated mechanisms by which they solubilize, sequester, and release iron within the cell. Bacterial pathogens have evolved to take advantage of the hosts iron and heme-containing proteins as a source of iron (1, 2). The ability to transport and utilize heme is a common mechanism employed by pathogenic bacteria in establishing and maintaining infection (3–6). Furthermore, a recent report highlighted a distinct preference for heme as an iron source during the initial stages of *Staphylococcus aureus* infections (7). However, the function of the proteins encoded within the heme transport operon of Gram-negative organisms has almost exclusively been based on genetic studies and sequence homology with the more well characterized iron-siderophore pathways (3, 4, 8–11). The heme transport systems in Gram-negative bacteria comprise a TonB-dependent outer membrane receptor critical for active transport of heme into the periplasm (12), where a periplasmic heme-binding protein then acts as a soluble receptor for the transport of heme to the cytoplasmic membrane. In addition, genes bearing significant homology to the previously characterized cytoplasmic permease and ATPase proteins facilitate active uptake of heme into the cytoplasm (13).

In the opportunistic pathogen *Pseudomonas aeruginosa*, two distinct heme uptake operons have been identified (10). The *pha* (Pseudomonas heme utilization) and *has* (heme assimilation system) heme-uptake systems are under the transcriptional regulation of the ferric uptake regulator (Fur) and are found in distinct loci within the genome. Both operons have genes encoding for outer membrane receptors *phaR* and *hasR*, respectively. In addition, the *pha* operon encodes genes for a periplasmic binding protein (*PhaT*), cytoplasmic heme-binding protein (*PhaS*), and the ATP-dependent permease proteins (*PhaUVW*) (10). The *has* operon encodes for an extracellular heme-binding protein or hemo-pher *HasA* that functions to deliver heme to HasR. The remaining genes in the operon, *hasD and -E*, are proposed to be components of an ABC-transporter for secretion of the hemoprotein (HasA) (14, 15).

Whereas the general concept of heme uptake is fairly well understood from genetic and biochemical studies of the iron-siderophore uptake systems, the fate of the heme once transported into the cytoplasm was based on speculation of a heme oxygenase-like activity. In *Yersinia enterocolitica* the cytoplasmic heme-binding protein encoded by the *hems* gene was essential in preventing heme toxicity and was proposed to be a heme-degrading enzyme (16). In contrast, the *shuS* gene of *Shigella dysenteriae* was not essential in the prevention of heme toxicity (17). It is unclear at this point whether this reflects functional differences in the ShuS and Hems proteins, or between their respective outer membrane receptors (18). The existence of degenerate systems for heme and iron acquisition in such organisms may also complicate identification of distinct phenotypes.

We have recently purified and characterized the ShuS protein from *S. dysenteriae* as a heme-binding protein and based on preliminary characterization it does not appear to be a heme oxygenase (19). Further-
more, the recent identification and characterization of iron-regulated heme oxygenases from *Corynebacterium diphtheriae* (HmuO) (20), *Neisseria meningitidis* (HemO) (21), and *P. aeruginosa* (pa-HO, PigA) (22) suggest that the function of the cytoplasmic heme-binding proteins is distinct from that of the heme oxygenases.

The recent identification of a heme oxygenase (BphO), which produces α-biliverdin as a chromophore for the sensor kinase (BphP) of a bacterial two-component signaling system (23), confirms the role of the δ-selective pa-HO in the mining of iron from exogenously acquired heme. These recent findings led us to hypothesize that the PhuS protein is an intracellular heme trafficking protein specific to the iron-regulated heme oxygenase (pa-HO).

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals (ACS grade or better), resins, DNA modifying enzymes, and oligonucleotides were purchased from commercial sources and used as received. Plasmid purification, subcloning, and bacterial transformations were carried out as previously described (24). Deionized, doubly distilled water was used for all experiments.

**Bacterial Strains**—Escherichia coli strain DH 5α (F-, ara D(lac-proAB) rpsL ø80d lacIq XylI -D-galactopyranoside. The cells were grown for 20 h at room temperature and harvested by centrifugation for 10 min at 10,000 × g at room temperature.

**Construction of the Expression Vector pEPhuS**—pCRphuS was generated by PCR amplification using Herculease DNA polymerase (Stratagene) and the following primers containing the listed restriction site: 5′ NdeI-GAACCGCATATGAGCAGCACTCCC and 3′ XhoI-GGCGCGCTTAAGGATTTC. Poly(A) overhangs were generated by the addition of 1 μl λDNA polymerase and 1 μl of Taq polymerase to the completed PCR and incubated in a water bath at 95 °C for 2 min. The PCR product was cloned into the pCR2.1-TOPO II vector (Invitrogen) to generate pCRphuS. The resultant phuS fragment was subcloned into pET21a expression vector by NdeI-XhoI digestion and re-ligation generating pEPhuS.

**Expression and Purification of the PhuS Protein**—The PhuS proteins were purified by a modification of the published procedure for ShuS (19). A 10-ml innoculum in Luria-Bertani (LB)-ampicillin (100 μg/ml) was prepared from LB-Amp (100 μg/ml) agar plates with freshly transformed colonies of pEPhus in *E. coli* BL21(DE3) pLYS5 (F' ompT hsdSb gal dcm) (DE3)) was used for expression of the wild type PhuS construct.

**Expression and Purification of the Heme Oxygenase Proteins**—The iron-regulated pa-HO was purified as described previously (22). The BphO protein was purified by modification of the previously described procedure for pa-HO (22). An overnight 10-ml innoculum in LB-kanamycin (50 μg/ml) was prepared from LB-kanamycin (50 μg/ml) plates with freshly transformed colonies of pET28b-BphO in *E. coli* BL21(DE3) cells. One-liter cultures were inoculated to an A600 of 0.06 from a 100-ml subculture grown to an A600 = 0.6 from the overnight growth. The cells were grown for a further 5–6 h at room temperature and harvested by centrifugation for 10 min at 8,000 × g. The cell debris was removed by centrifugation at 18,000 × g for 35 min. The soluble fraction was applied to a Sepharose-Q Fast Flow column (3 × 10 cm) equilibrated with 20 mM Tris-HCl (pH 8.0). The protein was washed with 10 column volumes of the same buffer containing 50 mM NaCl and eluted with a linear salt gradient from 50 to 350 mM NaCl. The protein eluted at 200 mM NaCl. The peak fractions were pooled and concentrated to 5 ml and stored at −80 °C.

**Absorbance Spectroscopy of the Heme-Phus Complex**—The millimolar extinction coefficient at the Soret band maximum (410 nm) of the heme-PhuS complex in 20 mM Tris-HCl (pH 7.5) was determined by pyridine hemochrome method (25). UV-visible absorbance spectra of the heme-PhuS complexes were recorded over the pH range of 6.0 to 10.5. This range was covered with 20 mM MES, 20 mM HEPES, and 20 mM CHES buffers. Spectra were recorded over this pH range and evaluated via global analysis to determine the speciation of the heme-PhuS complex and the corresponding pKₐ values. In a separate pH titration experiment, ΔA was monitored at 419 nm between pH 6.5 and 9.0 in 20 mM Tris-HCl. These data were fit to the Henderson-Hasselbalch equation, with the value of n held fixed at 1.

**Fluorescence Spectrophotometry**—Titrations of PhuS with heme were monitored by steady state fluorescence emission. Samples were excited at 295 nm, and the fluorescence was monitored at 337 nm. Solutions of 1.0 μM PhuS in 20 mM Tris-HCl (pH 7.8) were titrated with 1-μl aliquots (0.1–100 μM) of heme at 25 °C. All stock heme solutions were freshly prepared in dimethyl sulfoxide and used within 30 min. The binding constant (Kₐ) was calculated by Scatchard analysis, which was based on the decrease in Trp fluorescence intensity at 337 nm as a function of increasing heme concentration.

**Resonance Raman Spectroscopy**—Resonance Raman (rR) spectra were obtained with either 413.1 nm excitation from a Kr⁺ laser or 441.6 nm excitation from a HeCd laser. PhuS samples were 20 to 90 μM in heme. Raman spectra were recorded using a Raman spectrometer described previously (26). The spectrometer was calibrated using the Raman shifts of bands from spectra of neat toluene, dimethyl formamide, and methylene bromide as external frequency standards. Data were collected at ambient temperature from samples in spinning 5-mm NMR tubes using the 135° backscattering geometry. Laser powers ranged from 10 to 15 milliwatt at the sample with 413.1 nm excitation and from 441.6 nm excitation from a HeCd laser.
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FIGURE 1. Purification of the heme-PhuS complex. *A*, SDS-PAGE of the purified PhuS protein (10–15 μg) following ion-exchange chromatography: lane 1, molecular mass markers as shown; lane 2, purified PhuS. *B*, fast protein liquid chromatography of the purified PhuS on Superdex S200HR. The PhuS monomeric and dimeric forms, solid line; molecular mass markers, dashed line. A 50-μl sample of PhuS containing 200 μg of protein was loaded onto the column equilibrated in 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl at a flow rate 0.2 ml/min. Molecular mass markers in a final volume of 100 μl contained: apoferritin (50 μg), β-amylose (100 μg), alcohol dehydrogenase (50 μg), bovine serum albumin (100 μg), and carbonic anhydrase (50 μg).

15 to 20 milliwatt with 441.6 nm excitation. No spectral artifacts attributable to photoinduced chemistry were observed. UV-visible spectra were recorded before and after rR experiments to ensure that the samples were not irreversibly altered by laser irradiation. Samples for the pH study were prepared by dilution of a stock of PhuS into 50 mM buffer at the desired pH.

**Reaction of the Heme-PhuS Complex with Ascorbate or NADPH Cytochrome P450 Reductase**—To determine whether PhuS has heme oxygenase activity, the heme-PhuS complex was assayed in the presence of ascorbate (20–22). Ascorbate (500 μM final concentration) was added to the heme-PhuS complex (10 μM) in a final volume of 1 ml of 20 mM Tris-HCl (pH 8.0) at 25 °C. A second set of reactions with ascorbate were carried out in the presence of catalase (150 units) and superoxide dismutase so as to discriminate between heme degradation via coupled oxidation and that possibly arising from enzymatic heme oxygenation. The experiments described above were also carried out with the *P. aeruginosa* heme oxygenase (pa-HO) to establish a basis for comparison of the aforementioned activity with that of an authentic heme oxygenase.

**In Vitro Heme Transfer Experiments**—Absorbance spectra of pure heme-PhuS, apo-pa-HO, and BphO were recorded between 300 and 700 nm. Following addition of an equimolar amount of either apo-pa-HO or apo-BphO to heme-PhuS, the spectrum was recorded again. The extent of heme transfer from the heme-PhuS complex to pa-HO or BphO was verified by UV-visible absorbance (406 nm for heme, 280 nm for protein) and SDS-PAGE analysis of chromatographic fractions separated from the reaction mixture by gel filtration (Sepharose S-100, 1.5 × 120-cm column, equilibrated with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl).

**UV-visible Stopped Flow Measurements**—Rate measurements were carried out using a model SX18MV stopped flow spectrometer from Applied Photophysics. Determinations were made in both the single- and multichannel modes. Equimolar amounts of heme-PhuS and apo-pa-HO or apo-BphO were mixed to final concentrations of 10 μM in 20 mM Tris-HCl (pH 8.0). For each reaction, 60 μl of each protein were mixed and the increase in absorbance at 406 nm was tracked. Initial rates of the reactions of heme-PhuS with apo-pa-HO and BphO were determined from time courses of ΔA, which was followed for 50 and 1000 s, respectively.

**Protein-Protein Interaction as Determined by Surface Plasmon Resonance**—Kinetic evaluations of heme-PhuS (analyte) binding to immobilized pa-HO or BphO were carried out on a BIACORE 3000 instrument (Biacore, Upppsala, Sweden). All experiments were performed at 25 °C on carboxymethyl-dextran sensor chips (CM5), which were activated using equimolar amounts of N-hydroxysuccinimide and N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride for 7 min (Amine Coupling Kit, BIACore). The ligand, either pa-HO or BphO, was diluted in the acetate coupling solution to a concentration of 30 μg/ml at pH 5.0 and pumped over the activated surface at 5 μl/min. Approximately 2000 and 3500 resonance units of either pa-HO or

FIGURE 2. Heme titration and electronic absorbance spectrum of the heme-PhuS complex. *A*, emission spectrum of 1 μM apo-PhuS with increasing increments of heme (0–100 μM) (inset, change in fluorescence intensity as a function of heme concentration). *B*, heme-PhuS (10 μM) in 20 mM Tris-HCl (pH 8.0) (inset, 500–700 nm region, ×2).

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FIGURE 3. Spectrophotometric pH titration of PhuS. A, UV-visible absorbance spectra of heme-PhuS from pH 5.8 to 10.4. Inset shows data and fits from global analysis for titration curves at 395 (●), 408 (●), 413 (■), and 567 (▲) nm. B, speciation of PhuS as a function of pH, calculated by global analysis.
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BphO, respectively, were immobilized on the chip. The surface of the flow cell was then blocked with ethanolamine for 7 min to deactivate any unreacted esters and to remove any remaining unbound protein. A second flow cell was activated and deactivated to yield a blank surface. All analyte samples were diluted in degassed, filter-sterilized buffer (10 mM HEPES, pH 7.4, 0.15 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20). The same solution was used as running buffer during kinetics experiments.

For reactions of heme-PhuS with immobilized pa-HO, the concentration of heme-PhuS was varied between 0 and 350 nM in 50 nM increments. The analytic flow rate was 20 μl/min. The association with immobilized pa-HO was followed for 180 s, and the dissociation for 300 s at each concentration. Following completion of the dissociation step, surface regeneration was accomplished with a 5-μl injection of 50 mM NaOH at a flow rate of 100 μl/min. The data were fit to a 1:1 Langmuir model using the BIAevaluation Software provided by BIAcore. Control experiments were carried out at each analytic concentration by passage over the activated blank chip to determine any nonspecific binding of heme-PhuS to the surface. Any signal attributable to nonspecific binding was then subtracted from the actual data curves.

Similar experiments were carried out for the reaction of heme-PhuS with immobilized BphO. Heme-PhuS concentrations were varied from 0 to 150 μM. The flow rate was 30 μl/min and the association and dissociation rates were both measured over a period of 200 s. Regeneration of the surface was accomplished as described above.

RESULTS

Expression and Purification of PhuS—PhuS purifies as the apo-protein, as judged by the absence of heme features in its UV-visible absorbance spectrum. Following ion exchange, heme reconstitution, and size exclusion chromatography, the PhuS protein was judged 98% pure by SDS-PAGE with an apparent molecular mass of 39 kDa (Fig. 1A). The yield of purified PhuS ranged from 10 to 20 mg/liter of cell culture. Analysis of the heme-PhuS complex by size exclusion chromatography indicates that, in vitro, the protein exists as a mixture of dimer (78 kDa) and monomer (39 kDa) (Fig. 1B), and the ratio of dimer to monomer appears to be independent of whether the heme is present or not (data not shown). In addition, varying the salt concentration in the elution buffer up to 300 mM NaCl did not affect the ratio of dimer to monomer (data not shown), suggesting that the interaction is hydrophobic. It is unclear at the present time whether the dimerization of PhuS is physiologically relevant. However, the PhuS homolog, ShuS from S. dysenteriae, also forms dimers and oligomers in vitro (19).

Spectroscopic Characterization of the Heme-PhuS Complex—The heme binding constants (K_d) were measured by the addition of heme to aliquots of protein at pH 8.0 and monitored by the heme-induced decrease in tryptophan fluorescence (Fig. 2A). This method yielded K_d values of 0.18 ± 0.01, 0.72 ± 0.06, and 1.5 ± 0.1 μM for PhuS, pa-HO, and BphO, respectively. Upon reconstitution with heme the Soret maximum of the heme-PhuS complex occurs at 410 nm at pH 8.0 with maximum extinction coefficients. The extinction coefficient at 410 nm was determined to be 122 μM⁻¹ cm⁻¹. The visible spectrum of heme-PhuS at pH 8.0 is very similar to a number of heme-hydroxide complexes, such as those reported for Hb at pH 10.4, Mb at pH 10.4 (27, 28), various peroxidases (29, 30), and FixL at pH 10.7 (32).

The pH dependence of the absorbance spectrum is shown in Fig. 3A. These experimental spectra are comparable with the component spectra of the acidic, neutral, and alkaline species obtained from global analysis of the pH titration over the range of 6.0 to 10.5. The speciation of these heme-PhuS forms is shown in Fig. 3B. The pK_a values for the transitions involving these three species are at 7.2 ± 0.1 and 10.2 ± 0.5. The pK_a of 7.2 is assigned to the Fe(III)-H_2O to Fe(III)-OH transition. Least squares analysis of the pH titration of heme-PhuS monitored by changes in absorbance at 419 nm between pH 6.5 and 9.0 yields a similar pK_a of 7.1 ± 0.1 for this acid-base equilibrium (data not shown).

The pH-dependent changes in spin state and axial coordination of the heme identified via the spectrophotometric titration were further clarified by rR spectroscopy. Fig. 4A shows the rR spectra of heme-PhuS

![Image](https://example.com/image.png)

**FIGURE 4.** The pH dependence of PhuS over the pH range of 5.7 to 10.0 as monitored by resonance Raman spectroscopy, A, the high frequency region of the rR spectra of PhuS, B, the low frequency region of the rR spectra of PhuS. Spectra were collected with 413.1 nm excitation. Samples were in the following buffers: 50 mM CAPS, pH 10.0, 50 mM Tris/HCl (pH 8.0), 50 mM sodium phosphate (pH 7.2), 50 mM MES (pH 6.0 and 5.7).
A band at 321 cm\(^{-1}\) indicates that its effect on the out-of-plane vibrations are relatively small.

The insensitivity of the low frequency rR spectra to pH (Fig. 4B) indicates that its effect on the out-of-plane vibrations are relatively small. A band at 321 cm\(^{-1}\) grows in with increasing pH, and the band tentatively assigned to a propionate bending mode (\(\delta(C_3C_4C_5)\)) shifts from 376 cm\(^{-1}\) at acidic pH to 373 cm\(^{-1}\) under alkaline conditions.

Coordination of hydroxide to heme can be verified by isotope sensitivity of rR bands due to the Fe-OH stretching vibration. The rR spectra of heme-PhuS prepared in D\(_2\)O at pH 8.0 and 10.0 were examined for isotope-sensitive bands. None were observed in the range typical for HS or LS Fe-OH adducts (data not shown). However, distal coordination of heme-PhuS by hydroxide at pH 10 was confirmed by the \(^{18}\)O isotope-sensitive band at 546 cm\(^{-1}\) shown in Fig. 5. When the sample was prepared in H\(_2\)\(^{18}\)O, this band shifted 22 cm\(^{-1}\) to lower energy, which is close to the 23.8 cm\(^{-1}\) shift calculated for an isolated Fe-OH oscillator. Thus, the 546-cm\(^{-1}\) band is assigned to the \(v(\text{Fe-OH})\) mode of a 6-c LS heme-PhuS-\(^{18}\)O complex. This assignment is further supported by the similarity of its frequency to those assigned to \(\nu(\text{Fe-OH})\) in 6-c LS hydroxide adducts of Hb (27) and HO (37). The \(\nu(\text{Fe-OH})\) mode for 6-c HS hydroxide adducts typically falls between 450 and 495 cm\(^{-1}\) (38). No clearly discernable isotope-sensitive bands were seen in this range for heme-PhuS. A second isotope-sensitive band is observed at 376 cm\(^{-1}\) which water and hydroxide compete. Efforts to identify this ligand are ongoing. The persistence of 6-c LS and 6-c HS hemes under alkaline conditions is consistent with a distal hydroxide ligand, as heme-OH complexes often exist as HS/LS equilibria (27, 32, 36–38). Interestingly, some 5-c HS heme persists to the most alkaline conditions investigated here. One possible explanation for the presence of the alkaline 5-c HS species is that it is produced by the \(pK_{a1}\) transition. Alternatively, a small amount of nonspecifically bound heme from the reconstitution procedure may survive the purification process.

The low-frequency region of the resonance Raman spectrum of alkaline PhuS. Spectra were obtained for 21.2 \(\mu\)g PhuS in 25 mM CAPS (pH 10) in 100% H\(_2\)\(^{18}\)O and in 73% H\(_2\)\(^{16}\)O with 413.1 nm excitation. The difference spectrum obtained by subtraction of PhuS\(^{16}\)O from PhuS\(^{18}\)O shows the isotope-sensitive bands as difference features.

The sensitivity of the low frequency rR spectra of ferric hemes, the \(\nu_3\) band, often referred to as the heme spin-state marker, falls in characteristic frequency ranges for the following permutations of spin state and coordination number: 1490–1500 cm\(^{-1}\) for high-spin (HS) five-coordinate (5-c), 1475–1488 cm\(^{-1}\) for HS six-coordinate (6-c), and 1500–1510 cm\(^{-1}\) for low-spin (LS) 6-c hemes. The spectra at pH < \(pK_{a1}\) show that 5-c HS, 6-c HS, and 6-c LS heme species are present. Whereas the same spin states and coordination numbers are present at \(pK_{a1} > \text{pH} < pK_{a2}\), their relative populations differ with the 6-c LS heme being favored.

In conjunction with the visible titration, the acidic 6-c HS form is assigned to the Fe(III)-H\(_2\)O adduct of heme-PhuS, whereas the two 6-c species observed at neutral pH are assigned to 6-c LS and 6-c HS heme hydroxide adducts. The 5-c HS heme form observed at pH 7.2 and above could have either a histidine or a hydroxide as the fifth heme ligand; there is precedent for both (33–35). The persistence of 6-c LS heme at acidic pH suggests that the acid-base transitions involving an exogenous axial ligand do not account for all of the heme. This is consistent with a second endogenous ligand with which water and hydroxide compete. The \(pK_{a1}\) value of 9.26 for low-spin (LS) 6-c hemes under alkaline conditions is consistent with a distal hydroxide adduct. The 5-c HS heme form observed at pH 7.2 and above is assigned to the Fe(III)-H\(_2\)O adduct of heme-PhuS, whereas the two 6-c bands typical of propionate bending. Based on the difference spectrum, this band shifts 9 cm\(^{-1}\) in H\(_2\)\(^{18}\)O. Similar \(^{18}\)O shifts have been noted in previous R studies of heme-OH complexes (33, 39). The reason for this difference feature has not been determined, but it may be attributable to the slow exchange of carboxylate oxygens with \(^{18}\)O from H\(_2\)\(^{18}\)O.

Upon reduction of the ferric PhuS complex at pH 8 with a 10-fold excess of sodium dithionite, the Soret maximum shifts from 410 to 428...
In the visible region the \( \alpha/\beta \)-bands are seen at 559 and 535 nm, respectively. The spectrum is indicative of two ferrous heme-PhuS species. Peak-fitting analysis of the spectrum revealed two components having Soret maxima at 419 and 432 nm, consistent with 6-c LS and 5-c HS species, respectively (Fig. 6A). In the high frequency rR spectra of the Fe(II) heme-PhuS complex marker bands for both 5-c HS and 6-c LS species are observed at 1469 and 1490 cm\(^{-1} \), respectively (Fig. 6B). Furthermore, unlike the ferric heme-PhuS complex, which has a \( pK_a \) at neutral pH, the speciation of ferrous heme-PhuS shows no pH dependence between 5.7 and 8.0.

In the low frequency Soret-excited rR spectrum of the ferrous heme-PhuS, the \( \nu_{\text{Fe-His}} \) band at 222 cm\(^{-1} \) suggested the presence of histidine as the proximal ligand in 5-c HS ferrous PhuS (Fig. 6C). Its increase in relative intensity with 441.6 nm excitation further supports this assignment (Fig. 6D). In its ferrous form, heme-PhuS is prone to precipitation after approximately 1 h at room temperature. Moreover, attempts to measure the reduction potential revealed a lack of electrochemical reversibility (data not shown) that is consistent with slow decomposition of the reduced protein.

**Heme Degradation via Non-coordinated \( \text{H}_2\text{O}_2 \) Versus Heme Oxygenation**—The turnover of heme to Fe(III)-verdoheme in the heme oxygenase-catalyzed reaction proceeds through reduction and formation of an oxy-heme intermediate. A ferric hydroperoxide intermediate is formed upon accepting a second electron and a proton. This species rapidly undergoes an intramolecular hydroxylation reaction to form \( \alpha/-\text{meso} \)-hydroxyheme, as shown in Scheme 1A (40–42). In contrast, coupled oxidation of the porphyrin core can be facilitated by 2-electron reduction of free \( \text{O}_2 \) in solution to yield \( \text{H}_2\text{O}_2 \) (Scheme 1B) (43). The conjugate base of \( \text{H}_2\text{O}_2 \) can coordinate to the ferric heme-HO complex to give the aforementioned reactive Fe(III)-OOH intermediate. Even though this intermediate is not formed via the HO mechanism it can undergo intramolecular conversion to ferric \( \alpha/-\text{meso} \)-hydroxyheme, which is rapidly converted to Fe(III)-verdoheme in the presence of oxygen. In other words, it is possible for heme proteins that do not exhibit HO activity to produce verdoheme if they have access to free \( \text{H}_2\text{O}_2 \). In an effort to determine whether PhuS is itself a heme oxygenase, assays were carried out to clarify if heme degradation occurs via reduction of the ferric-heme-HO complex or by the coupled oxidation reaction. In the presence of ascorbate, the heme-PhuS complex degrades, as evident by the decrease in absorbance at the Soret maximum and the increase in absorbance at 660 nm (Fig. 7A). Addition of pyridine to the completed reaction yielded a spectrum typical of Fe(III)-verdohemochrome with its characteristic visible band at 644 nm (Fig. 7A, inset). In contrast, Fe(III)-verdoheme was not produced in the presence of catalase and superoxide dismutase, as judged by lack of the aforementioned changes in the visible absorbance spectrum (Fig. 7B). The validity of these conditions to assay HO activity was verified using heme-\( \alpha_/\beta_-\text{HO} \) in which the final reaction mixture gave a spectrum typical of iron-free biliverdin (Fig. 7C).

**In Vitro Heme Transfer from PhuS to \( \alpha_-\text{HO} \) and BphO**—Studies of inter-protein heme transfer originating from PhuS were carried out by adding an equimolar amount of either apo-\( \alpha_-\text{HO} \) or apo-BphO (50 \( \mu \text{M} \)) to the heme-PhuS complex (50 \( \mu \text{M} \)). Transfer of heme from PhuS to \( \alpha_-\text{HO} \) was evident from a shift in the Soret maximum from 410 to 406 nm, the disappearance of the \( \alpha/\beta \)-band signature of LS heme-PhuS, and the appearance of the HS ferric marker band at 630 nm (Fig. 8A).
To determine the extent of heme transfer from PhuS to \textit{pa}-HO, the mixture was applied to a Sephacryl S-100 column (0.5 cm x 100 cm) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. SDS-PAGE analysis of the fractions containing protein and heme absorbance confirmed the almost complete transfer of heme from PhuS to \textit{pa}-HO (Fig. 9A). Reactions of heme-loaded \textit{pa}-HO and apo-PhuS resulted in no measurable heme transfer. Gel filtration and SDS-PAGE showed that the heme remained associated with \textit{pa}-HO (data not shown). Stopped flow measurement of the rate of heme transfer from heme-PhuS to \textit{pa}-HO in a 1:1 mol ratio gave a rate constant of $(9.4 \pm 0.1) \times 10^3$ M$^{-1}$ s$^{-1}$.

The addition of apo-BphO to the heme-PhuS complex shifted the Soret from 410 to 409 nm with the appearance of only a small fraction of HS heme, as indicated by the increase in absorbance at 630 nm. Persistence of the \(\alpha\) and \(\beta\) bands at 538 and 570 nm revealed that a significant fraction of the heme remained LS (Fig. 8B). Comparison of the spectrum of the PhuS/BphO mixture with that of a pure 5-c HS heme-BphO complex having a Soret maximum at 405 nm and a HS marker band at 635 nm, suggests that the heme is most likely distributed between the two proteins in the PhuS/BphO reaction. Analysis of the difference spectra following transfer of heme from PhuS to \textit{pa}-HO and \textit{BphO} did not show an increase at 402 nm or the corresponding trough at 418 nm, but gave an intermediate absorbance at 415 nm, indicating partial transfer of the heme (Fig. 8C). Although the rate constant for heme transfer to BphO was not significantly smaller than that to \textit{pa}-HO, \((5.5 \pm 0.1) \times 10^3$ M$^{-1}$ s$^{-1}\), it was not complete, as judged by gel filtration and SDS-PAGE analysis (Fig. 9B). Furthermore, back transfer of heme...
upon mixing holo-BphO with apo-PhuS suggested that, at equilibrium, the relative populations of the holo-proteins are determined by their relative intrinsic affinities for heme.

**Protein-Protein Interaction as Determined by Surface Plasmon Resonance**—Surface plasmon resonance was used to determine whether the mechanism of heme transfer from PhuS to _pa-HO_ involves a specific protein-protein interaction. The surface plasmon resonance data (Fig. 10) recorded with PhuS flowing over _pa-HO_ or BphO immobilized on the biosensor chip reveal dose-dependent binding of the heme-PhuS complex to _pa-HO_ at physiological pH (pH 7.4). Further analysis gave association (_k_a_) and dissociation (_k_d_) rate constants of $1.68 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $1.07 \times 10^{-3} \text{s}^{-1}$, respectively. The corresponding _K_D_ of 64 nM is consistent with the association of PhuS with _pa-HO_ being specific and physiologically relevant (Fig. 10A). Similar analysis with BphO showed no discernible surface plasmon resonance response within physiological ranges of protein concentration. Only at PhuS concentrations in the micromolar range, 1000-fold higher than _pa-HO_ concentrations, was a response observed (Fig. 10B). Furthermore, the data were not accurately modeled by a simple Langmuir interaction model. The shape of the curves suggests some heterogeneity, which may be a result of the high concentrations of PhuS flowing over the sensor chip.

**DISCUSSION**

The function of several proteins encoded in the _P. aeruginosa_ heme uptake (phu) operon have been postulated based on their homology with known proteins (10). In contrast, the function of PhuS and the highly conserved family of cytoplasmic heme-binding proteins are not as well understood. An earlier genetic study with the _Y. entercolitica_ hemS knock-out strain attributed the observed heme toxicity to the inability to release iron from heme, and hence proposed that the protein was a heme oxygenase (16). Recently, a _S. dysenteriae shuS_ genetic knock-out was also shown to suffer heme toxicity at high heme concentrations (17). This toxicity could be reversed by a plasmid encoding ShuS. We have previously characterized the heme-ShuS complex by...
The data presented thus far suggest that PhuS is not a heme oxygenase or oxidative enzyme. This is in contrast to the recent report that the PhuS homolog ChuS from *E. coli* is a heme degrading enzyme (47). Like PhuS the *E. coli* homolog ChuS is a dimer and binds one heme per monomer. The heme degrading properties of the heme-ChuS complex were carried out in the presence of ascorbate or the NADPH-cytochrome P450 reductase system. However, the authors did not consider the possibility of nonspecific degradation of the heme by H$_2$O$_2$. This study has accounted for this possibility by demonstrating that heme degradation in heme-PhuS only occurs by reaction of the ferric protein with H$_2$O$_2$. As has been previously reported, catalase inhibits the non-specific degradation of heme via such coupled oxidation of heme proteins, implying that the oxidant in such systems is free H$_2$O$_2$ (48). These initial results have been extended by Rivera and co-workers (49) with axial ligand mutants of cytochrome b$_{5}$, in which they have shown that heme degradation in coupled oxidation reactions yields meso-hydroxyheme and that the mechanism is distinct from that of heme oxygenation. In contrast to heme oxygenation, via the HO mechanism the coupled oxidation reaction is a consequence of free H$_2$O$_2$ produced by direct reduction of molecular oxygen or dismutation of superoxide (Scheme 1B). The H$_2$O$_2$ generated by these mechanisms can then react directly with Fe(III)-heme to yield Fe(III)-meso-hydroxyheme (Scheme 1B).

This data suggests that the *in vivo* toxicity reported for the genetic knockouts of *hemS* (*V. enterocolitica*) and *shuS* (*S. dysenteriae*) are not attributable to their inability to utilize heme, but to unproductive and destructive trafficking of the heme within the cell. This leads us to suggest that, under conditions of iron deprivation, PhuS acts as a heme trafficking protein to deliver acquired heme to *pa*-HO. Heme oxygenation by *pa*-HO would then release the iron for subsequent utilization by the cell. This hypothesis is supported by the nanomolar dissociation constant of the heme-PhuSpa-HO complex. In other words, the high stability of this protein-protein complex lends credibility to the suggestion that heme-PhuS *specifically* targets *pa*-HO for heme transfer. It is interesting to further speculate on the mechanistic role of this specific inter-protein association. An attractive model would have changes in the heme state and/or the protein conformation that are required for dissociation of the heme-PhuSpa complex being energetically coupled to formation of the protein-protein complex. At neutral pH the heme-PhuSpa and heme-*pa*-HO complexes are 6-c LS and 5-c HS, respectively. Therefore, changes in both the spin state and axial heme coordination must occur during the transfer reaction. These transformations may be driven by the free energy yield on protein-protein complexation, thereby triggering heme transfer. The ready accessibility of both spin states in the heme-PhuSpa complex suggests this is a feasible triggering mechanism.

Although limited transfer of heme from PhuS to the non-iron-regulated heme oxygenase (BphO) occurs *in vitro*, the heme is distributed between the proteins at equilibrium and the rate of approach to equilibrium speciation is slow. Therefore, in contrast to the mechanistically specific transfer of heme from PhuS to *pa*-HO, the transfer of heme between PhuS and BphO is most likely governed by their relative intrinsic affinities for heme. BphO with its extremely low affinity for PhuS would appear to have no physiologically relevant interaction with heme-PhuS. These observations are consistent with its role in the biosynthesis of $\alpha$-biliverdin for the phytochrome sensor kinase protein BphP (23, 50). Although the regulation of BphO is not well understood, microarray analysis of *P. aeruginosa* mRNA levels argue that it is neither directly, nor indirectly involved in iron metabolism or metabolic adaptation to iron availability (51).
The Cytoplasmic Heme-binding Protein of P. aeruginosa

The identification and characterization of the α-selective BphO and its physiological function in phytochromophore biosynthesis also provides some insight into the evolution of a δ-selective iron-regulated pa-HO in contrast to those of N. meningitidis and C. diphtheriae, which are α-selective. Hence the evolution of the iron-regulated δ-selective heme oxygenase further supports the hypothesis that pa-HO and BphO have distinct and separate physiological roles in P. aeruginosa.

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