HHS Public Access

Author manuscript

*Biochemistry*. Author manuscript; available in PMC 2015 October 30.

Published in final edited form as:

*Biochemistry*. 2015 April 28; 54(16): 2601–2612. doi:10.1021/acs.biochem.5b00017.

# Spectroscopic Determination of Distinct Heme Ligands in Outer-Membrane Receptors PhuR and HasR of *Pseudomonas aeruginosa*

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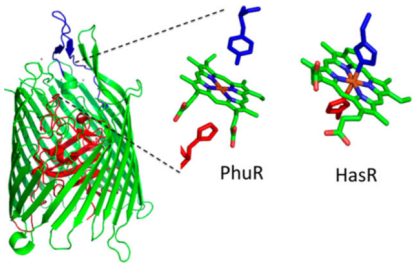
## Abstract

*Pseudomonas aeruginosa* PAO1 encodes two outer membrane receptors, PhuR (*P*seudomonas heme uptake) and HasR (heme assimilation system). The HasR receptor acquires heme through interaction with a secreted hemophore, HasAp. The non-hemophore-dependent PhuR is encoded along with proteins required for heme translocation into the cytoplasm. Herein, we report the isolation and characterization of the HasR and PhuR receptors. Absorption and MCD spectroscopy confirmed that, similar to other Gram-negative OM receptors, HasR coordinates heme through the conserved N-terminal plug His-221 and His-624 of the surface-exposed FRAP-loop. In contrast, PhuR showed distinct absorption and MCD spectra consistent with coordination through a Tyr residue. Sequence alignment of PhuR with all known Gram-negative OM heme receptors revealed a lack of a conserved His within the FRAP loop but two Tyr residues at positions 519 and 529. Site-directed mutagenesis and spectroscopic characterization confirmed Tyr-519 and the N-terminal plug His-124 provide the heme ligands in PhuR. We propose that PhuR and HasR represent nonredundant heme receptors capable of sensing and accessing heme across a wide range of physiological conditions on colonization and infection of the host.




Iron is an essential micronutrient for the survival and virulence of most bacterial pathogens, and as such, they have developed complex mechanisms to uptake and utilize heme as an iron source.[1,2] In the opportunistic pathogen *Pseudomonas aeruginosa*, two heme uptake systems

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The authors declare no competing financial interest.

have been characterized: the *Pseudomonas* heme uptake (*phu*) and the heme assimilation systems (*has*).[3] The *phu* system encodes a TonB-dependent outer-membrane (OM) receptor (PhuR) that transports heme to the periplasm, where a soluble heme binding protein (PhuT) acts as the receptor for an ATP-dependent permease (ABC-transporter) (PhuUV), which then translocates heme to the cytoplasmic heme binding protein (PhuS). In contrast the *has* operon encodes a soluble hemophore (HasA) that is secreted to the extracellular environment, where it extracts heme from hemoglobin and transfers it to the TonB-dependent OM receptor (HasR).[4–6] The HasR N-terminal domain on binding the heme-loaded HasA hemophore mediates signal transduction through the extracytoplasmic function $\sigma$ and anti-$\sigma$ factors HasI and HasS.[3] Thus, the hemophore-dependent HasR receptor in addition to transporting heme across the OM acts as a sensor of the extracellular heme signal in a Fur-independent manner.

The HasA hemophore coordinates heme through His-32 and Tyr-75 with picomolar affinity.[7,8] Recent kinetic and spectroscopic studies of the *P. aeruginosa* HasA protein have shown that heme acquisition from methemoglobin is relatively passive, occurring at a rate similar to that of heme dissociation.[9] Therefore, the capture of dissociated heme by HasA and its reported nanomolar binding affinity to the HasR receptor would allow the bacteria to access a wider range of heme concentrations within the host environment.[10] Sequence alignment of all TonB-dependent OM heme receptors, in combination with site-directed mutagenesis of *Yersinia enter-colitica* HemR, identified the highly conserved His residues located in the predicted N-terminal plug and conserved FRAP/PNPNL loop as being required for heme uptake.[11] Spectroscopic and structural characterization of HasR of *Serratia marcescens* and ShuA of *Shigella dysenteriae* have confirmed heme to be coordinated through a His on the face of the N-terminal plug occluding the membrane spanning $\beta$-barrel and a corresponding His on the extracellular FRAP/PNPNL loop (Figure 1).[12–14] Interestingly, the majority of heme receptors so far characterized, both hemophore-dependent and -independent, have the characteristic bis-His coordination. An exception to the bis-His coordination was the recently reported five-coordinate Tyr-ligation in the HmbR receptor of *Neisseria meningitides*.[15]

Many Gram-negative pathogens in addition to *P. aeruginosa* have multiple heme uptake systems, including *S. marcescens*,[16] *S. dysenteriae*,[17] *V. cholera*,[18] *Y. pestis*,[19] and *N. meningitides*.[20] In a *S. marcescens* heme auxotroph, *in vivo* studies have shown the *hasR* and the nonhemophore *hemR* genes are differentially regulated by heme and iron.[21] Although the HasA–HasR system could access heme at much lower concentrations than HemR, it could do so only when iron was extremely limiting. In contrast, the HemR receptor is expressed at higher concentrations of iron. It was further shown that HasR in the absence of HasA cannot access heme at concentrations attainable in the presence of the hemophore. In addition the ability to extract heme from the high-affinity heme binding protein hemopexin required the presence of HasA. The authors proposed that the differential regulation and spectrum of accessible substrates represented nonredundant uptake systems and further hypothesized that the high-affinity *has* system was absolutely required for vertebrate infection. In contrast, a recent transcriptome analysis of *P. aeruginosa* longitudinal CF lung isolates suggests that PhuR, and not the hemophore-dependent HasR,

is the major heme receptor in clinical infection.[22] It was shown that mutations within the *phuR* promoter increase PhuR expression levels, concomitant with decreased pyoverdine biosynthesis. Several independent studies have also concluded that genes involved in heme uptake are consistently expressed by *P. aeruginosa* colonizing the CF lung, whereas the pyoverdine (PVD) biosynthesis genes were intermittently expressed.[23] Utilizing isotopic-labeling studies, we have recently shown that upregulation of *phu* system genes is consistent with an increase in the metabolism of extracellular heme in clinical isolates.[24] As part of our long-term goal in understanding the relative contributions of the *P. aeruginosa has* and *phu* systems to heme utilization and infection, we have characterized the heme binding properties and substrate selectivities of the HasR and PhuR receptors. Herein, we report on the unique His-Tyr heme ligation in PhuR not previously observed in any TonB-dependent OM heme receptor. We propose that this unusual heme coordination together with the differential substrate specificity of the *phu* and *has* systems allows *P. aeruginosa* to access heme under a range of physiological conditions critical for infection within the host.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains

DNA manipulations were performed in *Escherichia coli* strain DH5$\alpha$ (F′, ara D (lac-proAB) rpsL $\phi$80dlacZ DM15 hasd R17), and *E. coli* strain BL21 (DE3) (B F– dcm ompT hsdS (rB–mB–) gal $\lambda$ (DE3)) was utilized for protein expression.

### PhuR and HasR Vector Construction and Site-Directed Mutagenesis

The *hasR* and *phuR* genes were PCR-amplified from *P. aeruginosa* PAO1 genomic DNA. The expression vector pET22b (Novagen) was chosen for expression of HasR and PhuR. The first 22 amino acid residues corresponding to a *P. aeruginosa* signal peptide were omitted from the final gene constructs as predicted by the signal peptide program SignalP. The forward primer (PhuRF, 5′-TAATATGGCCATGGCCCTGGCGGGGAAC-3′) incorporated an MscI site preceding the codon for Ala-23 of *phuR*. The reverse primer (PhuRR, 5′-ACCATCTCGAGGATGTCCCAGACCAGGTTGACC-3′) was designed to remove the stop codon at the end of the *phuR* gene to allow the utilization of the $His_6$ tag following the XhoI site in pET22b. Similarly, primer HasRF (5′-TATAGGATCCACAAGACGGGGCGGA-3′) was designed to omit the first 36 amino acids of the predicted native signal peptide of HasR and allow cloning at the BamH1 preceding the *E. coli* PelB signal peptide site. The reverse primer (HasRR, 5′-TAATGAGCTCGAACTGGTATTCGAGGGTGC-3′) removed the native stop codon to include the $His_6$ tag utilizing the pET22b SacI restriction site. The resulting constructs, pHasR22b and pPhuR22b, incorporated the N-terminal pelB leader sequence of *E. coli* for targeting to the periplasm and a $His_6$ tag on the C-terminus for rapid purification of the proteins by metal-affinity chromatography. Mutagenesis was performed by PCR utilizing the QuikChange mutagenesis kit (Agilent). All mutants were verified by DNA sequencing (Eurofins MWG Operon).

### Expression of the HasR and PhuR Proteins

*E. coli* BL21 (DE3) cells were freshly transformed with pHasR22b or pPhuR22b constructs and plated on LB agar containing ampicillin (Amp) (100 μg/mL) plates at 37 °C overnight. A single colony was selected to inoculate 100 mL of noninducing MDAG-135 media containing 100 μg/mL Amp.[25] The subculture was grown for 12 h at 37 °C and 225 rpm, following which 10 mL was then taken to inoculate 1 L of autoinducing ZYM-5052 media containing 100 μg/mL Amp.[25] Cultures (8 × 1 L) were allowed to grow for an additional 12 h at 25 °C and 200 rpm and were then harvested by centrifugation for 15 min at 7000 rpm in a Beckman JLA8.1 rotor. Harvested cells were frozen and stored at −80 °C until purification.

### Outer-Membrane Isolation

Cell pellets were resuspended in 4 mL $g^{-1}$ of cell pellet in buffer A (10 mM sodium phosphate, pH 7.5, 125 mM NaCl, 3 mM KCl) containing 10 mM benzamidine, 2 EDTA-free protease inhibitor cocktail tablets (Roche Diagnostic), 1 mM $MgCl_2$, 20 μg/mL DNase, and 10 μg/mL lysozyme. Cells were stirred at 4 °C for 1 h and then passaged twice through a Thermo Spectronic French pressure cell at 18 000 psi. The cell debris was removed by centrifugation at 12 000 rpm for 15 min in a Beckman JA17 rotor. The resulting lysate was then centrifuged for 1 h at 25 000 rpm in a Beckman JA25.50 rotor to pellet the total cellular membranes. The pelleted membranes were combined and resuspended in 1 mL $g^{-1}$ of Buffer A with an added EDTA-free protease inhibitor tablet, and the cytoplasmic membrane proteins were solubilized by addition of 2% (w/v) Triton X-100 (Sigma) and 0.5% (w/v) Sarkosyl (Teknova). The membrane fractions were stirred at room temperature for 1 h and repelleted at 25 000 rpm for 1 h in a Beckman JA25.50 rotor. The resulting supernatant containing only the cytoplasmic membrane proteins was discarded. The pelleted OM fraction was then resuspended in 30 mL of Buffer A containing a mini EDTA-free protease inhibitor cocktail tablet (Roche Diagnostic) and stirred at 4 °C overnight.

### HasR and PhuR Purification

OM protein concentrations were determined using the detergent-compatible RC DC protein assay (BioRad), and the total protein concentration was adjusted to 10 mg/mL with Buffer A. The OM receptors were solubilized by addition of 2% (w/v) Fos-Choline-15 (FC-15) (Anatrace), stirred at room temperature for 2 h, and then pelleted by centrifugation at 25 000 rpm for 1 h a Beckman JA25.50. FC-15 was previously determined to have the best extraction efficiency for HasR and PhuR based on screening against an 88 detergent kit purchased from Anatrace. Both PhuR and HasR were purified over a Ni-nitriloacetic acid (Ni-NTA) metal-affinity column (2.5 × 4 cm) equilibrated in Buffer B (20 mM Tris (pH 7.5), 200 mM NaCl, 5 mM Imidazole (Im), 0.07% (w/v) lauryl-*N*,*N*-dimethylamine oxide (LDAO) (Anatrace)). The flow through was collected, and the column was washed with 10 CV of Buffer B, followed by 3 CV Buffer C (20 mM Tris (pH 7.5), 100 mM NaCl, 20 mM Im, 0.07% (w/v) LDAO). The receptors (PhuR or HasR) were eluted with Buffer E (20 mM Tris (pH 7.5), 50 mM NaCl, 250 mM Im, 0.07% (w/v) LDAO). The peak fractions were analyzed by 10% SDS-PAGE, and fractions greater than 95% pure were pooled. Purified protein was concentrated on Ultracel YM-50 centrifugal filter units (Millipore) and

subjected to two subsequent dialysis steps in 50 mM Tris (pH 7.5), 50 mM NaCl, 0.07% (w/v) LDAO using Spectra/Por CE 50 kDa molecular weight cutoff dialysis membrane (Spectrum Laboratories). Protein yields varied from preparation to preparation but were typically 1 mg purified protein/g cell pellet.

For separation of the apoprotein fraction from that of the holoprotein, the HasR or PhuR receptors were purified over Q-sepharose. The solubilized protein was diluted 2× in 40 mM Tris, pH 7.5, and added to a Q-sepharose anion-exchange column (2.5 × 6 cm) at 4 °C equilibrated in Buffer Q1 (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.03% (w/v) *n*-dodecyl-*β*-D-maltoside (DDM) (Anatrace)). The flow through containing apo-PhuR or apo-HasR was collected, and the column was washed with 10 column volumes (CV) of Buffer Q1. Holo-HasR or holo-PhuR were eluted with Buffer Q2 (20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.03% (w/v) DDM), and the peak protein fractions were assessed for purity on a 10% SDS-PAGE gel. Fractions showing greater than 95% purity were pooled, concentrated, and dialyzed as described above.

The extinction coefficient of PhuR was calculated to be 148.5 $mM^{-1}$ $cm^{-1}$ using previously described methods[26] and agrees closely with the empirically calculated value of 134 $mM^{-1}$ $cm^{-1}$ (http://www.expasy.org). We were unable to calculate an extinction coefficient for the native HasR due to precipitation issues. HasR protein concentrations were determined by using the empirically calculated extinction coefficient of 126 $mM^{-1}cm^{-1}$ (http://www.expasy.org). Heme concentrations were determined by the pyridine hemochrome method of measuring the absorbances at 418.5, 526, and 555 nm following the addition of pyridine and dithionite using the extinction coefficients of 170, 17.5, and 34.4 $mM^{-1}$ $cm^{-1}$, respectively.[27] The molar percent heme bound to the HasR and PhuR proteins was calculated by comparing the heme concentration determined by pyridine hemochrome to the protein concentrations determined from the calculated or empirical extinction coefficient for PhuR and HasR, respectively.

## Purification of HasA

The *P. aeruginosa* HasA protein, referred to as HasAp to distinguish it from the previously characterized HasA of *S. marcescens*, was purified as previously described.[28] Briefly, pET11A harboring the *hasAp* gene was transformed into *E. coli* BL21 (DE3) cells. A single colony was used to inoculate LB media (50 mL) containing Amp (100 *μ*g/mL). Following growth overnight at 37 °C, 10 mL of the culture was taken to inoculate 1 L of LB media containing Amp (100 *μ*g/mL). Cultures were grown at 37 °C to an $OD_{600}$ ~ 0.8–0.9, induced with IPTG to a final concentration of 1 mM, and grown for an additional 3 h at 25 °C. Cells were harvested by centrifugation in a Beckman JLA8.1 rotor at 7000 rpm. Cells were lysed in 20 mM Tris-HCl (pH 7.6) containing 10 mM benzamidine, two protease inhibitor cocktail tablets (Roche Diagnostic), 1 mM $MgCl_2$, 20 *μ*g/mL DNase, and 10 *μ*g/mL lysozyme at 4 °C with stirring for 1 h followed by sonication on ice. Cellular debris removed by centrifugation at 25 000 rpm for 1 h in a Beckman JA25.50 rotor, and clarified supernatant was applied to a Q-sepharose anion-exchange column (2.5 × 10.0 cm) equilibrated in 20 mM Tris-HCl (pH 7.6). Following washing, the protein was eluted in the same Buffer over a 0−600 mM NaCl gradient. Peak fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 7.6).

Dialyzed protein was subjected to a second Q-sepharose anion-exchange purification as described above, and the peak fractions were concentrated to ~5 mL. The concentrated HasAp was purified over a Superdex S75 HR 10/300 column equilibrated in 20 mM Tris-HCl (pH 7.6) containing 100 mM NaCl. Purified fractions were pooled and concentrated to ~5 mL. Holo-HasAp was prepared by titrating the partially heme loaded HasAp with a fresh hemin solution. Residual heme was removed by passage of the holo-HasAp over a disposable desalting spin columns (Zeba, Thermo Scientific).

### Preparation of Hemoglobin

Hemoglobin (Hb) was prepared from outdated whole human blood obtained from the University of Maryland Blood Bank. Whole blood (10 mL) was centrifuged in a Beckman JA-12 rotor at 2000 g for 10 min. The supernatant was removed by aspiration, and the erythrocyte pellet was resuspended in 2 volumes of an isotonic Buffer consisting of 20 mM Tris-HCl (pH 7.4), 5 mM KCl, 2 mM $CaCl_2$, 140 mM NaCl, and 1 mM $MgSO_4$. Resuspended erythrocytes were washed a further three times and resuspended in 10 volumes of hypotonic Buffer consisting of 5 mM Tris-HCl (pH 7.4), 2 mM EDTA. The cells were stirred for at 4 °C for 20 min and lysed on ice by sonication. The cell debris was removed by centrifugation in a Beckman JA25.50 rotor at 25 000 rpm for 1 h. The supernatant was collected and analyzed by 4–15% SDS-PAGE. The isolated Hb was dialyzed overnight in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl or in 50 mM Tris (pH 7.4) and 500 mM NaCl. Stock solutions of hemoglobin were kept above 600 $\mu$M to prevent spontaneous dissociation into $\alpha\beta$ dimers and concomitant dissociation of heme upon rapid oxidation.[29] The UV–visible spectroscopic properties of the freshly isolated Hb were checked to ensure proper folding and functionality. Oxidized Hb (metHb) was generated by incubation with a 10-fold excess of $K_3Fe(CN)_6$ for 10 min at room temperature followed by removal of excess $K_3Fe(CN)_6$ over a Zeba desalting spin column (Pierce).

### HasR and PhuR Binding to Hemin Agarose

Purified apo-PhuR or apo-HasR (10 $\mu$M) in 20 mM Tris-HCl, (pH 7.5) 50 mM NaCl, and 0.07% LDAO in a final volume of 200 $\mu$L was incubated with hemin-agarose (2 $\mu$L). Samples were incubated with rotation at 25 °C for 1 h. Hemin- and hemoglobin-agarose beads were briefly centrifuged, and the supernatant was aspirated. Resin was washed four times in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.07% (w/v) LDAO. The resin was pelleted, resuspended in 50 $\mu$L of 1× SDS loading Buffer, boiled, and analyzed on a 10% SDS-PAGE gel. Removal of the His-tag by thrombin cleavage did not affect the binding of apo-PhuR and apo-HasR to hemin-agarose, confirming binding at the ligand binding site.

### Heme Transfer to HasR and PhuR from HasA and Hb

His-tagged apo-HasR or PhuR (5 $\mu$M) in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl was incubated with either holo-HasA or Hb (4 $\mu$M) in a final volume of 200 $\mu$L, respectively. The proteins were incubated for 10 min at 37 °C, following which the receptors were immobilized on Ni-NTA resin washed with 4 volumes of 20 mM Tris-HCl (pH 7.5) containing 250 mM NaCl and 5 mM Im followed by 4 volumes of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 20 mM Im. HasR and PhuR were eluted in the same Buffer

containing 250 mM Im. Im was removed over desalting spin columns, and the UV–visible spectrum for each fraction recorded to determine the extent of heme transfer from holo-HasA to HasR and metHb to PhuR. The fractions were further analyzed by SDS-PAGE for evidence of complex formation.

### Circular Dichroism (CD) Spectroscopy

Holo-HasR and holo-PhuR purified from heme-supplemented cultures were analyzed for secondary structure by CD. All samples were recorded at 25 °C in 20 mM potassium phosphate (pH 7.5) and 50 mM NaCl containing 0.07% LDAO on a JASCO J810 spectropolarimeter from 190 to 260 nm with 0.2 mm resolution and 1.0 cm bandwidth. The mean residue ellipticity (deg $cm^2$ $dmol^{-1}$) was calculated using CDPRO software supplied by the manufacturer.

### Magnetic Circular Dichroism Spectroscopy

Magnetic circular dichroism spectra were recorded at 4 °C and 1.41 T on a Jasco J815 spectropolarimeter fitted with a MCD-1B electromagnet and interfaced with a Gateway PC through a JASCO IF-815-2 interface unit. JASCO software was used for data acquisition and manipulation as reported previously.[30] UV–visible absorption spectra were recorded on a Cary 400 spectrometer before and after each MCD measurement to track sample integrity. Holo-PhuR and holo-HasR was obtained following ion exchange to remove the apoprotein fraction. Concentrations of the samples were determined (based on the pyridine hemochromogen method) for the Soret absorption peaks of ferric WT PhuR ($\varepsilon_{Soret}$ = 96.9 $mM^{-1}$ $cm^{-1}$), Y519A PhuR ($\varepsilon_{Soret}$ = 114.0 $mM^{-1}$ $cm^{-1}$), Y519H PhuR ($\varepsilon_{Soret}$ = 100 $mM^{-1}$ $cm^{-1}$), H124A PhuR ($\varepsilon_{Soret}$ = 105.8 $mM^{-1}$ $cm^{-1}$), and HasR (100 $mM^{-1}$ $cm^{-1}$). The cyanide ($CN^-$) adducts were prepared by adding aliquots of KCN stock solutions (1 M or diluted concentrations, pH adjusted to ~7) to the protein until apparent saturation was reached.

## RESULTS

### Purification of the Apo- and Holo-PhuR and HasR Receptors

Recombinant expression and isolation of the membrane fractions followed by SDS-PAGE confirmed that PhuR and HasR were successfully targeted to the OM with apparent molecular weights of ~85 and 95 kDa, respectively (Figure 2A,B). On the basis of pyridine hemochrome of the Ni-NTA purified receptors, both receptors were purified partially heme-loaded and ranged from 5 to 10% for HasR and 20 to 25% for PhuR (Figure 2C,D). The heme-loaded state of the receptors could be increased to 30–60% (based on pyridine hemochrome) on addition of 10 $\mu$M heme or freshly prepared Hb to the cultures 1 h before harvesting the cells (Figure 2C,D). As previously reported for the hemophore receptor HasR from *S. marcescens*, anion-exchange chromatography separated apo-PhuR and apo-HasR from the heme-loaded fraction.[10] The Soret maxima obtained on heme supplementation and separation of the holoprotein fraction by anion exchange were 405 and 411 nm for PhuR and HasR, respectively (Figure 2A,B). Interestingly, the 405 nm heme Soret observed following supplementation of the media with heme shifted to 402 nm when cells were supplemented with Hb as the heme source. The shift in heme Soret suggests some heterogeneity within the binding cleft depending on how heme is delivered to the receptor. The Soret maxima of

holo-PhuR or HasR are not affected on cleavage of the 6× His-tag at the C-terminus of the protein, ruling out any contributions from the tags (data not shown).

### *In Vitro* Heme Reconstitution of the Purified HasR and PhuR Receptors

Attempts to load the purified HasR and PhuR receptors by titrating with heme were not successful. Incremental addition of heme to purified HasR or PhuR showed blue-shifted Sorets from that of the whole cell isolated holoproteins and precipitation on reaching stoichiometric levels (data not shown). This is in keeping with previous studies on the *S. dysenteriae* ShuA[12] and recent characterization of the *N. meningitidis* HmbR receptor.[15] The addition of free heme to the detergent-solubilized proteins is due, in part, to nonspecific binding to the protein surface. However, we were able to show that apo-HasR is functional to bind hemin-agarose (Figure 3A). We further showed that the apo-HasR without the His-tag was capable of binding to heme in agarose. We next incubated apo-HasR (10% residual heme) with holo-HasA (100% heme loaded) and observed no shift in the heme Soret from 406 to 411 nm, indicating that heme transfer from HasA to HasR was not observed (Figure 3B). This is in stark contrast to the *S. marcescens* HasR receptor that was previously reported to extract heme from HasA, as determined by a shift in Soret from 406 to 412 nm.[10] Furthermore, HasR was reported to coelute over Ni-NTA resin as a stable 1:1 complex with HasA. In contrast, elution of His-tagged *P. aeruginosa* apo-HasR following incubation with its cognate holo-HasA did not result in a stable HasA–HasR complex (Figure 3C).

As for the HasR receptor, apo-PhuR with or without the His-Tag was able to bind to hemin-agarose (Figure 4A). Apo-PhuR (<5% residual heme) following incubation with Hb showed no evidence of complex formation following Ni-NTA purification (Figure 4B). Due to the overlap in the Soret of metHb (405 nm) with that of holo-PhuR (405 nm), we analyzed the heme content of PhuR prior to and following elution of PhuR from metHb over Ni-NTA agarose (Figure 4C). The residual heme (<5%) bound to PhuR prior to incubation with metHb showed no increase following incubation. Although the heme Soret shifts to 412 nm for metHb on elution in 250 mM Im, it is evident that the isolated PhuR is not competent to extract heme from metHb (Figure 4C).

To confirm that the inability of HasR and PhuR to extract heme from their respective substrates was not due to structural integrity of the detergent-purified receptors, we obtained a CD spectrum of purified apo-HasR and PhuR (Figure 5). In keeping with the predicted 22-stranded $\beta$-barrel structure from sequence identity to OM heme receptors of known structure, the CD spectra are consistent with secondary structures dominated by $\beta$-sheet. The CD spectrum of HasR, in comparison to that of PhuR, appears to have a higher $\alpha$-helical content, consistent with the extended extracellular loops and N-terminal cell surface signaling domain that have a higher $\alpha$-helical content (Figure 5).[3] In contrast to previous reports, the present data suggests that the OM receptors do not passively extract heme from their respective heme substrates, requiring the TonB energy transducing system for heme extraction and transport.

## Absorption and MCD Spectroscopy of WT HasR and PhuR

To determine the spectroscopic signatures of HasR and PhuR in their physiological state, initial characterization was performed on the proteins isolated from heme-supplemented cultures. The holo-PhuR or holo-HasR protein was separated from its respective apoprotein fractions by ion exchange as described in the Experimental Procedures. Holo-HasR, similar to that from *S. marcescens*, has a Soret absorption peak at 411 nm with bands in the visible region at 533 and 558 nm, typical of a ferric (Fe(III)) low-spin (LS) bis-His-coordinated heme (Figure 2). However, the spectrum of holo-PhuR is distinct from that all previously characterized TonB-dependent OM heme receptors, with a heme Soret peak at 405 nm and a distinct high-spin (HS) marker band at 606 nm. Visible bands at 527 nm and a slight shoulder around 564 nm also suggest that there is a small LS heme fraction (Figure 2B). The spectrum of holo-PhuR is more in keeping with that of the previously characterized Tyr-coordinated hemes in ShuT[26] and HasA.[8,31,32] On further analysis, the conserved His within the FRAP/PNPL loop that provides the ligand to the heme in the bis-His OM receptors is not conserved in PhuR (Figure 1B). However, the PhuR FRAP/PNPL extracellular loop contains potential coordinating ligands in Tyr-519 and Tyr-529.

The HasR and PhuR receptors were further characterized by MCD. MCD spectroscopic signatures for hemeproteins correlate with the axial ligands and ligand field strength. Therefore, such signatures can be valuable in determining the heme coordination in unknown heme environments through comparison with structurally determined hemeproteins or porphyrin model complexes. The MCD spectrum of HasR compared to that of bis-histidine-coordinated Cytochrome $b_5$ shows similar features, although some differences in intensity are evident (Figure 6A). On the basis of the absorption and MCD spectrum, we conclude that holo-HasR is six-coordinate low-spin (6CLS). This is consistent with the previously characterized *S. marcescens* hemophore-dependent HasR, with which the *P. aeruginosa* receptor has significant homology.[10]

Given the potential Tyr ligation in PhuR, the MCD spectrum of holo-PhuR was compared to that of phenol-bound Fe(III)-leghemoglobin A. As shown in Figure 6B, the absorption and MCD spectra of Fe(III) holo-PhuR in both the Soret and visible regions compare closely with those of phenol-bound leghemoglobin A. The slightly larger trough intensities in the MCD spectrum of Fe(III) holo-PhuR at ~570 and ~420 nm suggest that PhuR is significantly more LS than leghemoglobin A. Taken together, the absorption and MCD spectra suggest that, in contrast to HasR, the heme in PhuR is coordinated by histidine and an anionic O-bound ligand.

## Spectroscopic Properties of the PhuR FRAP/PNPNL Tyr Loop Mutants

Given preliminary spectroscopic evidence that heme in PhuR is coordinated by a histidine and an anionic O-ligand, in combination with the fact that His-124 of the N-terminal plug provides a ligand to the heme (see below), we proposed that either Tyr-519 or Tyr-529 most likely provides the anionic O-ligand to the heme. Although His-530 was unlikely to be the coordinating ligand, we additionally constructed the H530A PhuR mutant.

The absorption spectrum of the Y529A and H530A PhuR mutant remained unchanged following purification over Ni-NTA resin (data not shown). In contrast, the PhuR Y519A mutant upon metal-affinity purification gave a red-shifted Soret peak at 412 nm with visible bands at 534 and 553 nm (Figure 7A). However, upon dialysis and removal of exogenous Im, the Soret maximum shifted to 406 nm with the reappearance of the HS marker band at 612 nm, features similar to the spectrum of the WT PhuR (Figure 7A). The coordination of an exogenous Im within the heme binding site of the Y519A mutant suggests that in the absence of Tyr-519 an exogenous ligand, in this case Im, can coordinate to the heme.

The absorption spectrum of the Y519A PhuR mutant resembles that of the WT PhuR, suggesting that a water ligand may coordinate in place of Tyr-519. However, further analysis of the Y519A PhuR mutant by MCD showed distinct differences, as evident from the decreased intensity of the troughs at 420 and 570 nm, indicating that Y519A PhuR is more HS than the WT protein (Figure 7B). Similarly, while the Y519A PhuR mutant showed some broad similarities to the Fe(III) 6CHS met-aqua myoglobin (Mb), distinct differences are observed (Figure 7C). The environment of the Fe(III) heme in Y519A PhuR in the absence of a protein-donated ligand from the extracellular loop is likely to be more solvent-exposed, which may account for the unusual spectroscopic signatures observed. Consistent with a more exposed heme binding cleft, titration of Y519A with cyanide ($CN^-$) did not result in a completely LS system (Figure 8A). Titration with $CN^-$ shifts the Soret band from 407 to 418 nm with a LS visible peak at 541 nm. Similarly, the MCD spectra showed a 2-fold increase in the Soret peak to trough intensities, consistent with the increase in LS character (Figure 8A). However, the HS charge transfer band around 610 nm in the absorption spectrum and the 630 nm trough in the MCD spectra are not completely lost even in the presence of excess $CN^-$.

In contrast to the Y519A mutant, the Y519H variant displays a Soret peak at 411 nm, with visible bands at 533 and 558 nm, and significant decrease in the HS marker feature at 610 nm (Figure 8A). The spectrum of the Y519H protein was unchanged following dialysis, suggesting that the heme is coordinated through endogenous His ligands, namely, His-124 on the N-terminal plug and His-519 on the extracellular loop. Consistent with bis-His ligation, the MCD spectrum of Fe(III) Y519H PhuR overlays well with that of Cytochrome $b_5$ and the bis-Im-coordinated H93G Mb (Figure 8B). Furthermore, the absorption and MCD spectra are similar to those of HasR reported herein and to the structurally characterized OM heme receptors that contain heme coordinated by two histidines.[10,12–14]

## Spectroscopic Characterization of the H124A N-Terminal Plug Mutant

To further confirm His-124 as the N-terminal heme ligand, the H124A mutant was constructed and characterized. The purified protein following dialysis has a blue-shifted Soret peak at 402 nm and a HS marker at 607 nm. The spectrum is reminiscent of that of tyrosine-ligated proteins, which typically exhibit characteristic Soret maxima around 400 nm with bands in the visible region close to ~500, ~520, and ~615 nm. Absorption and MCD comparison of the H124A mutant to those of the structurally characterized 5CHS Tyrligated holo-ShuT showed close similarities, with the exception of a decrease in the trough at ~540 nm (Figure 9).[26]

## DISCUSSION

The ability of bacterial pathogens to access and acquire iron is critical to their virulence and survival within the host. Central to this adaption is the ability to utilize heme, an abundant iron source within the host. *P. aeruginosa* encodes two OM receptors in order to maximize its ability to acquire heme under a wide range of physiological conditions. In an effort to understand the contributions of PhuR and the hemophore-dependent HasR to *P. aeruginosa* heme acquisition, we report the purification and characterization of the OM receptors. As previously reported for the *S. marcescens* HasR receptor, the *P. aeruginosa* HasR and PhuR receptors were purified partially heme-loaded.[10] Furthermore, the *S. marcescens* HasR receptor was reported to form a stable complex with the hemophore HasA and transfer heme from HasA to HasR. In the current studies, neither HasR nor PhuR was competent in extracting heme from HasA or metHb, respectively. The inability to extract heme from HasA or metHb was not due to diminished structural integrity of the receptors, as judged by the CD spectra and the ability of the receptors to bind to hemin-agarose. Additionally, incubation of HasR with HasA or PhuR with metHb did not result in the formation of a stable protein–protein complex. We cannot reconcile these results with those for the *S. marcescens* HasA–HasR system, although the present data is consistent with the substoichiometric heme levels and inability to extract heme from metHb reported for the heterologous expressed OM heme receptors ShuA and HmbR of *S. dysenteriae* and *N. meningitides*, respectively.[10,15] Conflicting data on the isolated receptors is further complicated by the fact that previous in vivo reports on the *S. marcescens* system determined that heme release from HasA to HasR required interaction with the TonB-dependent energy transducing system.[33] Our current studies are consistent with the latter observation that the TonB-dependent energy transducing system is critical for heme extraction from the hemeprotein substrate.

*In vitro* heme reconstitution of the purified receptors has proven to be remarkably challenging for several reasons. First, the addition of free heme to the purified OM receptor is complicated by the hydrophobicity of the protein and the presence of detergent, often yielding spectra more consistent with nonspecific binding of heme to the protein or precipitation.[12,15] Second, heme binding at the OM is likely to depend on specific interactions of the receptor with its cognate hemeprotein substrate as well as the TonB-dependent energy transducing system. In order to obtain information on the physiologically relevant heme binding state of the OM receptors, spectroscopic characterization of the HasR and PhuR wild-type and mutant receptors was performed on the as-isolated proteins following heme and hemoglobin supplementation of the cultures. Site-directed mutagenesis and spectroscopic characterization of the isolated receptors following heme supplementation confirmed that PhuR coordinates heme through the N-terminal plug His-124 and Tyr-519 on the FRAP/PNPL extracellular loop. This is in contrast to almost all previously characterized OM receptors, including the structurally characterized *S. marcescens* HasR, which have a bis-His coordination.[14] An exception to the bis-His motif is the recently characterized OM heme receptor of *N. meningitides*, HmbR, that has a 5CHS heme bound through a Tyr residue.[15] Interestingly, the conserved His found in the N-terminal plug of all previously characterized OM receptors is absent in HmbR and is replaced with a Tyr. Similarly, within

the HmbR FRAP/ PNPL-loop, there are potential heme ligands, including Tyr-519, Tyr-522, and His-524. Although the authors did not confirm if the heme-coordinating Tyr residue is contributed from the N-terminal plug domain or the FRAP/PNPL-loop, resonance Raman and EPR data are conclusive for a protein in which the proximal heme ligand is the phenol side chain of Tyr.[15] Interestingly, Tyr ligation has emerged as a common motif across a spectrum of high-affinity heme acquisition proteins and systems. These include the secreted *S. marcescens* and *P. aeruginosa* HasA hemophores,[8,31] lipid-anchored surface-exposed Isd heme binding proteins of Gram-positive pathogens,[34–36] and the soluble periplasmic binding proteins in Gram negatives.[26,37] Recently, Rivera and co-workers, in a series of structural and spectroscopic studies of *P. aeruginosa* HasA, have proposed a model whereby heme binding is driven largely by hydrophobic and $\pi$–$\pi$ stacking interactions, with the heme affinity being attributed to a slow off rate. The axial Tyr ligation is stabilized by hydrogen-bond interactions from a neighboring residue, such as a His in the HasA hemophores,[8,31,32] a Tyr in the Isd heme receptors,[34–36] or an Arg in the case of the periplasmic binding proteins.[37] Heme release occurs on binding of the protein to its cognate receptor and causes a conformational rearrangement and disruption of the Tyr-ligand hydrogen bond that triggers heme transfer.[32,38] The fact the *S. marcescens* HasR in the absence of HasA is less efficient in acquiring heme would suggest that the emergence of the His-Tyr motif in surface-exposed heme receptors provides an advantage in the scavenging and acquisition of heme over that of the bis-His-coordinated OM receptors.

It is difficult to directly attribute heme uptake efficiency by PhuR or HasR to the thermodynamic and kinetic parameters of heme binding to the purified receptors due to the difficulties in working with the purified receptors and the absence of the TonB-energy transducing system. Despite the difficulties in translating such *in vitro* observations to the relative roles of PhuR and HasR in heme uptake, several *in vivo* lines of evidence support PhuR being the primary heme receptor in infection. First, *phuR* is divergently transcribed from the same promoter as *phuSTUV* genes that encode all of the components required for translocation of the heme into the cell. In contrast, the hemophore-dependent HasR requires the *phu* encoded periplasmic ABC-transporter PhuT-UV for translocation of heme into the cytoplasm. Also, it has recently been reported that *P. aeruginosa* clinical isolates are more efficient at metabolizing extracellular heme and do so as a result of upregulation of the PhuR receptor.[22,39] Finally, mutations in the promoter of PhuR that lead to increased expression also coincide with the loss of pyoverdine biosynthesis and increased dependence on heme.[22] We have recently addressed the contributions of PhuR and HasR to heme uptake and utilization through *in vivo* bacterial genetic and $^{13}$C-heme isotopic labeling studies.[43] The phuR deletion strain was unable to utilize heme as efficiently as the WT strain, despite a ~10-fold increase in HasR protein levels. In contrast, on deletion of *hasR*, the bacteria were able to utilize heme as well as that of the wild-type strain, with no significant increase in PhuR protein levels. The *in vivo* studies suggest that the PhuR receptor is the major heme transporter in *P. aeruginosa*, with the HasA–HasR ECF system functioning primarily as a heme sensor for the activation of heme uptake and utilization. The present studies along with our recent finding that PhuR is the major heme transporter in *P. aeruginosa* support the growing consensus within the literature that hydrogen-bond-stabilized Tyr coordination is a conserved motif in bacterial heme acquisition systems. Further insight into the mechanism

of heme acquisition and trans-location by the His-Tyr coordinated PhuR receptor will require spectroscopic, structural, and *in vivo* translocation studies presently underway.

## ACKNOWLEDGMENTS

We thank Dr. Mario Rivera for the gift of the *P. aeruginosa* HasA expression plasmid and Dr. Masanori Sono for helpful discussions with the MCD analysis.

Funding

This work was supported by the NIH (AI85535 and AI102883 to A.W. and GM26730 to J.H.D.).

## ABBREVIATIONS

| | |
|---|---|
| **phu** | *Pseudomonas* heme uptake |
| **has** | heme assimilation system |
| **HemO** | heme oxygenase |
| **OM** | outer membrane |
| **Amp** | ampicillin |
| **LB** | Luria–Bertani |
| **MCD** | magnetic circular dichroism |
| **Im** | imidazole |
| **Isd** | iron surface determinant |
| **LS** | low spin |
| **HS** | high spin |
| **Ni-NTA** | Ni-nitriloacetic acid |

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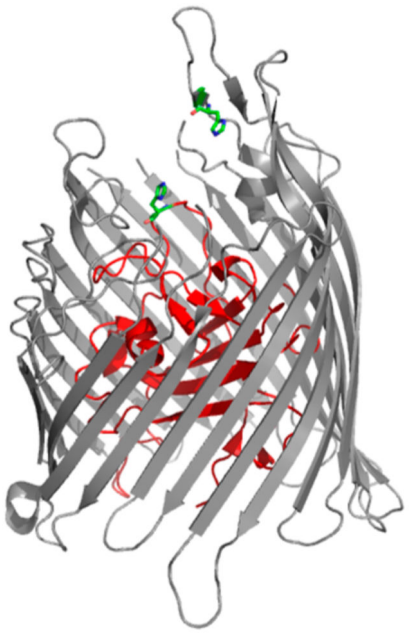
A.



B.

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Ye HemR     443 YAQAFRAPTMGEMYNDSKHFSMNIMGNTLTNYWVPNPNLKPETNETQEY 491
Sd ShuA     430 YAQAFRAPTMGEMYNDSKHFSIGRFYTNYWVPNPNLRPETNETQEY 475
Vc HutA     456 ISQGFRAPDFQELYYSFGNPAHGYVFKPNPNLEAEDSVSYEL 497
Pg HmuR     416 YAEGYRAPSLQEMYFFFNHGAFFIYGNPDLKPEKSRMLSY 455
Sm HasR     610 YGKSWRPPAITETLTNGSAHSSSTQYPNPFLQPERSRAWEV 658
Pa HasR     605 YGKGWRPPAVTESLITGRPHGGGAENMYPNPFLSPERSKAWEV 647
Pa PhuR     506 YAQGFRTPTAKALYGRFENLQAGYHIEPNPNLKPEKSQSFET 547
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**Figure 1.**

(A) Structure of apo-ShuA showing the N-terminal plug (red) and $\beta$-barrel (gray). The heme-coordinating residues His-86 (plug) and His-420 (extracellular loop, L7) are shown as sticks. Image generated from PDB file 3FHH in PyMOL.[40] (B) Sequence alignment of the heme-coordinating extracellular loop of known heme recpetors. Yp, *Yersinia pestis*; *Ye*, *Yersinia entercolitica*; Sd, *Shigella dysenteriae*; Vc, *Vibrio cholerae*; Pg, *Porphyromonas gingivalis*; Sm, *Serratia marcescens*; and Pa, *Pseudomonas aeruginosa*.

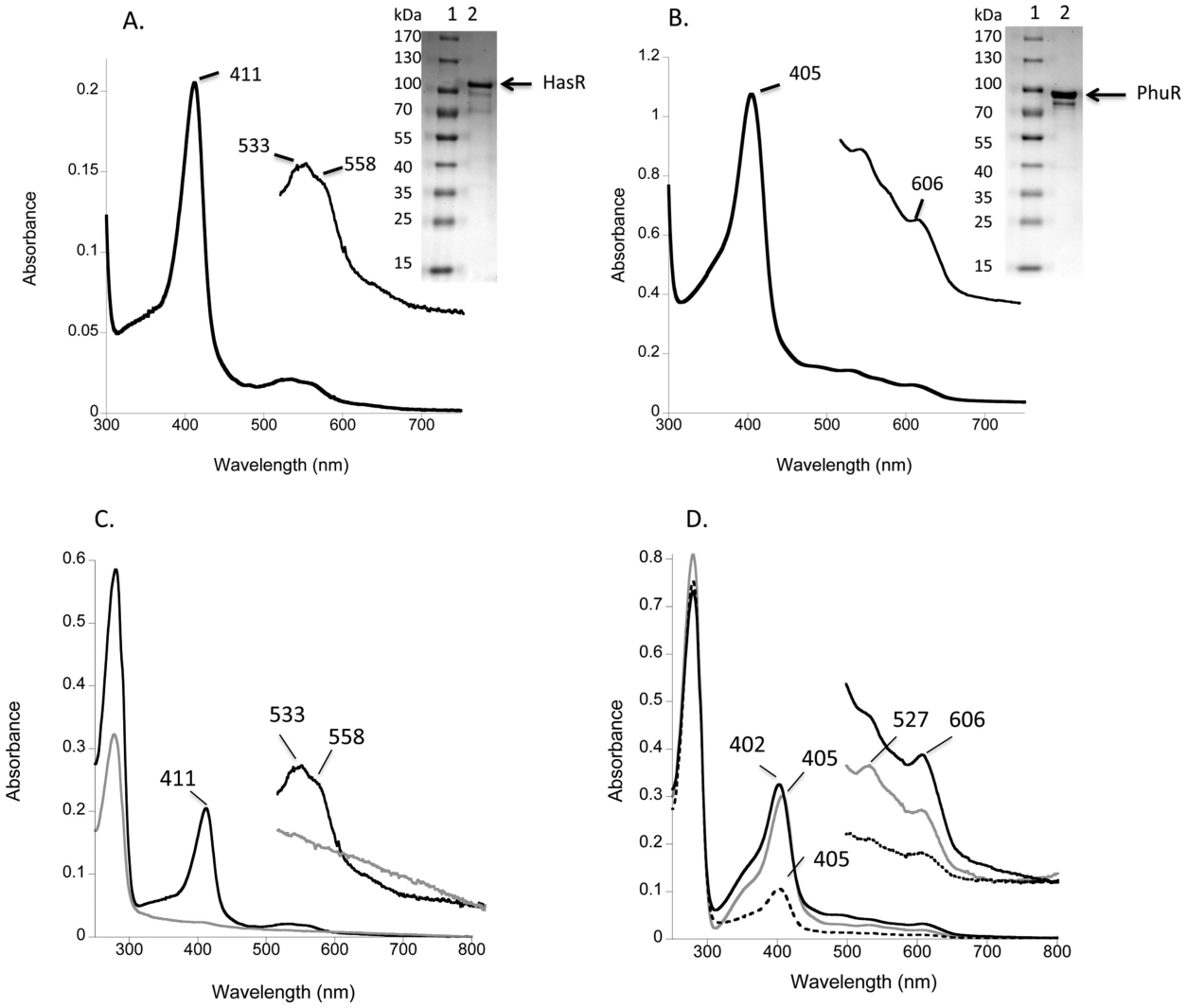


**Figure 2.**

Purification of HasR and PhuR from cultures supplemented with heme or Hb. (A) Absorption spectrum of the purified partially heme-loaded Fe(III) holo-HasR receptor (5 $\mu$M). Inset: SDS-PAGE of purified HasR. Lane 1, molecular weight markers; Lane 2, 10 $\mu$g of purified HasR. (B) Absorption spectrum of the purified partially heme loaded PhuR receptor (20 $\mu$M). Inset: SDS-PAGE of purified PhuR. Lane 1, molecular weight markers; Lane 2, 10 $\mu$g of purified HasR. (C) Spectra of HasR purified from cultures supplemented with (black line) or without (gray line) 10 $\mu$M heme. (D) PhuR purified from nonsupplemented cultures (black dashed line), cultures supplemented with 10 $\mu$M heme (gray solid line), or cultures supplemented with 10 $\mu$M Hb (black solid line). All visible region 500–800 nm insets were magnified 5×.

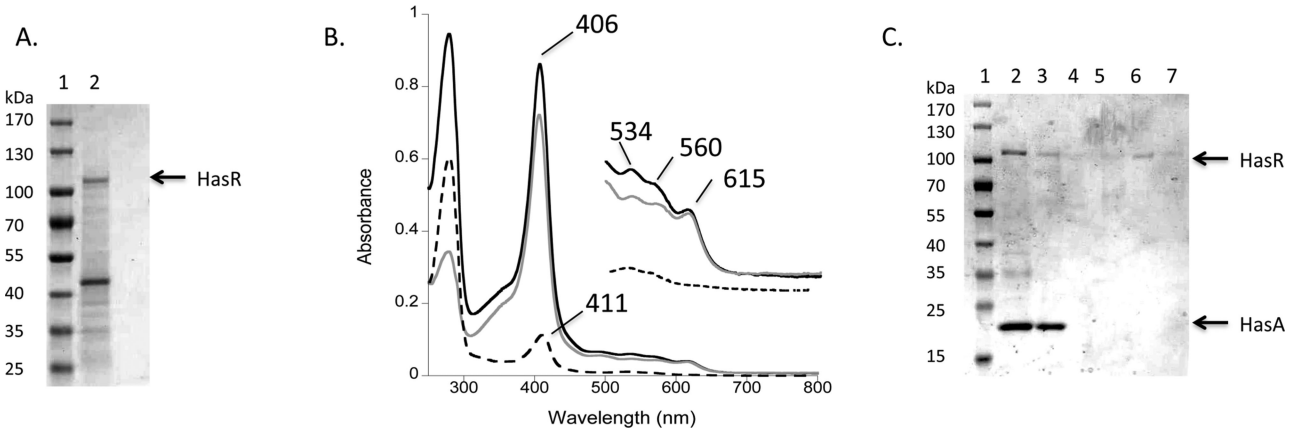


**Figure 3.**

Heme binding properties of purified HasR. (A) Apo-HasR (10 $\mu$M) following elution from hemin-agarose in SDS-loading Buffer (50 $\mu$L). HasR (10 $\mu$L) was analyzed on a 12% SDS-PAGE gel. (B) Spectra of 5 $\mu$M apo-HasR (black dashed line) prior to incubation with holo-HasA, 4 $\mu$M holo-HasA (gray solid line), holo-HasA, and apo-PhuR following incubation (black solid line). Inset: 5× magnification of the visible region, 500–800 nm. (C) Elution of HasR (5 $\mu$M) from Ni-NTA resin following incubation with HasA (4 $\mu$M). Lane 1, molecular weight markers; Lane 2, Ni-NTA flow through; Lane 3, wash in 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5 mM Im; Lane 4, wash in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 20 mM Im; Lanes 5–7, elution in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and 250 mM Im.

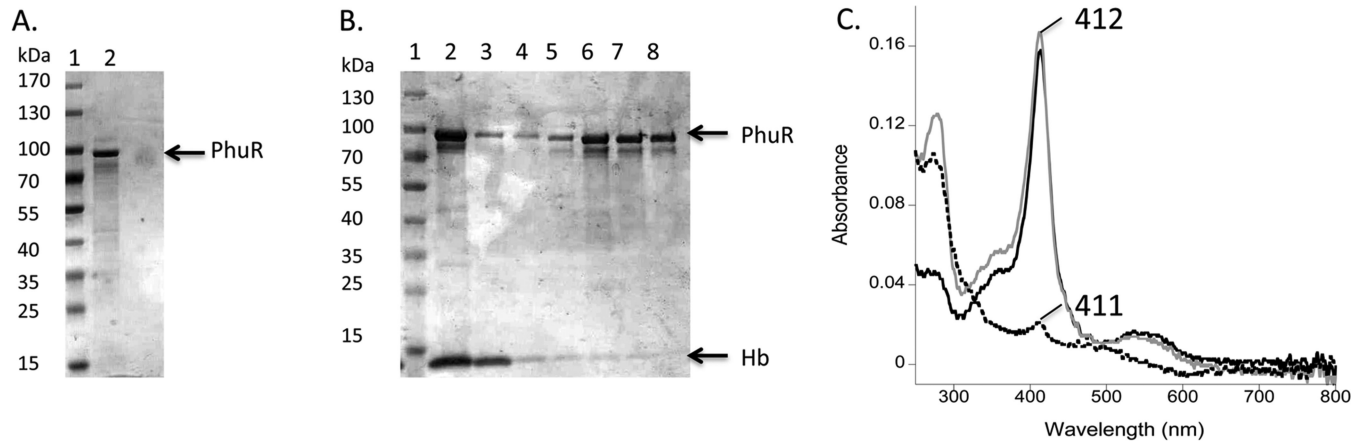


**Figure 4.**

Heme binding properties of PhuR. (A) Apo-PhuR (10 $\mu$M) following elution from hemin-agarose in SDS loading Buffer (50 $\mu$L). PhuR (10 $\mu$L) was analyzed on a 12% SDS-PAGE gel. (B) Elution of PhuR (5 $\mu$M) from Ni-NTA resin following incubation with metHb (4 $\mu$M). Lane 1, molecular weight markers; Lane 2, apo-PhuR and metHb prior to loading; Lane 3, Ni-NTA flow through; Lane 4, wash in 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5 mM Im; Lane 5, wash in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 20 mM Im; Lanes 6–8, elution fraction in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and 250 mM Im. (C) Spectra of metHb with apo-PhuR in 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5 mM Im (gray solid line), wash fraction in 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5 mM Im (black solid line), and elution fraction in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and 250 mM Im (black dashed line).

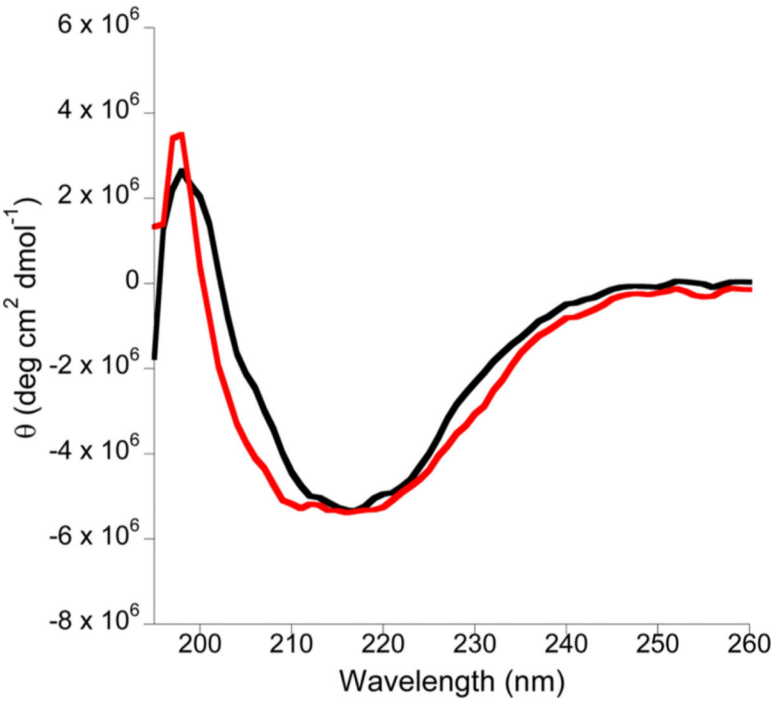


**Figure 5.**

CD spectra of holo-HasR and holo-PhuR proteins. Spectra of holo-HasR (red line) and holo-PhuR (black line) were recorded at 25 °C in 20 mM potassium phosphate (pH 7.5) and 50 mM NaCl containing 0.07% LDAO at a final protein concentration of 5 $\mu$M.

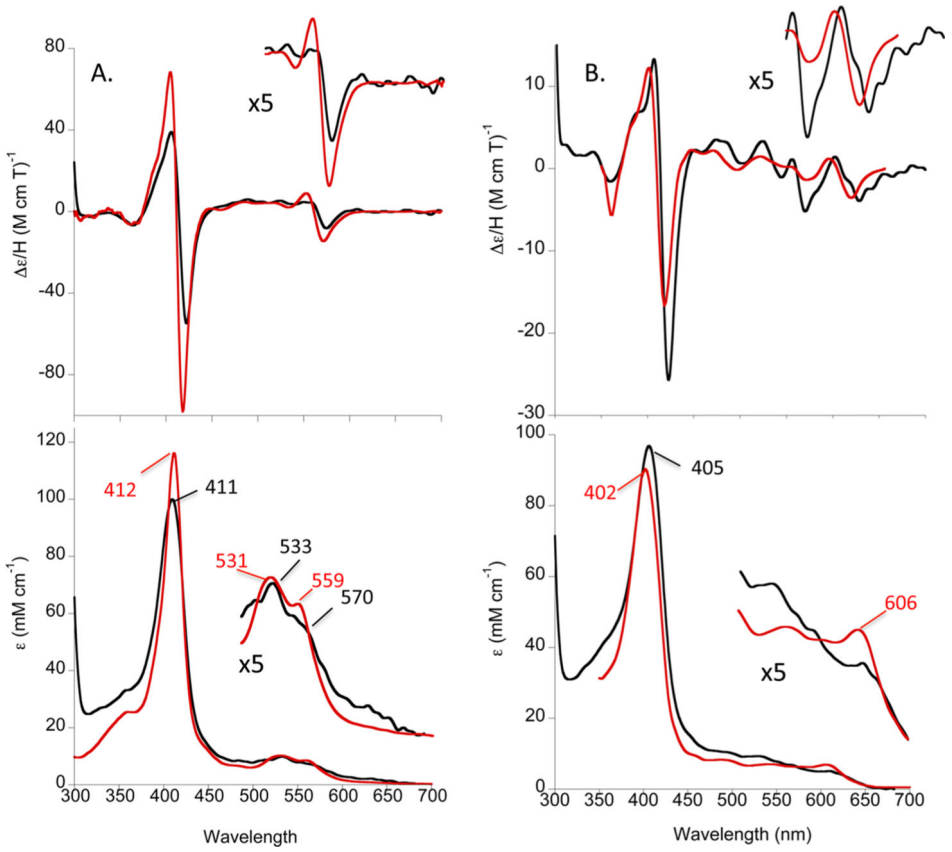


**Figure 6.**

Absorption and MCD spectra of the Fe(III) holo-HasR and PhuR receptors. (A) Absorption (lower panel) and MCD (upper panel) of HasR (black line) recorded at 4 °C in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.07% LDAO and Fe(III) Cytochrome $b_5$ (red line) in 50 mM potassium phosphate (pH 7.0). The Cytochrome $b_5$ spectrum was replotted from ref 41. (B) Conditions as in (A) were used for PhuR (black line) and phenol-bound leghemoglobin A (red line).

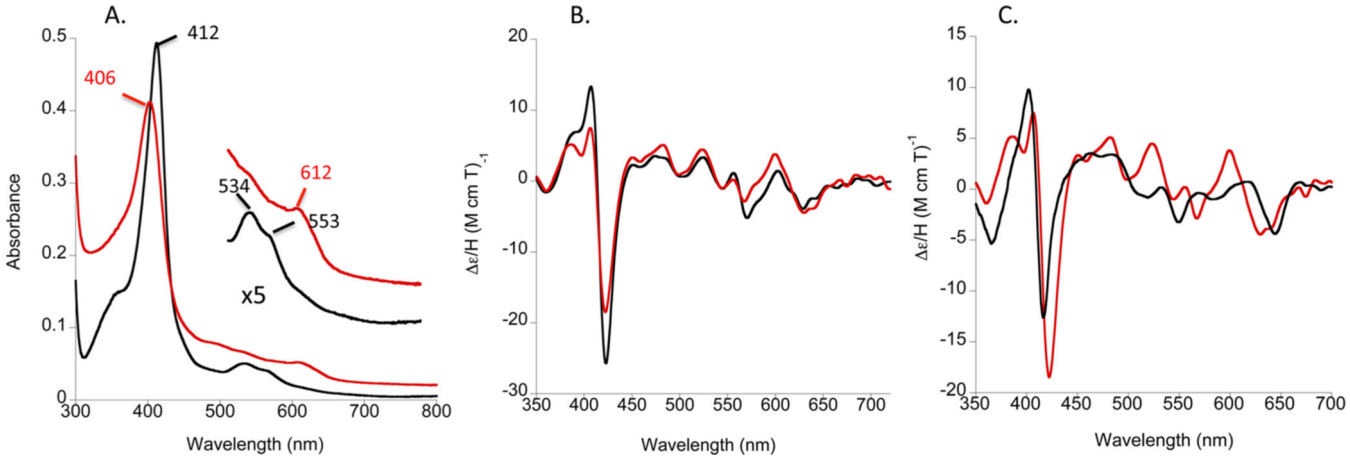


**Figure 7.**

Absorption and MCD spectra of the PhuR Y519A mutant. (A) Absorption spectra of imidazole-coordinated PhuR Y519A (8.5 $\mu$M) (black line) and following dialysis (red line) at 4 °C in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.07% LDAO. (B) MCD spectra of the wild-type (black line) and Y519A (red line) PhuR as in (A). (C) MCD spectra of Fe(III)-Mb (black line) in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and Y519A PhuR (red line) under the same conditions as those in (B).

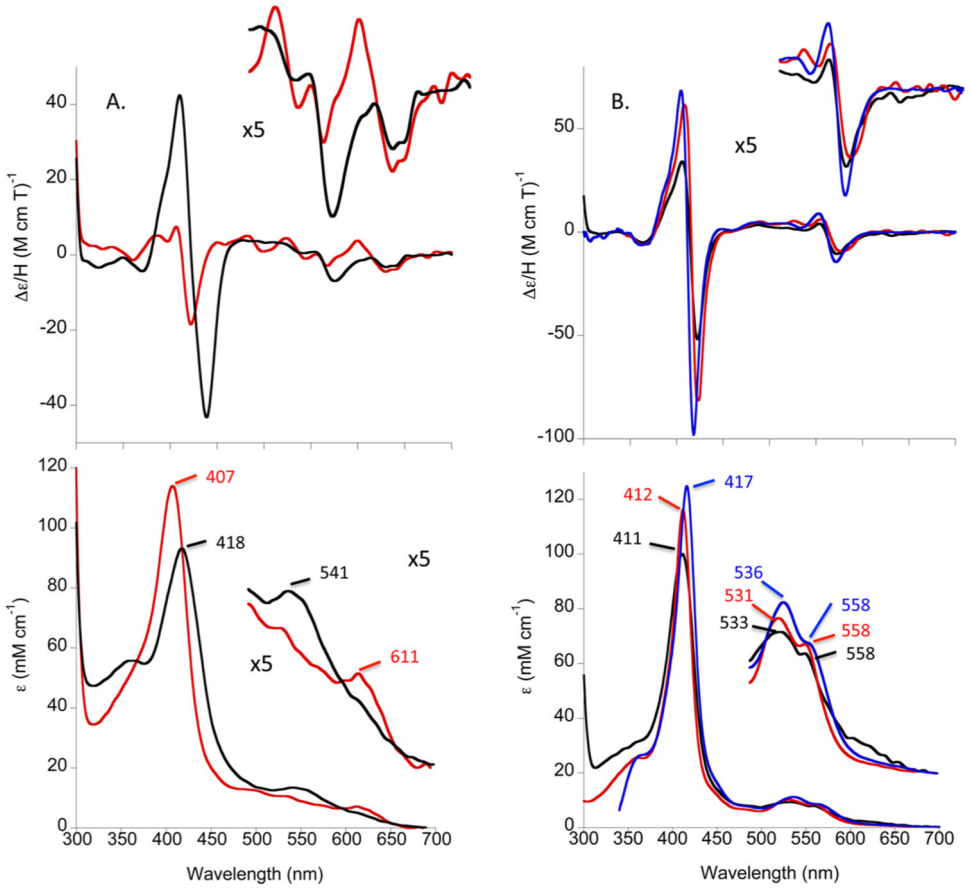


**Figure 8.**

Absorption and MCD spectra of the PhuR Y519A and Y519H mutants. (A) Absorption (lower panel) and MCD (upper panel) of PhuR Y519A (red line) and Y519A-CN (aliquots of 1 mM KCN until apparent saturation) (black line) at 4 °C in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.07% LDAO. (B) Absorption and MCD spectra of PhuR Y519H (black line), Fe(III) Cytochrome $b_5$ (red line), and Fe(III) Mb H93G–bis-Im complex (1 mM Im) (blue line) in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl. The Fe(III) Mb H93G–bis-Im complex spectrum was replotted from ref 42. Copyright 2011 American Chemical Society.

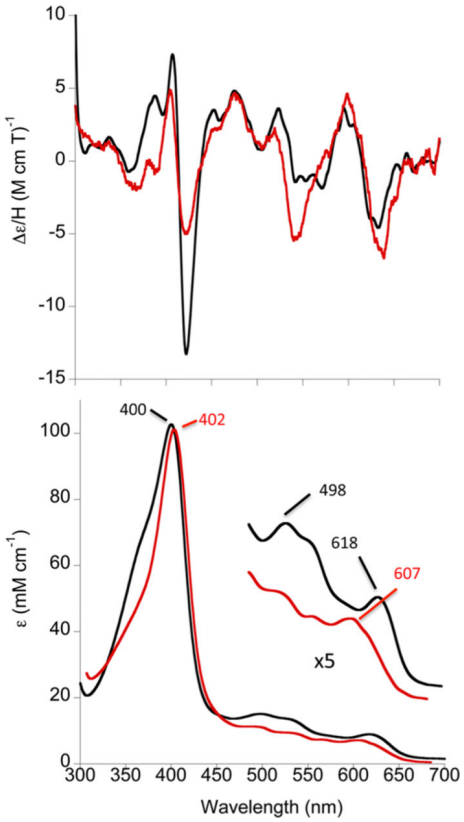


**Figure 9.**

Absorption and MCD spectra of the PhuR H124A mutant. Absorption (lower panel) and MCD (upper panel) of PhuR H124A (red line) and holo-ShuT (black line) at 4 °C in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.07% LDAO in the case of the PhuR H124A receptor.