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The Asp99-Arg188 Salt Bridge of the *Pseudomonas aeruginosa* HemO is Critical in Allowing Conformational Flexibility During Catalysis.

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Abstract

The *P. aeruginosa* iron-regulated heme oxygenase (HemO) is required within the host for the utilization of heme as an iron source. As iron is essential for survival and virulence HemO represents a novel antimicrobial target. We recently characterized small molecule inhibitors that bind to an allosteric site distant from the heme pocket, and further proposed binding at this site disrupts a nearby salt bridge between D99 and R188. Herein, through a combination of site-directed mutagenesis and hydrogen-deuterium exchange mass spectrometry (HDX-MS), we determined disruption of the D99-R188 salt-bridge leads to significant decrease in conformational flexibility within the distal and proximal helices that form the heme binding site. The RR spectra of the resting state Fe(III) and reduced Fe(II)-deoxy heme-HemO D99A, R188A and D99/R188A complexes are virtually identical to those of wild type HemO, indicating no significant change in the heme environment. Furthermore, mutation of D99 or R188 leads to a modest decrease in the stability of the Fe(II)-O₂ heme complex. Despite this slight difference in Fe(II)-O₂ stability we observe complete loss of enzymatic activity. We conclude the loss of activity is a result of decreased conformational flexibility in helices previously shown to be critical in accommodating variation in the distal ligand and the resulting chemical intermediates generated during catalysis. Furthermore, this newly identified allosteric binding site on HemO represents a novel alternative drug design strategy to that of competitive inhibition at the active site or via direct coordination of ligands to the heme iron.

Graphical Abstract

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GH generated, purified and characterized the D99 and R188 mutants in the WT and HemO_α background. WH performed all of the HDX-MS experiments. ER performed the in vitro activity assays. PML performed and interpreted the resonance Raman experiments. GH, WH, AW and PML wrote the manuscript. All authors contributed to final editing of the manuscript and have given approval to the final version of the manuscript.

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Keywords

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Introduction

Canonical heme oxygenases (HO) catalyze the oxidative cleavage of heme to biliverdin (BVIX), and have been identified in animals [1-6] plants [7, 8], and bacteria [9-12], where they play a central role in iron recycling [13], antioxidant defense [14], signaling [15], iron acquisition [9-11] and synthesis of light-sensing bilins [12, 16], respectively. While non-canonical HO's have recently been identified in Gram-positive pathogens the overall structure and oxidative cleavage of heme is distinct from that of the canonical HO's [17-19]. The canonical HO's in Gram-negative pathogens and higher eukaryotes display an overall similar helical fold, where heme is bound between the proximal (I and II) and distal helices (VII and VIII), respectively (Fig 1A) [20, 21].

Over the past two decades many groups have contributed to the mechanistic understanding of oxidative heme cleavage to biliverdin IX α (BVIX α) with the concomitant release of Fe³⁺ and carbon monoxide (CO_(g)) [22-31]. Through these studies several reaction intermediates have been spectroscopically and structurally characterized, including the reduced oxy (Fe²⁺-O₂) species, the ferric hydroperoxide (Fe³⁺-OOH) intermediate, α -meso-hydroxyheme, α -verdoheme, and the terminal product biliverdin [32-35].

In contrast to all other canonical HO's the oxidative cleavage of heme by the *Pseudomonas aeruginosa* HemO leads to the formation of BVIX β and BVIX δ [9]. The altered regioselectivity was shown to be a consequence of a 90° in-plane rotation of the heme within the binding pocket, mediated by alternate interactions of the heme propionates with the protein scaffold [21]. However, as for all HO's a highly-ordered network of water molecules within the heme-binding pocket is required for catalytic activity [20, 21, 36]. The structural water network is critical in providing the hydrogen bond network required for stabilizing the Fe(II)-O₂ ligand, and in providing the proton relay in generating the activated Fe(III)-OOH intermediate [37-39]. Previous H/D NMR studies of the HemO WT Fe(III)-CN, Fe(III)N₃ [40] and HemO R80L Fe(III)-CN and Fe(II)-CO complexes [41] concluded the structural water network is integral to long-range communication and conformational flexibility in helices distant from the active site. The authors proposed such conformational plasticity is required for accommodating changes in axial ligand coordination and chemical intermediates during catalysis. Therefore, disruption of conformational flexibility and/or the

structural water network, offers an alternative drug design strategy in addition to directly competing with heme binding at the active site, or by coordination to the heme iron [42, 43].

In keeping with this hypothesis, we have recently shown by HDX-MS that a series of iminoguanidine compounds inhibit HemO by binding to a newly-discovered site on the back side of the protein rather than through competitive inhibition at the active site [44]. In these studies Site-Identification by Ligand Competitive Saturation (SILCS) analysis [45] highlighted a binding site in close proximity to R188, which forms a salt bridge with D99 connecting helix XII with the loop connecting helices VI and VII on the back side of HemO [44]. Interestingly, these helices have been implicated in long range motions required to accommodate changes in the distal ligand and chemical intermediates generated during catalysis. While computational and biophysical characterization of the ligand-protein interaction identified the binding site of the iminoguanidine inhibitors, the mechanism of HemO inhibition has not been elucidated [44]. We hypothesized based on the proximity of the iminoguanidine inhibitor binding site to the D99-R188 salt bridge that disruption of the salt-bridge on binding may result in changes in conformational flexibility and/or a disruption of the structural water network. To further probe this hypothesis, we undertook a site-directed mutagenesis approach creating the D99A, R188A and D99A/R118A HemO mutant proteins. Biochemical and spectroscopic analysis of the D99 or R188 to Ala mutant proteins show that despite retention of the overall structural fold, the D99A and R188A mutants show significant changes in conformational flexibility within distal helices and those involved in direct hydrogen bonding interactions with the active site structural water network. Furthermore, the decrease in conformational dynamics of the active site helices in the D99 and R188 mutant proteins results in a complete loss of enzyme activity. The data is consistent with previous H/D-NMR studies that concluded long-range communication of the active site hydrogen bond network with helices distant from the active site is critical for conformational flexibility during catalysis [40, 41]. Furthermore, the current studies provide a plausible mechanism of action for the iminoguanidine inhibitors, and offers an attractive alternative approach to competitive inhibition of heme binding at the active site of HemO.

Materials and Methods

Bacterial strains.

Genetic engineering and plasmid replication were performed in *Escherichia coli* strain DH5 α (F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* ϕ 80d*lacZ* *M15* (*lacZYA-argF*) U16, hsdR17(*r_K⁻m_K⁺*), λ^-). HemO WT and mutant protein expressions were performed in *E. coli* strain BL21 (DE3) (B F⁻ *ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)* λ (DE3 [*lacI lacUV-T7p07 ind1 sam7 nin5*])) [*malB⁺*]_{K-12}(λ^S)).

Site-directed mutagenesis.

HemO mutants were constructed in the pET21a vector used previously to express HemO [9, 44]. Primers were synthesized by Sigma and mutations were introduced by PCR using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. Primers used to introduce the D99A mutation were 5'-GTACCGGAGGGCGCGCAGAGCGTGCGCG-3' and 5'-CGCGCACGCTCTGCGCGCCCTCCGGTAC-3';

primers used to introduce the R188A mutation were 5'-CGCCAGCGATGCCTTCAATGCTTTCGGCGACC-3' and 5'-GGTCGCCGAAAGCATTGAAGGCATCGCTGGCG-3': the underlined codons represent the introduced mutations. Isolated plasmids (pET21a) containing the mutations were transformed into *E. coli* Dh5 α cells for replication and into BL21 (DE3) cells for overexpression. The BVIX α regioselective mutant of HemO (HemO α) (N19K/K34A/F117Y/K132A) incorporating the D99A mutation, R188A mutation, or both were constructed in a similar manner using the same primers with the gene cloned into the pBAD33 vector [46].

Protein expression and purification.

The HemO WT and the respective D99 and R188 variants were expressed and purified as previously described [9, 44]. Briefly, an overnight culture of transformed BL21 (DE3) cells grown in Luria-Bertani broth (LB) and treated with 30 μ g/mL kanamycin was used to inoculate 4 1-liter cultures at OD₆₀₀ = 0.05. Cultures were treated with 30 μ g/mL kanamycin and incubated at 37°C with shaking until OD₆₀₀ = 0.6. Expression was induced with 1 mM isopropyl-D-thiogalactopyraniside (IPTG) and cells continued shaking at 25°C for four hours. Cells were pelleted at 12,000 *g* for 12 min and pellets were stored at -80°C until purification. For purification, pellets were thawed on ice and lysed in 50 mL lysis buffer (50 mM tris, pH = 8.0, 50 mM NaCl, 100 μ M phenylmethanesulfonyl fluoride (PMSF), lysozyme, DNase I, protease inhibitor cocktail (complete mini, Roche Diagnostics)) for 1 h. Lysate was sonicated then centrifuged at 21,000 *g* at 4 °C for 1 h. The resulting supernatant was applied to a Ni-NTA column pre-equilibrated with 50 mM tris (pH = 8.0), 300 mM NaCl, 2 mM imidazole. Column was washed with 50 mM tris (pH = 8.0), 300 mM NaCl, 20 mM imidazole, and IFP1.4-His₆ was eluted with 50 mM tris (pH = 8.0), 300 mM NaCl, 250 mM imidazole in 10 mL fractions. Fractions were analyzed by SDS-PAGE and fractions containing IFP1.4-His₆ were pooled and exhaustively dialyzed against 20 mM tris (pH = 8.0). SDS-PAGE and UV-vis analysis determined protein was sufficiently pure and concentrated for use. Expressions typically yielded 1.8 mg protein per 1 L culture.

UV-vis spectroscopy.

Purified HemO (WT or mutants) was incubated with 1.5 equivalents of heme from freshly prepared hemin solution in 0.1 M NaOH brought up with 20 mM sodium citrate buffer (pH = 7.0). Excess hemin was removed by passage of the holo-HemO protein over a Q-Sepharose column (1.5 cm $\times$ 1.0 cm) and the holo-HemO eluted with 250 mM NaCl in 20 mM sodium citrate buffer (pH = 7.0). Holo-proteins were diluted to 10 μ M (using ϵ_{280} = 13980 M⁻¹ cm⁻¹ for apo-HemO) into 20 mM sodium citrate buffer (pH = 7.0) and absorption spectra were recorded on an Agilent Cary 300 UV-vis spectrophotometer. Extinction coefficients were determined using the pyridine hemochrome method (ϵ_{418} = 170 mM⁻¹ cm⁻¹; ϵ_{525} = 17.5 mM⁻¹ cm⁻¹; ϵ_{555} = 34.4 mM⁻¹ cm⁻¹) [47]. For heme titration experiments, proteins were diluted to 15 μ M in 20 mM sodium citrate buffer (pH = 7.0). Hemin was prepared as described above and added in 0.5 μ M increments to final concentrations of 25 μ M. Heme binding was analyzed by the increase in the Soret band (405 nm). The ferrous oxy (Fe(II)-O₂) and CO (Fe(II)-CO) HemO complexes were obtained following saturation with CO(g) and addition of excess sodium dithionite. The absorption

spectrum of the Fe(II)-CO HemO complex was obtained following dilution in CO_(g) saturated 20 mM sodium citrate buffer (pH = 7.0). Fe(II)-O₂ HemO complexes were generated by passage over a Sephadex G-25 desalting column (1.5 cm × 5 cm) to remove excess reductant. The Fe(II)-O₂ HemO complexes were analyzed immediately following elution in 20 mM sodium citrate buffer (pH = 7.0). The decay and stability of the Fe(II)-O₂ HemO D99A and R188A complexes were monitored by recording the spectra at 1 min intervals for 60 min. The rate of decay was analyzed by fitting the visible α -band at 541 nm to an exponential decay model and the half-life was determined using initial spectra as 100% oxy-complex and final spectra as 0% oxy-complex.

Circular dichroism.

Purified apo- and holo-HemO (WT or mutants) was buffer exchanged into 50 mM sodium phosphate (pH = 7.2) and diluted to 2.5 μ M (using $\epsilon_{280} = 13980 \text{ M}^{-1} \text{ cm}^{-1}$ for apo-HemO) in the same buffer. Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter using a JASCO PFD-425S temperature controller. Spectra were recorded at 25°C from 180 nm to 260 nm in triplicate, and then spectra were averaged and smoothed. Each protein was analyzed three times. For determination of melting temperature (T_m), temperature was increased from 15 to 90°C at a rate of 1°C per min, and proteins were monitored at 222 nm. T_m was determined by analysis of linear range of unfolding and solved for 50% folded, using 15°C as 100% folded and 90°C as 0% folded.

Fluorescence spectrophotometry.

Heme binding affinities of the WT and mutant HemO mutants were determined by fluorescence quenching as previously described. Briefly, fluorescence experiments were performed on a Synergy HI hybrid microplate reader using flat-bottom black 96-well plates. Protein concentration was 1 μ M in 20 mM Tris-HCl (pH = 8.0). Freshly prepared heme was added to final concentrations of 0.05-100 μ M. Solutions were excited at 295 nm and emission spectra were recorded from 300 to 400 nm. Decrease in maximum emission (332 nm) was fit to a one-site binding model as a function of increasing heme concentration.

Resonance Raman.

RR spectra were obtained using a custom McPherson 2061/207 spectrograph (0.67 m with variable gratings) equipped with a Princeton Instruments liquid N₂-cooled CCD detector (LN-1100PB). Excitations at 407 and 442 nm were provided by a krypton laser (Innova 302, Coherent) and a helium-cadmium laser (Liconix 4240NB). Kaiser Optical edge filters were used to attenuate Rayleigh scattering. Spectra were collected at room temperature using a 90° scattering geometry on samples mounted on a reciprocating translation stage. Frequencies were calibrated relative to indene and CCl₄ and are accurate to $\pm 1 \text{ cm}^{-1}$; CCl₄ was also used to check the polarization conditions. The integrity of the RR samples, before and after laser illumination, was confirmed by direct monitoring of their UV-vis spectra in the Raman capillaries. All RR measurements were conducted on $\sim 100 \mu$ M protein solutions in 20 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, and 10 % glycerol. The reduced protein samples were prepared by addition of sodium dithionite (3 mM final concentration) under anaerobic conditions.

In vitro HemO enzyme activity measurements.

Turnover of the holo-HemO WT or mutant proteins was performed in the presence of the NADPH-dependent ferredoxin reductase (*pa*-FPR) and NADPH as described previously [48]. Briefly, reactions were monitored spectrophotometrically in a 1-cm path length cuvette. The reactions contained 8-10 μ M HemO, D99A or R188A mutants, 20 μ M FPR in a final volume of 1 ml. Catalase (final concentration of 0.1 mg/ml) was used as a H₂O₂ scavenger. The reactions were initiated on addition of NADPH at a final concentration in the cuvette of 200 μ M. Heme turnover was monitored between 300-800 nm with repeated scanning every 30 secs for 30 min. To obtain the spectrum of iron free BVIX the reaction was acidified with 3 N HCl (10 μ l).

IFP1.4 in cell fluorescence activity.

The fluorescence assays were performed as previously described with some minor modifications [44]. *E. coli* BL21 (DE3) were transformed with pBAD plasmid expressing either the HemO α or HemO α D99A, R188A, or D99/R188A mutants and pET28a expressing the bacterial phytochrome IFP1.4-His₆. Protein expression was performed at 37°C in LB media containing 25 μ g/mL chloramphenicol and 30 μ g/mL kanamycin to OD₆₀₀ = 0.8. IFP1.4 was induced with IPTG (final concentration 1 mM) and cells expressed at 25°C for 2 h. HemO (wild-type or mutants) was then induced on addition of arabinose (final concentration 0.02%) and cells were aliquoted as 200 μ L cultures in a black, clear-bottom 96-well plate. Cells were maintained at 25°C with orbital shaking monitoring both OD₆₀₀ and fluorescence emission on formation of the IFP1.4- BVIX α conjugate was then monitored at 700 nm following excitation at 630 nm. Fluorescence was monitored every twenty minutes over a twenty-hour period on a Synergy H1 hybrid microplate reader. Fluorescence change over time was corrected to account for differences in OD₆₀₀ between samples. Negative controls were performed in the absence of arabinose.

Hydrogen-deuterium exchange mass spectrometry (HDX-MS).

Purified HemO mutants D99A and R188A were analyzed by hydrogen-deuterium exchange mass spectrometry as previously described for the wild-type HemO [44]. The heme concentration required for 95% saturation was estimated using a K_D of 1 μ M. Therefore, to account for heme exchange on dilution of the sample 100 μ M apo-HemO was reconstituted with 114 μ M heme during the sample preparation, and heme was added to the deuteration buffer to a final concentration of 21 μ M. HemO protein samples were prepared by diluting the HemO protein to a final concentration of 100 μ M with 20 mM HEPES buffer in H₂O (pH 7.4). Deuterium exchange was initiated by diluting the HemO sample 50-fold with 20 mM HEPES buffer in D₂O (pD 7.4, deuteration buffer) at 23 °C. One hundred pmol of protein was removed from the reaction at 30 min, and the deuteration reaction immediately quenched by lowering the pH to 2.5 with ice-cold HCl. Quenched samples were frozen on dry ice prior to analysis. One hundred pmol of protein was removed from the reaction at 30 min, and the deuteration reaction immediately quenched by lowering the pH to 2.5 with ice-cold HCl. Quenched samples were frozen on dry ice prior to analysis. Following thawing, samples were immediately injected into a nanoACQUITY UPLC system with HDX manager (Waters). The protein was digested online at 10 °C using an Enzymate BEH Pepsin Column

(2.1 × 30 mm, Waters) in 1 min. The digest was trapped and desalted online on an ACQUITY Vanguard BEH C18 pre-column (2.1 × 5 mm, Waters) at 0 °C for 4 min at a flow rate of 125 µl/min in 0.1 % formic acid. Peptides were separated on an ACQUITY UPLC BEH C18 column (1.7 µm, 1 × 100 mm, Waters) at 0 °C by a 15 min linear acetonitrile gradient (5 – 50%) with 0.1 % formic acid at a flow rate of 40 µl/min. The eluent was directed into the ion source of a coupled SYNAPT G2 HDMS mass spectrometer (Waters). Mass spectra were acquired in the MS^E mode over the m/z range of 50–2000. Mass spectrometer parameters were as follows: electrospray ionization positive (ESI+) mode, capillary voltage 3 kV, collision energy 20–30 eV, sampling cone voltage 30 V, source temperature 80°C, desolvation temperature 175 °C and desolvation gas flow 500 L/h. To generate a peptide list for ion search, 100 pmol of undeuterated protein in 2 mM HCl in H₂O was injected. The undeuterated peptides were identified using Waters ProteinLynx Global Server software. The peptide list generated was imported into Waters DynamX software to guide the search of deuterated peptides, and the relative deuterium incorporation levels for each deuterated peptide were calculated using the time zero sample as reference.

Results and discussion

Spectroscopic characterization of the holo-HemO D99A, R188A and D99A/R188A complexes.

HemO WT and the D99A, R188A and D99A/R188A mutants following purification and reconstitution with heme were analyzed by absorption and CD spectrometry. The CD spectra of the D99A, R188A and D99/R188A holo-HemO mutants show a similar overall helical fold as for the WT HemO (Fig 2A). The melting temperature (T_m) for the apo- and holo-HemO mutant proteins as monitored by CD, show a slight decrease in stability compared to the WT (Table 1), presumably due to loss of the D99-R188 salt bridge between helix VII and helix XII (Fig 1B). However, the D99A, R188A and D99/R188A HemO proteins bind heme with similar affinities to the WT (Table 1). Furthermore, the resting state ferric (Fe³⁺) complexes of the HemO mutants show similar overall spectra, with Soret bands at 404 nm compared with 405 nm for the WT, and visible bands consistent with a six-coordinate high-spin heme (Figure 2B).

To characterize further the coordination and spin states of the D99-R188 salt bridge mutants, high-frequency RR spectra of the ferric and ferrous proteins were obtained using Soret excitation (Figure 3). These spectral data are remarkably similar to those of the WT protein, with oxidation marker band ν_4 at 1376 and 1354 cm⁻¹ in the ferric and ferrous proteins, respectively. In the ferric proteins, the ν_3 and ν_2 spin-state marker bands show two sets of frequencies that are indicative of a six-coordinate low-spin/high-spin mixtures consistent with occupancy of the distal coordination site by an aqua/hydroxy ligand.

After reduction with excess dithionite, all proteins adopt a pure five-coordinate high-spin configuration with unique ν_3 and ν_{10} modes at 1469 and 1602 cm⁻¹. These high-frequency RR spectra also identify the vinyl groups stretching vibrations $\nu_{C=C}$, which are observed at 1624 and 1620 cm⁻¹ in the ferric and ferrous proteins, respectively. The characteristics of the proximal histidine coordination to the Fe(II)-deoxy heme were also monitored by the acquisition of low-frequency RR spectra with a 442-nm excitation known to specifically

enhance the $\text{Fe}^{\text{II}}\text{-N}_{\text{His}}$ stretching mode in high-spin heme proteins. All spectra show an intense band at 220 cm^{-1} readily assigned to the $\nu(\text{Fe}^{\text{II}}\text{-N}_{\text{His}})$ mode (Figure 4).

Deformations modes from the peripheral propionate and vinyl modes observed between 350 and 440 cm^{-1} are also unchanged by the salt bridge mutations (Figure 4). Taken together the spectroscopic data indicates mutation of D99 and/or R188 have no effect on the overall structure or heme binding properties of the protein.

Stability of the reduced Fe(II)-O_2 holo-HemO D99A, R188A and D99A/R188A complexes.

The HemO wild-type Fe(II)-CO and Fe(II)-O_2 complexes on reduction with sodium dithionite were isolated as previously reported [9], and the respective Soret and visible-bands are listed in Table 2. HemO mutants D99A, R188A, and D99A/R188A all form Fe(II)-CO and Fe(II)-O_2 complexes, albeit with slightly altered Soret and visible bands. The Fe(II)-O_2 complexes of all three mutants showed blue-shifted Soret bands compared to the WT protein, suggesting that the distal O_2 ligand is experiencing minimal perturbation of the heme environment as a result of the mutations. The visible bands of the Fe(II)-CO and Fe(II)-O_2 complexes of the D99 and R188 mutants are also slightly shifted consistent with no significant changes in heme coordination or spin-state.

However, despite the fact the overall structural fold and heme environment of the D99A and R188A and D99/R188A mutants is similar to WT HemO, the stability of the Fe(II)-O_2 complexes in all three mutants was slightly decreased. Following generation of the Fe(II)-O_2 complexes and removal of excess reductant the decay of the Fe(II)-O_2 complex was monitored. In the absence of reductant, over time the Soret and visible bands shift back to the Fe(III) resting state. However, the rate of decay of the WT HemO Fe(II)-O_2 complex (12.6 min) (Figure 5A) was modestly slower than the decay observed for the three mutants, with half-lives of 7.6 min, 6.3 min, and 5.2 min for D99A, R188A, and D99A/R188A, respectively (Figure 5B-D). The increased rate of decay of the Fe(II)-O_2 holo-HemO mutant complexes is indicative of a decrease in the stability of the bound O_2 ligand. Previous NMR studies have shown structural elements surrounding the heme pocket undergo changes in conformational freedom as a function of the ligand allowing flexibility of the active site hydrogen bonding network to accommodate changes in the ligand [41]. As we observe no apparent changes per se in the heme binding environment of the D99A, R188A and D99/R188A mutants based on the RR, we hypothesized the decreased stabilization of the Fe(II)-O_2 ligand may be result of changes in the conformational flexibility of the protein.

Structural analysis of HemO D99A and R188A mutants by hydrogen-deuterium exchange mass spectrometry (HDX-MS).

To determine the effect of disrupting the D99-R188 salt-bridge on protein conformational flexibility we performed HDX-MS on the holo-HemO D99A and R188A mutants. The peptides obtained following deuterium exchange and pepsin digestion showed greater than 95% coverage for the HemO D99 and R188 proteins (Fig S1) as previously reported for HemO WT [44]. Analysis of the HDX at 30 min showed that in contrast to the wild-type HemO, both D99A and R188A mutants show a decreased deuterium incorporation in the proximal (I and II) and distal helices (VII and VIII) of the heme binding pocket (Figure 6

and 7). Specifically, in the D99A HemO mutant residues 12-18 of proximal helix I and residues 117-128 of distal helices VII and VIII showed decreased exchange (Figure 6A and 6B). Similarly, the R188A HemO mutant shows decreased exchange in proximal helices I and II (residues 12-43) and in distal helices VII-XI (residues 117-161) (Figure 7A and 7B). Interestingly, the pattern of decreased flexibility in the D99 and R188 HemO mutant proteins map to the same regions reported to show the greatest flexibility by H/D NMR when accommodating changes in the distal ligand during catalysis [40, 41]. On the backside of HemO the loop between helices VI and VII which contains D99 also shows decreased exchange in both mutants (as evidenced by residues 67-109 and 69-107 for D99A and R188A, respectively). In contrast to the decreased exchange in the proximal and distal helices, an increase in deuterium incorporation is seen in helices III (residues 55-62 and 54-62 of D99A and R188A, respectively) and XII (residues 182-192 and 175-192 in D99A and R188A, respectively). Overall, the global reduction in deuterium incorporation in helices around the distal pocket is more pronounced in the R188A mutant. However, a similar pattern of decreased conformational flexibility is observed in both holo-HemO D99A and R188A in helices VI, VII and VIII and increased flexibility in helix III. Interestingly, these helices all contribute at least one direct hydrogen bond to a structural water molecule within the active site.

Enzymatic activity of the HemO D99A, R188A, and D99A/R188A mutants.

The rate limiting step in heme degradation by HO is the release of product. In mammalian HO's this is circumvented by coupling BVIX α reduction to bilirubin IX α (BRIX α) through biliverdin reductase [2, 6]. The turnover of the bacterial holo-HemO complex results in Fe(III)-BVIX bound in the active site, which in the absence of further reduction of the Fe(III) or the presence of a BVIX-binding protein does not allow for multiple enzyme turnover *in vitro* [9-11]. Furthermore, the final *in vitro* product of heme degradation by the bacterial HemO enzymes is not released to the eukaryotic BVIX reductase enzymes. Compounding the issue of measuring enzymatic heme degradation *in vitro* is the use of chemical reductants such as ascorbate as the electron donor, which can result in reduction of O₂ to non-coordinated H₂O₂ leading to non-enzymatic coupled oxidation of heme [49, 50]. Therefore, the D99A, R188A and D99A/R188A mutants in which the Fe(II)-O₂ intermediate is destabilized may lead to the rapid formation of H₂O₂ and non-enzymatic heme degradation. To circumvent this complication we performed *in vitro* single turnover enzyme assays utilizing the HemO specific redox partner NADPH ferredoxin reductase (FPR) in the presence of catalase to consume H₂O₂ produced as a consequence of enzymatic uncoupling of FPR and HemO *in vitro* [48]. As shown in Fig 8A initiation of the reaction with NADPH results in a shift in the Soret from 406 nm to 413 nm with a concomitant increase in bands at 541 and 577 nm on formation of the Fe(II)-O₂ heme complex (Fig 8A; inset). The formation of the Fe(III)-BVIX complex is indicated by the decrease in the visible bands over time and a shift of the Soret peak back to 406 nm. Acidification of the reaction to release the iron from the BVIX complex results in broad bands at ~680 nm and 380 nm typical of BVIX. Interestingly, the D99A and R188A mutants on initiation of the reaction with NADPH result in a shift in the Soret toward 411 nm and a subsequent decay in the intensity of the Soret band (Fig 8B and C). However, in contrast to the turnover of the holo-HemO WT complex, we do not observe formation of a stable Fe(II)-O₂ complex as evident by the reduced

intensity of the visible bands at 541 and 576 nm (Fig 8B and C; inset). This is consistent with the previous data where the holo-HemO D99A and R188A Fe(II)-O₂ complexes autoxidized more rapidly than the HemO WT Fe(II)-O₂ complex (Fig 5). Despite the presence of catalase in the assays for both the D99A and R188A holo-HemO complexes we observe a decrease in the intensity of Soret and visible bands over time consistent with heme degradation (Fig 8B and C). However, on acidification of the D99A and R188A holo-HemO reactions rather than the broad featureless band around 680 nm consistent with formation of biliverdin, we noted a peak at 660 nm more indicative of verdoheme [51]. This is consistent with the fact that in contrast to HemO WT the HemO D99A, R188A and D99A/R188A mutants in *E. coli* do not give rise to the green pigmented cell pellets indicative of BVIX β and BVIX δ (Fig 9A). Therefore, we attribute the degradation of heme in the reconstituted *in vitro* assay to autooxidation of the unstable Fe(II)-O₂ complex generating H₂O₂ within the heme active site leading to coupled oxidation of heme to verdoheme. To determine if heme degradation in the D99A and R188A mutants is a consequence of non-specific coupled oxidation of heme to verdoheme we employed a cell-based assay recently developed in our laboratory that takes advantage of the fact expression of HemO in *E. coli* leads to the production of endogenous BVIX α , that can be coupled to a fluorescence readout on co-expression of the BVIX α -dependent bacterial phytochrome IFP1.4 [44, 52-54]. As the *in vivo* assay relies on the increased fluorescence properties of the natural IFP1.4 ligand BVIX α , this required utilizing the previously characterized HemO α variant that switch the regioselectivity to yield BVIX α through an in-plane rotation of the heme [55]. The inplane rotation placing the α -meso-carbon in position for oxidative cleavage does not alter the enzymatic activity of the HemO α variant compared to HemO WT [55]. Similar to the HemO WT, the expression of the HemO α variant in *E. coli* gives rise to green pigmented cells as a consequence of BVIX α accumulation, whereas the corresponding HemO α D99 and R188 mutants are colorless (Fig 9A). We further confirmed the inactivity of the HemO α D99 and R188 mutants in the previously developed in cell *E. coli* assay expressing both HemO α and the BVIX α dependent bacterial phytochrome IFP1.4 [44]. As expected for the HemO α variant we observe an increase in fluorescence over time as the IFP1.4-BVIX α chromophore accumulates in the bacterial cells (Fig 9B). However, the D99A, R188A, or D99A/R188A mutants in the HemO α background show no increase in fluorescence over time (Fig 9B). Taken together the data suggests the decreased conformational dynamics of the D99A, R188A and D99A/R188A HemO mutants does not allow for the accommodation of changes in ligand coordination and chemical structure during catalysis. Furthermore, in the *in vitro* activity assay the decrease in stability of the Fe(II)-O₂ is sufficient to lead to non-enzymatic heme degradation as a result of the generation of H₂O₂ within the active site.

Conclusions

The *P. aeruginosa* HemO enzyme is essential for heme utilization within the host, and together with the emerging role of BVIX β in adaptation and virulence, represents a novel drug target [55-57]. We recently identified a series of iminoguanidine inhibitors that bind on the back side of HemO close to a salt-bridge formed between D99 and R188. However, the mechanism of enzyme inhibition by the iminoguanidine ligands is not known. Interestingly, D99 is within a loop connecting helix VI to distal helix VII which forms the

heme binding site (Figure 1A). Furthermore, distal helix VII is critical for maintenance of the structural integrity of the active water network required for catalysis [21]. We previously hypothesized the mechanism of inhibition may be a result of disrupting the D99-R188 salt bridge, leading to long range allosteric effects on distal helix VII. To further probe if the iminoguanidine inhibitors are potentially disrupting the D99-R188 salt bridge we constructed the D99A, R188A and D99/R188A site directed mutants. Analysis of the secondary structure of the apo and holo-HemO D99 and R188 proteins by CD show no significant differences from the WT (Fig 2A) [55]. As previously reported the secondary structure of the HemO α variant is indistinguishable from that of the WT [55]. Analysis of the D99A, R188A, and D99A/R188A mutants by CD also showed similar profiles to the HemO α variant (data not shown). Furthermore, the heme binding affinities and absorption spectra of the resting state holo-HemO mutant proteins are indistinguishable from that of the WT HemO (Fig 2B and Table 1). A more detailed analysis of the heme environment of the holo-HemO D99A, R188A and D99/R188A mutants by RR indicated that like the resting state holo-HemO WT, the heme is six coordinate high-spin with a water molecule as the sixth ligand to the heme. The conserved RR spectra in the resting state Fe(III) and reduced Fe(II)-deoxy D99A, R188A and D99/R188A HemO mutant complexes are inconsistent with a significant disruption of the active site structure. Furthermore, the rate of decay of the Fe(II)-O₂ complex for the HemO D99A, R188A and D99/R188A mutants was only slightly increased over that of WT. However, despite the fact the heme environment and the stability of the initial Fe(II)-O₂ complex were not significantly affected in the HemO D99A, R188A and D99/R188A mutants they are catalytically inactive (Fig 8 and 9).

Interestingly, previous HDX-MS analysis of HemO in the presence of iminoguanidine inhibitors shown to bind in close proximity to the D99-R188 salt bridge induced significant changes in conformational flexibility within structural elements surrounding the active site [44]. Similarly, our current HDX analysis on mutation of either D99 or R188 show similar changes in conformational dynamics within the same structural elements as observed with the inhibitors (Fig 6 and 7). Specifically, we see increased conformational flexibility in helix III and XII and decreased disorder in helices VII and VIII that surround the active. Our data is also consistent with previous HDX-NMR studies of the *P. aeruginosa* HemO WT protein in which the authors concluded the conformational dynamics of the protein are critical in allowing the changes in the coordinated ligand as well as the electronic and structural changes to the heme during catalytic turnover [40, 41]. Specifically, the authors show the Fe(III)-N₃ ligand as a mimic of the activated Fe(III)-OOH intermediate induced chemical shift perturbations throughout structural elements surrounding the heme, with the greatest changes being observed within proximal helix II, distal helix VII, as well long range effects in helices V, VI, X and the loop preceding helix X (Figure 1A). Subsequent studies on the HemO R80L mutant which disrupts the structural water network also showed an overall increase in HD exchange and conformational dynamics in these same structural elements [41]. Interestingly, the structural elements shown to undergo conformational flexibility in accommodating ligand changes by H/D-NMR in HemO are those also shown to be affected in the current HDX-MS analysis of the salt bridge mutants (particularly those in proximal helix II, distal helix V, VI and VII) (Figures 6 and 7). Our current data when taken in the context of previous studies suggests the D99-R188 salt bridge is critical in modulating the

conformational dynamics and long range effects on the heme active site required for catalysis. Furthermore, the current studies provide a rationale for the mechanism of inhibition by the previously characterized allosteric iminoguanidine inhibitors [44]. We propose allosteric HemO inhibitors that target protein conformation and dynamics rather than competitive inhibition at the heme active site, offer an alternative and complementary approach to the design of novel antimicrobial agents targeting *P. aeruginosa*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CD	circular dichroism
HDX-MS	hydrogen-deuterium exchange mass spectrometry
RR	resonance Raman spectroscopy
DM	double mutant
BVIX	biliverdin
HemO	<i>Pseudomonas aeruginosa</i> iron-regulated heme oxygenase
HemOα	biliverdin IX α selective mutant of <i>P. aeruginosa</i> iron-regulated heme oxygenase
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	heme oxygenase
HO-1	human heme oxygenase 1
HO-2	human heme oxygenase 2
HDX-MS	hydrogen deuterium exchange mass spectrometry
IFP	infra-red fluorescent protein
IPTG	isopropyl β -D-thiogalactopyraniside
IsdG	iron-regulated surface determinant protein G

IsdI	iron-regulated surface determinant protein I
LB	Luria-Bertani
MhuD	<i>Mycobacterium</i> heme utilization degrader
Ni-NTA	Nickel-nitriloacetic acid
PMSF	phenylmethanesulfonyl fluoride
rHO-1	rat heme oxygenase 1
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
WT	wild type

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Fig 1.

Structure of the *P. aeruginosa* HemO in the resting ferric state (PDB:1SK7). A. The heme is held between proximal helices (I and II) and distal helices (VII and VIII) with the Gly-Gly kink connecting helix VII and VIII. Heme is ligated through proximal His-26 shown in blue; B. Rotation 90° showing a side view with the D99-R188 salt bridge between the loop connecting helix VII and VIII and helix XII shown in stick format.

Fig 2.

CD and absorption spectra of the HemO WT and D99A, R188A and D99A/R188A mutant proteins. A. CD spectra of the apo-HemO proteins. B. Absorption spectra of the holo-HemO proteins. C. CD spectra of the holo-HemO proteins. CD spectra were recorded with 2.5 μM apo- or holo HemO proteins in 50 mM potassium phosphate buffer (pH=7.2) and absorption spectra in 20 mM sodium citrate (pH 7.0) at 25°C as described in the Materials and Methods.

Fig 3. High-frequency region of the RR spectra of HemO WT and D99A, R188A and D99A/R188A mutant proteins in the ferric (left panel) and ferrous (right panel) states obtained with a 407-nm excitation. Spectra are listed from the upper spectrum for HemO WT, followed by D99A, R188A and D99A/R188A.

Fig 4. Low-frequency region of the RR spectra of HemO WT and D99A, R188A and D99A/R188A mutant ferrous proteins obtained with a 442-nm excitation.

Fig 5.

Decay of the Fe(II) O₂-complexes for HemO wild-type and mutant proteins. Visible bands of the Fe(II)-O₂ HemO complexes (A) WT, (B) D99A, (C) R188A, and (D) D99A/R188A mutants were observed for 1 h following complex formation. The decrease in absorbance of both the α -band (577 nm) and β -band (541 nm) of the Fe(II)-O₂ holo-HemO complexes was followed by absorption spectroscopy (left panel). The rate of decay at 577 and 541 nm was plotted for each protein (right panel). Samples were prepared as described in Materials and Methods.

Fig 6. HDX-MS of the resting state Fe(III) holo-HemO D99A. Deuterium incorporation was compared to HemO wild-type and region showing the greatest change in deuterium uptake are color coded and mapped on the HemO crystal structure (PDB 1SK7). Peptide regions with increased deuterium incorporation are colored in red, while decreased exchange is colored in blue. A. Holo-HemO viewed from the front with the proximal H26, D99 and R188 shown in stick format. B. Holo-HemO viewed from the back. C. Relative deuterium uptake for helices showing the most significant changes in deuterium uptake at 30 min (Student *t*-test p , < 0.05, $n=3$). Samples were prepared and analyzed as described in the Materials and Methods.

Fig 7.

HDX-MS of the resting state Fe(III) holo-HemO R188A. Deuterium incorporation was compared to HemO wild-type and region showing the greatest change in deuterium uptake are color coded and mapped on the HemO crystal structure (PDB 1SK7). Peptide regions with increased deuterium incorporation are colored in red, while decreased exchange is colored in blue. A. Holo-HemO viewed from the front with the proximal H26, D99 and R188 shown in stick format. B. Holo-HemO viewed from the back. C. Relative deuterium uptake for helices showing the most significant changes in deuterium uptake at 30 min (Student *t*-test *p*, < 0.05, *n*=3). Samples were prepared and analyzed as described in the Materials and Methods.

Fig 8.

In vitro turnover of (A) holo-HemO WT, (B) D99A, and (C) R188A proteins. Spectral changes on addition of 200 μ M NADPH to a solution containing HemO (8-10 μ M), FPR (20 μ M) in 20 mM Tris-HCl pH 7.4. The inset (x10) shows the increase in intensity of the visible bands on formation of the Fe(II)-O₂ complex which subsequently decreases on formation of the Fe(III)-BVIX complex. Following completion of the assay acidification with 3 N HCl releases BVIX (dashed line) as judged by the band at 680 nm (A) or a spectrum more typical of verdoheme with a peak at 655 nm (B and C). See Materials and Methods for more details.

Fig 9.

A. Cell pellets of HemO WT and HemO α and their corresponding D99A, R188A, D99A/R188A mutants harvested at 6 hour post-induction with 1 mM IPTG. The HemO WT cell pellet shows less intense green pigmentation than the HemO α variant as BVIX β and BVIX δ are more readily excreted into the extracellular medium than BVIX α . B. HemO activity as monitored by fluorescence of the IFP1.4-BVIX α chromophore in cells expressing HemO α , or the corresponding D99A, R188A and D99A/R188A mutants. Following induction of HemO α and IFP1.4 cultures were incubated at 25°C with shaking and the OD₆₀₀ and fluorescence of the IFP1.4-BVIX α complex (ex. 630 nm, em. 700 nm) was monitored over 20 hours on a Synergy H1 hybrid microplate reader as described in the Materials and Methods.

Table 1.

Heme binding constants and melting temperatures of HemO WT and D99A, R188A and D99A/R188A apo- and holo-HemO proteins.

HemO Mutant	Heme binding affinity K_D (μM)	apo-HemO T_m ($^{\circ}\text{C}$)	holo-HemO T_m ($^{\circ}\text{C}$)
WT	0.87 ± 0.17^a	56.6 ± 1.2^a	63.6 ± 1.7
D99A	0.83 ± 0.21	$53.2 \pm 1.1^*$	$59.7 \pm 1.2^*$
R188A	0.79 ± 0.22	$49.6 \pm 1.0^{**}$	$54.5 \pm 1.9^{**}$
D99A/R188A	0.99 ± 0.21	$51.7 \pm 0.6^{**}$	$60.2 \pm 0.8^*$

^aStandard deviation,

* $p < 0.05$ vs WT,

** $p < 0.005$ vs WT.

Table 2.

Spectroscopic properties of the holo-HemO WT, D99A, R188A and (Fe(III) and D99A/R188A resting state Fe(II) CO- and O₂-complexes.

Protein Complex	Soret Band (nm)	Q-bands (nm)	Q-band Ratio
Fe(III)			
HemO	406	-	-
HemO(D99A)	404	-	-
HemO(R188A)	404	-	-
HemO(D99A/R188A)	404	-	-
CO-Fe(II)			
HemO	420	540, 570	1.05
HemO(D99A)	421	540, 570	1.02
HemO(R188A)	421	539, 570	1.02
HemO(D99A/R188A)	421	539, 570	1.02
O₂-Fe(II)			
HemO	413	541, 577	1.05
HemO(D99A)	412	541, 576	1.09
HemO(R188A)	409	541, 576	1.13
HemO(D99A/R188A)	411	540, 575	1.11