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Spectral characterization of a novel NO sensing protein in bacteria: NosP

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Abstract

A novel family of bacterial hemoproteins named NosP has been discovered recently; its members are proposed to function as nitric oxide (NO) responsive proteins involved in bacterial group behaviors such as quorum sensing and biofilm growth and dispersal. Currently, little is known about molecular activation mechanisms in NosP. Here, functional studies were performed utilizing the distinct spectroscopic characteristics associated with the NosP heme cofactor. NosPs from *Pseudomonas aeruginosa* (*Pa*), *Vibrio cholerae* (*Vc*) and *Legionella pneumophila* (*Lpg*) were studied in their ferrous-unligated forms as well as their ferrous-CO, ferrous-NO, and ferric-CN adducts. The resonance Raman (rR) data collected on the ferric forms strongly support the existence of a distorted heme-cofactor, which is a common feature in NO sensors. The ferrous spectra exhibit a 213 cm⁻¹ feature, which is assigned to the Fe-N_{his} stretching mode. The Fe-C and C-O frequencies in the spectra of ferrous-CO NosP complexes are inversely correlated with relatively similar frequencies, consistent with a proximal histidine ligand and a relatively hydrophobic environment. The rR spectra obtained for isotopically labeled ferrous-NO adducts provide evidence of formation of a 5-coordinate NO complex, resulting from proximal Fe-N_{his} cleavage, which is believed to play a role in biological heme-NO signal transduction. Additionally, we found that of the three NosPs studied, *Lpg* NosP contains the most electropositive ligand binding pocket, while *Pa* NosP has the most electronegative ligand binding pocket. This pattern is

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Author Contributions

E.M.B. and B.A.B. designed experiments related to NosP purification, LC/MS, electronic absorbance spectra, NO dissociation rate constants and heme redox potentials. B.A.B. performed these experiments. Y.L. J.R.K. designed rR experiments. Y.L. performed these experiments. E.M.B., B.A.B., Y.L. and J.R.K. wrote the manuscript.

Supporting Information

NosP sequence alignment; NosP mass spec. demonstrating that NosP has non-covalent bound heme B; *Pa* NosP reduction potential determined by co-reduction with 5,5',7,7' indigo tetrasulphonate. This material is available free of charge via <http://pubs.acs.org>.

Accession Codes

Pa NosP: Q9I2D0, *Vc* NosP: Q9KS17 and *Lpg* NosP: Q5ZYT2

also observed in the measured heme reduction potentials for these three proteins, which may indicate distinct functions for each.

Graphical Abstract

Introduction

While at high concentrations nitric oxide (NO) is a cytotoxic gas, at low concentrations this molecule plays an important role in signal transduction.^{1, 2} In mammals, NO is generated from L-arginine by nitric oxide synthase (NOS)^{3, 4} and diffuses across cell membranes to activate the NO sensor soluble guanylate cyclase (sGC).⁵ This is important for a variety of signaling pathways including vasodilation and neurotransmission.⁶ Recently it has been reported that bacteria also respond to sub-micromolar concentrations of NO, such as in symbiotic signaling between *V. fischeri* and *E. scolopes*.²

So far, two classes of bacterial NO sensing proteins have been reported: heme-nitric oxide/oxygen binding domains (H-NOXs) and nitric oxide sensing proteins (NosPs). H-NOX proteins were discovered based on sequence homology with the sGC heme-binding domain.⁷ NO-ligated H-NOX effectively regulates cyclic di-GMP and histidine kinase signaling pathways in a variety of bacterial species; H-NOX proteins have been reviewed extensively.^{8–10}

In bacteria that lack H-NOX domains, NosP proteins may serve as the primary NO sensor, as demonstrated in *P. aeruginosa*.¹¹ NosP proteins are predicted to be particularly important in the regulation of biofilm formation in bacteria,¹² a bacterial group behavior that involves the secretion of a protective polysaccharide matrix and is associated with chronic infection and antibiotic drug resistance.^{13–17} Understanding structural mechanics that underlie NosP ligand binding and initiation of signal transduction is a vital step in understanding this novel class of proteins and in the potential development of future antibiotic applications.

NosP proteins from *P. aeruginosa*, *V. cholerae*, and *L. pneumophila* were chosen for this study as they are predicted to, or have been shown to, participate in pathways that modulate biofilm or quorum sensing behaviors. NO-bound *Pa* NosP has been shown to regulate the activity of a co-cistronic histidine kinase that is required for NO-dependent biofilm reduction.¹¹ NO-bound *Vc* NosP has been demonstrated to regulate the autophosphorylation activity¹⁸ of the quorum sensing kinase VpsS,^{19–21} and *Lpg* NosP modulates a co-cistronic kinase (Fischer, *et al.* unpublished data) whose cognate response regulator has been shown to result in a hyper-biofilm phenotype when deleted.^{21, 22}

Resonance Raman (rR) spectroscopy is an effective tool to detect the structures of heme proteins and their intermediates.²³ Model compounds and isotope labeling have been used to identify heme macrocycle modes.^{24, 25} The high-frequency region of a rR spectrum contains marker bands that are sensitive to oxidation state and spin state changes,²⁶ whereas the low-frequency region provides information on the porphyrin macrocycle structure and its peripheral substituents,²⁷ including both in-plane and out-of-plane features that are sensitive to the distinction of propionate and vinyl groups.^{27, 28} This is important because documenting the interaction between these peripheral groups and the protein can elucidate the structural effects resulting from heme-ligand binding.^{29, 30} Of special import for this work, it is possible to interrogate the status of Fe-X-Y fragments of CO, NO and O₂-ligated forms.³¹ The results obtained here suggest that upon binding of the NO molecule, NosP proteins form a 5-coordinate NO species by displacing a proximal histidine ligand.

Experimental Procedures

Materials and general methods.

All reagents were obtained in their highest available purity and used as received. DEA NONOATE was purchased from Caymen Chemical (CAS 372965-00-9; purity 98%). Carbon monoxide (40%, balanced with hydrogen) was introduced from a gas tank directly bubbled into dithionite solution to create a saturated CO solution (Airgas, 99.3% pure). Sodium dithionite was purchased from Sigma Aldrich, in technical grade. Sodium nitrite was purchased from Fisher Scientific, in laboratory grade, and sodium nitrate-¹⁵N (99% atom %) was purchased from MSD isotopes (Division of Merck Frost Canada INC.)

Gene cloning.

NosP from *Pa*, *Vc* and *Lpg* (Accession numbers Q9I2D0, Q9KS17 and Q5ZYT2) were cloned as described previously¹¹ with 6xHis tag. All plasmids were confirmed by DNA sequencing.

Protein expression and purification.

NosP from *Pa*, *Lpg*, and *Vc* plasmids were transformed into BL21 cells and grown in 1L yeast extract media (45 grams yeast extract, 90mM sodium phosphate pH 7.4) at 37° C. At OD=0.5, the media was supplemented with 20μM hemin (Sigma) and at OD=0.6 the protein was induced with 10μM IPTG at 16°C overnight. Cells were pelleted and lysed in lysis buffer (20mM Tris pH 8.0 at 4.0°C, 300mM NaCl, 1mM PMSF, 5mM 2-mercaptoethanol, 10% glycerol, 0.1mM EDTA, 0.3mM hemin, 1% Triton X-100) by sonication. The protein was purified from cleared lysate by Ni-NTA (Qiagen) using wash buffer (20mM Tris pH 8.0 at 4.0°C, 300mM NaCl, 1mM PMSF, 5mM 2-mercaptoethanol, 10% glycerol, 0.1mM EDTA, 4μM hemin) with increasing steps of imidazole (10, 20 and 50 mM imidazole). NosP was eluted with wash buffer and 250mM imidazole and desalted into storage buffer (20mM Tris pH 8.0 at 4.0°C, 100mM NaCl, 10% glycerol, 5mM 2-mercaptoethanol) and incubated in 0.3mM hemin (Sigma) over night at 4°C. The next day, NosP is concentrated in a 10kDa concentrator and unbound hemin is removed by FPLC (AKTA) gel filtration (Superdex200, GE). Fractions are analyzed by comparing A410/A280 to assess heme incorporation and stored at -80°C.

Mass Spectrometry.

Pa NosP purified from BL21 cells not supplemented with exogenous heme during purification was tested by LC/UV/MS to confirm the presence and character of its heme cofactor. Samples were run on AerisPeptide-XB-C18 (2.6 μ m, 300Å, 150×2.1mm) with a linear gradient from 95% H₂O (0.1% Ac, 0.02% TFA) 5% CH₃CN (0.1%CN 0.2% TFA) to 5% H₂O (0.1% Ac, 0.02% TFA) 95% CH₃CN (0.1%CN 0.2% TFA) over 30 min at 0.4 mL/min, 35 °C. Mass data was acquired using TOF 4Ghz HiRes 3200, ESI m/z=250–3200, 1Hz. Absorbance was acquired with a diode array 4nm slit, 2nm step 0.2mAU threshold, 5Hz acquisition.

Complex preparation for absorbance spectroscopy.

NosP complexes are prepared in an anaerobic chamber (COY). Samples were incubated with 10 mM potassium ferricyanide for 10 min to oxidize the heme and then desalted by PD-10 gel filtration (GE) to remove ferricyanide. A 600 mM dithionite solution was prepared anaerobically by dissolving 52 mg of solid dithionite (stored in a desiccator) in 500 μ L of oxygen-free tris buffered saline (20 mM Tris, pH 7.5, 100 mM NaCl). The protein was then incubated with 60 mM sodium dithionite for 45 min followed by desalting to remove dithionite. The protein was then exposed to either excess nitric oxide (NO) donor DEA-NONOate (Cayman Chemical) which has a 1/2 life of 16 min at 25°C and desalted to remove DEA and excess DEA-NONOate or was exposed to excess carbon monoxide (CO) gas continually passed through the headspace of a gas-tight reactivial. For CN⁻ complexes, ferric protein was incubated in 10 mM potassium cyanide for 40 min. At each stage of preparation, a small amount of sample was saved for UV-Vis spectroscopic analysis (CARY).

Determination of k_{off} for NO-NosP.

NO complexes were prepared as described above. The NO dissociation rate constant was measured using a dithionite trap as described previously.^{11, 32} Briefly, a dithionite trap was prepared by bubbling CO gas through protein storage buffer with varying concentrations of dithionite. The CO solution was injected into a gas tight cuvette containing the NosP-NO complex at various concentrations between ~200 nM to ~500 nM. Dissociation of NO from NosP was measured by scanning kinetics (CARY) of the change in UV-vis absorbance from the NO peak (395 nm) to the CO peak (420 nm) over time at 20 °C. Because fitting the data to either peak alone results in the same fit, the difference was used to maximize the signal, as has been reported in similar measurements as previously described.^{11, 33} CO association is expected to be much faster than NO dissociation, as is observed with other heme proteins.³⁴ The change in absorbance over time was fit to two parallel exponentials, each with the form $\text{Abs}(t) = A(1 - e^{-k \cdot t})$. Supplemental Table 1 describes the graphing software used and further elaboration on the kinetic determinations is in the results section below. All rate constants reported are independent of CO and dithionite concentration (3, 30, and 300 μ M tested).

Resonance Raman sample preparation.

The ferric NosP proteins were measured in NMR tubes. To prepare the ferrous samples, 75 μL of 150 μM NosP (from *P. aeruginosa*, *V. cholerae* and *L. pneumophila*) proteins were transferred into NMR tubes (WG-5 Economy, Wilmad), respectively, sealed with a rubber septum, and reduced by a stoichiometric amount of sodium dithionite solution under anaerobic condition; i.e., the sample was titrated with a sodium dithionite solution until the α/β bands at ~ 526 and 533 nm had appeared, as monitored by a special device (model CHEM2000-VIS, Ocean Optics Inc., Dunedin, FL) that is capable of recording absorption spectra from samples contained in NMR tubes. The ferrous CO complexes were prepared by saturating the sample with CO gas prior to the injection of excess sodium dithionite solution. The preparation of the nitric oxide adducts of ferrous NosP was achieved by anaerobic decomposition of the proper isotope-labelled (^{15}N or ^{18}O) sodium nitrite with dithionite. Specifically, 50 μL of NosP protein was placed in an NMR tube which was sealed with a rubber septum. After being degassed with argon for 15 min, the proteins were reduced by injecting a 50-fold excess of sodium dithionite followed by 20-fold excess of sodium nitrite to form the nitric oxide adducts; the concentration of the sodium dithionite solution was determined by electronic absorption spectrophotometry (employing a molar absorptivity of $8043\text{ M}^{-1}\text{cm}^{-1}$),³⁵ as described previously.^{36, 37} It is noted that the sample preparation employed here for the rR measurements used a 20-fold excess of NO. These conditions may possibly give rise to a 5-coordinate NO complex, where the NO is positioned in the proximal pocket, but confirmation of this would require more elaborate rR studies with carefully controlled relative concentration of enzyme and nitric oxide, or possibly time-resolved rR studies demanding rather large quantities of protein.³⁸

Resonance Raman measurement.

The spectra of ferric and ferrous nitrosyl NosP complexes were obtained using 406.7 nm excitation line, while the spectra of ferrous and their CO complexes were measured using 413.1 nm excitation line. Both lines are provided by a Kr^+ laser (Coherent Innova Sabre Ion Laser) with a Spex 1269 spectrometer equipped with a Spec-10 LN liquid nitrogen cooled detector (Princeton Instruments). The slit width was 150 μm (a spectral resolution of 8 cm^{-1}) for all the measurements except for high-frequency measurement on ferrous CO samples, where it was increased to 250 μm (a spectral resolution of 14 cm^{-1}), owing to the weaker intensity of the $\nu(\text{C-O})$ modes. The laser power for ferric and ferrous samples was kept around 10 mW whereas for the nitrosyl and ferrous CO complexes, it was adjusted to ~ 1 mW to avoid photodissociation. All samples were measured in a spinning NMR tube and a cylindrical focusing lens, providing a linear image on the sample, to avoid local heating and protein degradation. Spectra were calibrated with fenchone and acetone- d_6 (for calibration with high frequency above 1700 cm^{-1}) and processed with Grams 32/AI software (Galactic Industries, Salem, NH).

Measurement of heme redox potential.

Heme redox potential was measured using a slight modification of the method described by Massey³⁹ with the modifications of Efimov et al,⁴⁰ which measure reduction potential by measuring equilibrium co-reduction of the protein and a dye with a known reduction

potential as a reference. Methylene blue was selected as the dye for this experiment as it was determined to have a reduction potential closest to that of the NosP proteins. Oxidized protein is prepared in a gas tight cuvette in an anaerobic chamber in a solution with 5 µg/mL Catalase, 5 mM Glucose and 50 µM Glucose Oxidase to ensure that the solution remains anaerobic, phosphate buffer at the appropriate pH at 50 mM, protein and dye are added such that they give absorbance ~1 AU, and xanthine (Sigma) to 300 µM is added as an electron source. Immediately before beginning reading, 3.5×10^{-6} units of xanthine oxidase (Sigma) is added. The sample is scanned from 700–350nm once per min for 40 min. Next, a few crystals of sodium dithionite are added to fully reduce the sample and scans are continued for another 15 min to ensure maximal reduction. While preparation of anaerobic solution is a more common way to produce reduced proteins to avoid pH shock and product of reactive oxygen species, for this experiment the established methods add solid dithionite crystals to the sample to avoid changes to the absorbance amplitude from sample dilution.^{39, 40} The Soret is monitored during this time and no loss of absorbance is detected that might indicate any sort of damage to the protein. To determine the net reduction potential, the modified Nernst equation is used $E^{\circ} = \frac{RT}{nF} * \ln\left(\frac{[ox]}{[red]}\right)$, where the ratio of oxidized to reduced species is

calculated from absorbance values: $\frac{A_t - A_{min}}{A_{max} - A_t}$ where A_t is the absorbance value at a given

time point, and $A_{max/min}$ are the maximum and minimum observed absorbance values for the peak of the oxidized form of the protein/dye. This transformation is applied to the protein and dye separately, with $n=1$ for the protein and $n=2$ for the dye. The data are plotted against each other for each time point, which should give a line with slope of 1 under equilibrium conditions. As is described by Efimov *et al*, data points at the beginning and end of the experiment are excluded until the slope is between 0.99 and 1.1, as these points are more likely to be out of equilibrium for oxidized:reduced species. The line's intercept is the difference in reduction potential between the protein and dye, so using the dye's known reduction potential at a given pH, the protein's reduction potential is extrapolated. Reported values are calculated for averages and standard error of the mean from at least four repetitions for pH 7.0 samples and at least 2 samples for pH 8.0 samples.

Results and Discussion

NosP from *P. aeruginosa* was the first NosP hemoprotein to be characterized; it was demonstrated to be involved in NO-mediated biofilm regulation.¹¹ A molecular-level understanding of ligand binding and heme-based signaling are entirely lacking in this family, however. In this study, we use electronic and resonance Raman spectroscopies to characterize various ligation and oxidation states of three homologous NosP hemoproteins (Figure S1) and to explore the molecular features of the gas-ligand binding pocket.

NosPs contain heme B as a cofactor.

To confirm the heme cofactor present in NosP is heme B, as expected, we purified *Pa* NosP without supplementing hemin at any point during the expression/purification process for analysis by liquid chromatography mass spectrometry with diode array (LC/MS/DAD). *Pa* NosP has an elution peak at 17.8 min, which like a heme B standard, has $m/z = 616$ and gives an absorbance spectrum with a Soret peak at 398 nm (Figures S2a and S2b). The second

elution peak at 20.5 min has high 280 nm absorbance and gives a deconvoluted mass of 43,107 da, which corresponds to His-tagged *Pa* NosP (Figure S2c). Furthermore, titration experiments between apo-NosP and heme (Figure S3) are consistent with a single heme binding site per NosP monomer.

NosP proteins interact with CO, NO and CN⁻.

NosP proteins purify bound to heme as demonstrated by their characteristic Soret absorption peak. Electronic absorbance spectra for *Pa* NosP¹¹ and *Vc* NosP¹⁸ have been reported previously, demonstrating that NosP ligand binding properties are consistent with known NO sensors, such as sGC.⁴¹ The ligand binding properties of *Lpg* NosP are consistent with the NosP proteins characterized to date (Figure 1a and Table 1). *Lpg* NosP has resolved α and β peaks in the ferrous unligated state and a Soret maximum at 417 nm, which is similar to 6-coordinate heme proteins such as CooA.^{42–44} Upon NO binding, the Soret peak shifts to 395 nm, while the α/β peaks shift to 525 and 553 nm, respectively, consistent with a 5-coordinate {FeNO}⁷ heme nitrosyl complex. Upon CO binding, there is little shift in the Soret maximum, but the extinction coefficient is significantly higher and the α/β peaks shift to 533 and 566 nm respectively, similar to what is observed for other NosP proteins and sGC. Interestingly, in the ferric-CN complex, *Lpg* NosP has a single α/β at 544 nm, similar to *C. eugametos* cyanomet-hemoglobin (546nm),⁴⁵ while in sGC and the other NosP proteins there is a single α/β peak observed at 538 nm (Table 1 and Figure 1b).⁴⁶

NO dissociates slowly from NosP proteins.

The dissociation rate constant of NO from *Lpg* NosP is comparable to those observed in other NosP proteins as well as those reported for other sub-micromolar NO sensors (Figure 2 and Table 2). Dissociation was measured by the CO-dithionite trap method described previously.^{11, 32, 33, 47} Briefly, NO bound protein is incubated in a solution saturated with carbon monoxide and a large excess of sodium dithionite. As NO dissociates from heme it is reduced by dithionite. CO then rapidly associates with heme in a process expected to be much faster than NO dissociation. Dithionite and CO are both present to prevent NO-rebinding, which is typically very fast, and would artificially lower the apparent NO dissociation rate constant. The data were fit to a parallel two term exponential-associative function. The slower step is reported as k_{off} because it is the slowest step in NO-dissociation, not because we can assign it to the molecular step of Fe-N bond scission. The faster rate constant is possibly the result of association of the axial protein ligand after NO dissociation. Another possibility is that because excess NO was used in preparing the NO complex, the 5-coordinate NO species observed for NosP has NO bound in the proximal pocket, as is observed in cytochrome c'⁴⁸ and some H-NOX proteins.⁴⁹ Thus, the two rate constants could represent dissociation from the heme via a 6-coordinate ferrous intermediate to which CO binds more slowly than expected, although we note we see no dependence on CO in our measured rate constants. It is also possible that there could be a mixture of proximal and distal 5-coordinate NO complexes, and the two rate constants represent dissociation from these two different species.

Typically, NO dissociation measurements reach a plateau within 1 hour,^{11, 33, 34, 47} while in this case, even after data collection for 3 hours, a plateau was not observed for *Lpg* NosP.

Due to this, we cannot report a precise NO dissociation rate constant, but if this data is constrained to assume it has plateaued, we can report the dissociation rate constant is no faster than 2×10^{-4} per second for the slower rate constant and 100×10^{-4} for the faster rate constant. These rate constants are both independent of CO and dithionite concentration. We can thus conclude that the NO dissociation is quite slow. We are currently working on obtaining NO association kinetics, but as typical histidine-ligated hemoproteins have NO association rate constants that falls between 10^4 – 10^8 $\text{M}^{-1}\text{s}^{-1}$, we expect the K_D to fall in the nanomolar to picomolar range.⁵⁰

Ferric NosP hemes are mixtures of 5-coordinate high- and 6-coordinate low-spin states.

The high-frequency rR spectrum of the ferric NosP protein from *P. aeruginosa* (Figure 3a) has a peak near 1467 cm^{-1} attributable to glycerol; the relatively lower intensity of this feature in Figure 3c is the result of the lowered glycerol concentration. The ν_4 mode is seen at 1374 cm^{-1} , which indicates the sample is in the ferric state, while the intensity of the ν_3 mode at 1505 cm^{-1} , compared to the feature at 1492 cm^{-1} , attributable to a 5-coordinated high spin state, shows that the dominant form is the 6-coordinated low spin state.⁵¹ This spin state mixture is also reflected by alterations in the ν_2 and ν_{10} bands; i.e., the ν_2 bands occur at 1579 cm^{-1} and 1588 cm^{-1} , while the ν_{10} bands occur at 1628 cm^{-1} and 1640 cm^{-1} . It is also important to note that a vinyl stretching mode is expected to occur near 1620 cm^{-1} , though definitive confirmation of its presence would require careful determination of depolarization ratios to confirm the assignment.

The high frequency region of the ferric NosP protein from *V. cholerae* (Figure 3b) exhibits features characteristic of the ferric state, with the ν_4 oxidation state marker occurring at 1374 cm^{-1} . Again, the ν_3 spin state marker bands are indicative of the presence of a mixture of 5-coordinated high spin (the ν_3 mode at 1492 cm^{-1}) and 6-coordinated low spin (the ν_3 mode at 1505 cm^{-1}) species, with the latter showing an increased relative population. The ν_2 and ν_{10} mode for 5-coordinated high spin (1579 and 1628 cm^{-1}) and 6-coordinated low spin (1588 and 1640 cm^{-1}) species are also observed, at expected positions, in this region.

Figure 3 (trace C) shows the high frequency region of the ferric NosP protein from *L. pneumophila*, with its ν_4 band occurring at 1374 cm^{-1} , consistent with the ferric state. Two ν_3 modes, observed at 1492 cm^{-1} and 1505 cm^{-1} , show that this protein also exists in equilibrium between 5-coordinated high spin and 6-coordinated low spin forms, respectively. The intensity of the high spin ν_3 (1492 cm^{-1}) band is quite a bit larger relative to the low spin ν_3 (1505 cm^{-1}) mode, indicating a higher percentage of high spin species. This spin state mixture is also reflected by alterations both in the ν_2 and ν_{10} bands; i.e., the ν_2 bands occur at 1579 cm^{-1} and 1588 cm^{-1} and the ν_{10} bands occur at 1628 cm^{-1} and 1640 cm^{-1} .

The NosP heme is distorted.

The low-frequency rR spectra of ferric NosP proteins from the various sources are shown in Figure 4. In trace A, the assigned heme skeletal modes for the protein from *P. aeruginosa* are observed; ν_7 (678 cm^{-1}), ν_{15} (754 cm^{-1}) and ν_8 (345 cm^{-1}). A shoulder of ν_7 appears at 693 cm^{-1} , which can be attributed to γ_{20} . According to previous work with many other heme proteins, including globins and cytochromes P450,^{52, 53} the bands observed between

365–380 cm⁻¹ are typically assigned to the bending modes of the heme propionate groups,⁵⁴ though they are properly interpreted as heme deformation modes that involve significant contributions from the propionate bending coordinates as well as δ (C β -CH₃) motions, as indicated by substantial shifts observed for samples containing -CD₃ instead of -CH₃ group.⁵⁵ Likewise, bands appearing between 400–440 cm⁻¹ are assigned to “vinyl bending” modes, which also contain C β -CH₃ bending contributions.^{52, 53} It is noted that a mode occurring at 391 cm⁻¹ is most reasonably attributed to C β CH₃ bending motions.⁵² Other than an apparently small percentage of glycerol in samples from *Lpg* compared to *Pa* and *Vc* (415 and 485 cm⁻¹), there are no significant differences in traces B and C compared to A.

Significantly, all three NosP samples showed a high intensity feature at 693 cm⁻¹ (γ_{20}) in the low-frequency region, and the 5-coordinated high spin feature occurs at 1492 cm⁻¹ in the high-frequency region, suggesting a highly distorted heme cofactor.⁵⁶ The biological significance of distorted heme cofactors has been discussed for many other heme proteins; ^{57–61} for example, heme ruffling distortion in cytochrome c is important for controlling the electron transport mechanism.⁶² The crystallographic structure of the H-NOX domain from *Caldanaerobacter subterraneus* (*Cs* H-NOX, formerly identified as *Thermoanaerobacter tengcongensis*, *Tt* H-NOX) contains a highly distorted heme.⁶³ It is demonstrated that such distortion is maintained by a proline residue near the heme in the proximal pocket, and mutating this residue to alanine can eliminate the distortion, leading to a flattened heme, which lowered the redox potential of heme iron and caused notable conformational changes of the N-terminus of the protein.⁶⁴ Significantly, this flattened heme effectively mimics the model of the activated NO bound form,⁶⁵ suggesting that NO binding causes heme relaxation and induces the overall protein conformational changes which regulate signal transduction.

Ferrous NosP exists in equilibrium between 5-coordinate high spin and 6-coordinate low spin states.

As shown in Figure 5, all three ferrous proteins exhibit similar spectral patterns in both the low and high frequency regions. The high-frequency region exhibits the oxidation band (ν_4) at 1358 cm⁻¹, indicating the presence of ferrous iron. The spin state markers, ν_3 and ν_2 , occur at 1494 cm⁻¹ and 1588 cm⁻¹ respectively, which indicate a 6-coordinate low spin ferrous heme, consistent with the split α/β observed in the absorbance spectrum. The presence of 6-coordinated low spin state could be attributed to coordination of water or an unidentified residue from the distal heme pocket. The $\nu(\text{Fe-His})$ for ferrous histidyl-ligated heme is typically observed in the 200 to 230 cm⁻¹ spectral region.^{166, 67} The 213 cm⁻¹ feature present in all three ferrous NosP samples suggests an iron histidine vibrational mode in this region.

¹The abbreviations used are: NosP, nitric oxide sensing protein; *Pa*, *Pseudomonas aeruginosa*; *Vc*, *Vibrio cholerae*; *Lpg*, *Legionella pneumophila*; NO, nitric oxide; CO, carbon monoxide; CN, cyanide; rR, resonance Raman; His, histidine; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; H-NOX, heme nitric-oxide binding domain; cyclic-di-GMP, bis-(3'-5')-cyclic dimeric guanosine monophosphate; 5c or 6c, 5 coordinate or 6 coordinate (referring to the coordination number of the heme iron); OD, optical density; IPTG, isopropyl β -D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; FPLC, fast protein liquid chromatography; LC/UV/MS or LC/MS/DAD, liquid chromatography mass spectrometry with UV/visible diode array absorbance spectrometer, DEA-NONOate, diethylamine NONOate (a NO donor molecule); nm, nanometers; m/z, mass/charge number; HS or LS, high-spin or low-spin; mV, millivolts.

The suggestion that the 213 cm^{-1} band could be the $\nu(\text{Fe-N}_{\text{his}})$ stretching mode would require the presence of the ferrous high spin marker near 1470 cm^{-1} , which can be difficult to confirm in the presence of glycerol, which exhibits a band at this energy. Thus, all ferrous NosP samples were depleted of glycerol by buffer exchange techniques. As shown in Figure 5, the glycerol-free spectra all exhibit a feature at 1470 cm^{-1} , assignable to the ferrous high spin ν_3 mode. It is also noted that the high spin population seems to vary among the proteins from various sources, being relatively weak in NosP from *Pa*, but relatively strong in NosP from *Vc* and *Lpg*. It is important to note that these intensity changes are consistent with the changing intensities of the low frequency 213 cm^{-1} band (Figure 5, left panel). Subsequent experiments were performed to confirm an, albeit small, H/D shift similar to that seen for myoglobin,⁶⁸ but this shift was not observed. Despite failing to see the H/D isotope shift, the behavior in the high frequency region strongly supports the conclusion that the ferrous form is histidine ligated. This assignment is further supported in the spectrum obtained with 441.6 nm excitation line, from a He-Cd laser, where the enhancement of the 213 cm^{-1} band was observed to increase relative to the heme modes for all three samples, as shown in Figure 5, inset.⁶⁹

CO binds to NosPs to form a 6-coordinate complex where histidine does not dissociate.

Heme proteins readily bind exogenous diatomic gaseous ligands (XY) such as CO, NO and O_2 to trigger key biological processes.⁷⁰ Interactions with surrounding amino acid residues affect the bond strength of the Fe-X and X-Y linkages, which is reflected in changes of the intensity and frequencies of vibrational modes detected by resonance Raman spectroscopy. Well-established backbonding correlations exist, resulting from electrons being donated from the Fe $d\pi$ orbitals to the ligand π^* orbitals. The resulting negative linear correlations between the $\nu(\text{Fe-X})$ and $\nu(\text{X-Y})$ modes can provide useful information regarding the heme-protein interactions, such as H-bonding and electrostatic effects in the distal pocket and the trans axial ligand properties.^{31, 71} The CO adducts of heme proteins are particularly useful in probing the protein environment, as they are relatively chemically stable and there is a well-established spectra-structure correlation existing for these adducts.^{71, 72} The CO ligand has an empty π^* orbital and thus forms a linear adduct for which $d\pi-\pi^*$ overlaps in a perpendicular directions to maximize the backbonding interactions.⁷³

The rR spectra of the ferrous-CO NosP complexes are shown in Figure 6. All three samples exhibit spectra corresponding to a 6-coordinated low spin species, with the ν_4 , ν_3 , and ν_2 modes occurring at 1373 , 1498 and 1590 cm^{-1} , respectively, consistent with previously published data for other heme proteins.^{74, 75} No evidence of a band at 1358 cm^{-1} is detected, which is a marker band of unligated ferrous heme, demonstrating that the spectra are not contaminated with modes of photo-dissociated species. All three samples exhibit quite similar spectral patterns, with the $\nu(\text{Fe-C})$ frequency observed at the range of 496 to 497 cm^{-1} , with its correlated C-O stretching mode at $1955\sim 1959\text{ cm}^{-1}$. The feature observed at 574 cm^{-1} is assigned to the $\delta(\text{Fe-C-O})$ bending mode of Fe-C-O fragment. These assignments are within the typical range of well-documented frequencies of other heme proteins, which have generally been verified by ^{13}CO isotope substitution.⁷⁵⁻⁷⁷

A plot of the inverse back-bonding correlation line between $\nu(\text{Fe-C})$ and $\nu(\text{C-O})$ frequencies for many hemoproteins is shown in Figure 7. Three distinct correlations are evident, each corresponding to the proteins bearing different axial ligands; i.e., the three lines corresponding to the histidine-ligated hemoproteins ($\text{L}=\text{His}$),^{31, 78, 79} the P450cam-type hemoproteins ($\text{L}=\text{thiolate}$),^{80–82} and nitric oxide synthase enzymes ($\text{L}=\text{thiolate}$, but H-bonded with a nearby Trp residue).^{78, 79} The points determined for the CO adducts of the NosP proteins fall between the line generated for the thiolate-ligated enzymes and that derived for proteins bearing a trans axial histidine ligand, these rR data strongly supporting the presence of a histidine proximal axial ligand for these NosP proteins.

Heme-CO vibrational modes have previously been used to characterize the protein environment of the H-NOX family, specifically for *Cs* H-NOX and its mutants.⁸³ Interestingly, a quite high C-O stretching frequency was observed at 1987 cm^{-1} in wild-type *Cs* H-NOX, with a $\nu(\text{Fe-C})$ mode at 492 cm^{-1} . Similar behavior has also been observed in the H64V/V68T myoglobin mutant, which has a highly negatively charged polar environment in its distal heme pocket due to reorientation of residues upon mutation (i.e., $\nu(\text{C-O}) = 1984\text{ cm}^{-1}$).^{84, 85} Thus, it has been suggested that the high C-O frequency in wild-type *Cs* H-NOX is attributable a negatively charged polar environment, likely due to the interaction between the CO molecule and lone pair electrons on the $-\text{OH}$ group of Tyr-140. As expected, mutation of Tyr140 in *Cs* H-NOX to the more hydrophobic phenylalanine residue lead to a diminished negative polarity and the C-O stretching mode lowered to 1950 cm^{-1} . Given this result, we therefore conclude these three NosP proteins, which exhibit a C-O stretching mode between $1955\text{--}1959\text{ cm}^{-1}$, are likely exposed to a relatively nonpolar environment in the NosP distal pocket.

NO binds to ferrous NosPs, causing dissociation of the trans axial histidine and formation of a 5-coordinate complex.

As NosP proteins are predicted to function as receptors for gaseous NO, which is a key regulator of bacterial biofilm formation, characterization of the interaction between NosP and NO is essential to understanding how these proteins fulfill this important biological role. Similar to the ferrous CO adducts, the ferrous NO adducts have an Fe(II) to $\text{NO}(\pi^*)$ back-bonding interaction and a negative correlation between the $\nu(\text{Fe-N})$ and $\nu(\text{N-O})$ rR frequencies, which is dependent on interactions between the bound ligand and the protein environment, where an increased Fe-N bond strength leads to a weakened N-O bond. However, the NO molecule has a single electron in the π^* orbital, which leads to an inherently bent geometry (the Fe-N-O angle is $\sim 140^\circ$). The assigned 5- and 6-coordinate ferrous NO adducts of myoglobin exhibit the $\nu(\text{Fe-N})$ stretch at 521 and 552 cm^{-1} .⁸⁶ In many ferrous nitrosyl complexes, however, NO binding promotes the ejection of the proximal ligand as NO exerts a strong trans effect on ferrous iron, promoting 5-coordinate NO complexes in many hemoproteins, such as sGC and some H-NOX proteins; this process is considered as essential in activating the function of these proteins.⁵⁰ A good number of $\nu(\text{Fe-N})/(\text{N-O})$ frequencies have been collected for Fe(II)-NO adducts of heme proteins and model compounds in both 5-coordinate and 6-coordinate forms. While relatively low $\nu(\text{Fe-N})$ modes are seen for 5-coordinate NO complexes, occurring near $\sim 510\text{--}530\text{ cm}^{-1}$, with associated N-O frequencies near $\sim 1660\text{--}1700\text{ cm}^{-1}$, 6-coordinate NO adducts exhibit higher

Fe-N frequencies (in the range of ~ 550 – 580 cm^{-1}) along with higher N-O frequencies (~ 1620 – 1650 cm^{-1}).^{31, 87}

The rR spectra of NosP from *V. cholerae* complexed with NO are shown in Figure 8, where the left panels show spectra for low frequency while the right panels are those for high frequency. Within each panel, the top and bottom traces correspond to the ^{14}NO and ^{15}NO complex, respectively, and the middle trace is the difference spectrum. In the high frequency region (Figure 8, right panel), the oxidation state marker band ν_4 is observed at 1375 cm^{-1} and spin state markers ν_3 , ν_2 and ν_{10} occur at 1507 cm^{-1} , 1591 cm^{-1} and 1642 cm^{-1} , respectively, which are consistent with values typically seen for a 5-coordinated low spin heme. Distinctive difference patterns for Fe(II)- ^{14}NO and Fe(II)- ^{15}NO adducts (Figure 8, middle traces) are seen both in the high frequency and low frequency regions. A clear difference pattern is seen at $1669/1635\text{ cm}^{-1}$, a pattern consistent with a 5-coordinate low spin complex. Though generally of lower quality, the low frequency spectra also produce a slightly less clear difference pattern with components at $\sim 527/511\text{ cm}^{-1}$, also consistent with a 5-coordinate low spin complex, noting that the derived isotopic shifts on these two patterns are those expected. It is noted that the intensity of the $\nu(\text{Fe}-^{15}\text{N})$ is relatively higher than $\nu(\text{Fe}-^{14}\text{N})$; this is attributable to the fact that the Fe(II)- ^{14}NO spectrum was contaminated by the photo-dissociated species, as evidenced by the detected shoulder peak at 1358 cm^{-1} , which is a marker band of ferrous heme. Thus, the frequencies occurring at 527 cm^{-1} and 1669 cm^{-1} are assigned as $\nu(\text{Fe}-\text{N})$ and $\nu(\text{N}-\text{O})$ stretching modes, respectively. These results provide further evidence that a 5-coordinated heme complex was formed upon NO binding.

In the case of NosP from *P. aeruginosa*, the resonance Raman spectrum in the region between 1300 – 1750 cm^{-1} (Figure 9, right panel) reveals the oxidation marker occurs at 1374 cm^{-1} and spin state marker bands ν_3 , ν_2 and ν_{10} occur at 1508 cm^{-1} , 1591 cm^{-1} and 1642 cm^{-1} , respectively, which are typical of 5-coordinate low spin heme. The $\nu(\text{Fe}-\text{N})$ frequency is identified at 525 cm^{-1} from its 15 cm^{-1} downshift with ^{15}NO , while the isotope replacement reveals that the $\nu(\text{N}-\text{O})$ vibration occurs at 1671 cm^{-1} with the expected 35 cm^{-1} isotope shift. Again, the spectrum of the ^{14}N - isotopomer reveals evidence of photodissociation, as reflected by the appearance of the 1358 cm^{-1} ferrous photoproduct.

Turning attention to the NO adducts of NosP from *L. pneumophila*, it is first noted that during spectral acquisition for these samples, more extensive sample precipitation occurred. In the high frequency region (Figure 10, right panel), the ν_4 oxidation state marker band is located at 1375 cm^{-1} , while the spin state marker bands, the ν_3 and ν_2 modes, are seen at 1508 and 1593 cm^{-1} , respectively, spectra again characteristic of a low spin 5-coordinate NO adduct. The $\nu(\text{N}-\text{O})$ bands appear at 1665 cm^{-1} for $^{14}\text{N}-\text{O}$ and 1632 cm^{-1} for the ^{15}N analogue, in close agreement with the shift expected for a diatomic harmonic oscillator. In the low frequency region the raw spectra show relatively high background (small S/N), with a difference trace exhibiting discernible positive and negative features. The difference feature revealing peaks at 530 cm^{-1} (^{14}N) and 516 cm^{-1} (^{15}N) yields the expected isotopic shift for a $\nu(\text{Fe}-\text{N})$ mode. Two positive features, seen at 345 , 675 and 754 cm^{-1} , appear in the difference trace, owing to a slightly larger concentration (less precipitation) of the ^{14}N isotopomer.

***Lpg* NosP has the most electropositive heme environment relative to *Vc* and *Pa* NosP.**

Figure 11 shows well-established inverse correlation plots for 5-coordinate NO proteins, which are useful for probing the environment of the distal pocket. All three of these NosP proteins exhibit frequencies close to the back-bonding line, within which the NosP from *L. pneumophila* shows higher frequencies (530 cm^{-1}) than NosP from *V. cholerae* (527 cm^{-1}) or *P. aeruginosa* (525 cm^{-1}). It has been shown that H-bonding to O_{NO} will strengthen back-bonding in ferrous nitrosyl proteins, yielding higher $\nu(\text{Fe-N})$ and lower $\nu(\text{N-O})$ frequencies.³¹ This would indicate that the NosP from *L. pneumophila* possibly forms stronger H-bonds to the surrounding protein residues than the NosPs from *V. cholerae* or *P. aeruginosa*. Alternatively, the NosP from *L. pneumophila* may have a relatively more positive electrostatic environment in the distal pocket than the other two proteins, a hypothesis that we tested as described below.

We measured the reduction potentials for each NosP protein in this study. Heme redox potential were measured using the Efimov et al. adaptation⁴⁰ of Massey's method.³⁹ Massey proposed that heme redox potentials could be determined using the oxidation of xanthine to uric acid as an electron donor in a solution with the heme protein and a dye with a known reduction potential. From the difference in percent reduction of the protein and of the dye in equilibrium, the difference in their reduction potentials is extrapolated. An ideal dye for this experiment has a reduction potential as close as possible to that of the heme, with the assay becoming less reliable at $> 30\text{ mV}$ separation.

In order to determine which dye would be appropriate for NosP, we tested several dyes until we found one with a reduction potential closest to that of the NosPs, methylene blue. Methylene blue is a well characterized dye with reduction potentials characterized across the pH range.⁸⁹ The reduction potential for *Pa* NosP at pH 7.0 is near the edge of the 30 mV boundary, so we also confirmed its reduction potential using 5,5',7,7'-indigotetrasulphonate (reduction potential at pH 7.0 = -46 ,⁹⁰ supplemental Figure 4). Reduction potentials of heme proteins shift with pH, as side chains and heme propionate groups change protonation states.^{91, 92} It has been suggested that effects of pH on reduction potential will be mitigated in proteins where the heme is deeply buried away from solvent effects.⁷⁰

Using the reduction potentials for methylene blue, 11 and -20 mV for pH 7.0 and 8.0, respectively, we determined reduction potentials for NosP proteins. Figure 12 (panels A-C) illustrates characteristic plots of $\frac{RT}{nF} \ln([\text{Dye}_{\text{ox}}]/[\text{Dye}_{\text{red}}])$ against $\frac{RT}{nF} \ln([\text{NosP}_{\text{ox}}]/[\text{NosP}_{\text{red}}])$ with a slope equal to 1, indicating the electron transfer is in equilibrium. At pH 7.0, the reduction potentials for *Lpg*, *Vc* and *Pa* NosPs were measured to be -1 ± 3 , -9 ± 3 and $-31 \pm 7\text{ mV}$, respectively, while at pH 8.0 they were -13 ± 2 , -29 ± 10 and $-59 \pm 6\text{ mV}$, respectively (Figure 12, panel D). This trend is consistent with what was observed in the Fe(II)-NO resonance Raman backbonding energies, where *Lpg* NosP has the strongest Fe-N bond (530 cm^{-1}) and therefore the weakest N-O bond (1665 cm^{-1}). *Lpg* NosP also showed the smallest change in reduction potential with the shift of one pH unit, which may indicate more protein-heme interactions are present, including a more deeply buried heme, more H-bonding to residues in the heme pocket, or a more hydrophobic heme binding pocket.

In summary, it is proposed that upon binding NO, NosP proteins form a 5-coordinated heme with NO as the axial ligand, displacing a histidine ligand. This behavior is similar to NO sensing observed in sGC and H-NOX proteins.^{93–96} Finally, it has been widely reported that the nature of the iron histidine stretching mode $\nu(\text{Fe-His})$ is closely correlated with ligand-induced dissociation of proximal histidine.^{97, 98} Native myoglobin, which forms a 6-coordinate NO adduct, exhibits a $\nu(\text{Fe-N})$ structure mode at 220 cm^{-1} .⁵³ Conversely, sGC forms a 5-coordinate NO complex, which exhibits a low Fe-N_{his} stretching mode that occurs at 204 cm^{-1} . There are exceptions, however, H-NOX from *V. cholerae* has a high energy iron-histidine bond ($\nu(\text{Fe-N}) = 224\text{ cm}^{-1}$), but forms a 5-coordinate NO complex. The frequency of the Fe(II)-His stretching mode of NosP (213 cm^{-1}) is between those of Mb and sGC, and NosP forms a 5-coordinate NO adduct. Collectively, these data suggest the correlation between the Fe-His bond strength and coordination number of the NO complex must also be influenced by different architectures in both the distal and proximal pockets, such as H-bonding interactions or steric effects on the Fe-His bond, as well as interactions of the heme peripheral groups with heme pocket protein residues that cause out-of-plane heme distortions.^{49, 60, 65}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Soret peaks of *Lpg* NosP with inlay of α/β peaks in ferrous unligated, ferrous CO and Ferrous NO complexes. (B) NosP Fe(III)-CN complexes. Soret peaks with inlay of α/β peak for ferric cyanide complexes for NosP from *P. aeruginosa*, *L. pneumophila* and *V. cholerae*.

Figure 2.

Dissociation of NO from *Lpg* NosP. NO bound NosP was incubated with a dithionite solution saturated with CO. As NO dissociates, rapid binding of CO occurs giving a large shift in Soret absorbance, while NO reacts with dithionite to prevent rebinding. The difference in the absorbance change due to NO (395 nm) dissociation and the absorbance change due to CO association (420 nm), over time, was fit to a two-term exponential function. The dissociation rate constants determined are independent of dithionite and CO concentration.

Figure 3.

High-frequency rR spectra of homologous ferric NosP proteins. Spectra were measured at room temperature with an excitation line at 406.7 nm. The 1467 cm^{-1} band is attributed to glycerol. (A) NosP from *P. aeruginosa* (B) NosP from *V. cholerae* (C) NosP from *L. pneumophila*.

Figure 4.

Low-frequency rR spectra of homologous ferric NosP proteins. Spectra were measured at room temperature with an excitation line at 406.7 nm. (A) NosP from *P. aeruginosa* (B) NosP from *V. cholerae* (C) NosP from *L. pneumophila*.

Figure 5.

Low-frequency (left panel) and high-frequency (right panel) rR spectra of homologous ferrous NosP proteins (in the absence of glycerol). The inset shows bands in the low frequency region acquired with the 441.6 nm excitation line from a HeCd laser. (A) NosP protein- from *V. cholerae* (B) NosP protein- from *P. aeruginosa* (C) NosP protein- from *L. pneumophila*.

Figure 6. rR spectra of ferrous CO NosP complexes. The left panel shows the low frequency region, the middle panel shows the high frequency region, and the right panel exhibits the region above 1900 cm^{-1} where the C-O stretching mode occurs. The 2049 cm^{-1} band is a combination of ν_7 and ν_4 . Spectra were normalized to ν_7 and ν_4 in low-frequency and high-frequency regions, respectively. (A) NosP from *P. aeruginosa* (B) NosP from *V. cholerae* (C) NosP from *L. pneumophila*.

Figure 7.

Diagrams showing an inverse correlation for cytochromes P450 (purple; all contain heme-thiolate linkages), nitric oxide synthases (grey; all contain a heme-histidine linkage where this His is H-bonded to a Trp), and myoglobins (red; heme-histidine linkages). The blue dot represents the NosP from *P. aeruginosa*, the green dot is the NosP from *V. cholerae* and the yellow dot is NosP from *L. pneumophila*.

Figure 8.

Resonance Raman spectra of nitrosyl complexes of NosP from *V. cholerae*. Left panel shows the low-frequency region while the right panel shows the high frequency region. Middle traces represent nitrosyl isotope (^{14}NO – ^{15}NO) difference spectra.

Figure 9.

Resonance Raman spectra of nitrosyl complexes of NosP from *P. aeruginosa*. Left panel shows the low-frequency region while the right panel shows the high frequency region. Middle traces represent nitrosyl isotope (^{14}NO – ^{15}NO) difference spectra.

Figure 10.

Resonance Raman spectra of nitrosyl complexes of NosP from *L. pneumophila*. Left panel shows the low-frequency region while the right panel shows the high frequency region. Middle traces represent nitrosyl isotope (^{14}NO – ^{15}NO) difference spectra. It is noted that during spectral acquisition for these samples, more extensive sample precipitation occurred.

Figure 11.

The NO back-bonding correlation line of 5-coordinated NO proteins. The numbers represent proteins listed in Table 3.

Figure 12.

Linear plots of (A) *Pa* NosP (B) *Vc* NosP and (C) *Lpg* NosP during equilibrium reduction with methylene blue. Slopes of 1 indicate the equilibrium state and the y-intercept is the difference between the reduction potential of methylene blue and the reduction potential of the relevant NosP. (D) Summary of reduction potentials measured for NosPs at pH 7.0 and 8.0

Table 1.Soret and α/β peaks of NosP proteins compared to sGC

Fe (II)		Soret (nm)	α (nm)	β (nm)	Reference:
	<i>Lpg</i> NosP	417	526	553	This work
	<i>Vc</i> NosP	415	529	553	18
	<i>Pa</i> NosP	420	524	554	11
	sGC	431	555		41
Fe(II)-NO					
	<i>Lpg</i> NosP	395	533	566	This work
	<i>Vc</i> NosP	395	533	563	18
	<i>Pa</i> NosP	396	534	574	11
	sGC	398	537	571	41
Fe(II)-CO					
	<i>Lpg</i> NosP	417	535	563	This work
	<i>Vc</i> NosP	416	535	563	18
	<i>Pa</i> NosP	416	538	565	11
	sGC	423	541	567	41
Fe(III)-CN					
	<i>Lpg</i> NosP	419	544		This work
	<i>Vc</i> NosP	420	538		This work
	<i>Pa</i> NosP	418	538		This work
	sGC	418	538		46

Table 2.

NO dissociation rate constants for NosPs and sGC

Protein	NO _{off} Rate Constant	Reference
<i>Lpg</i> NosP	$< \sim 2 \times 10^{-4} \text{ s}^{-1}$	This work
<i>Vc</i> NosP	$(4.6 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$	18
<i>Pt</i> NosP	$(1.8 \pm 0.5) \times 10^{-4} \text{ s}^{-1}$	11
sGC	$(3.6 \pm 0.8) \times 10^{-4} \text{ s}^{-1}$	41

Table 3.RR frequencies of 5-coordinate NO hemoproteins.⁸⁸

Protein	$\nu(\text{N-O}) \text{ cm}^{-1}$	$\nu(\text{Fe-N}) \text{ cm}^{-1}$
<i>Alcaligenes xylosoxidans</i> Cytochrome c' (AXCP)	1664	531
Cytochrome c' R124K	1657	533
Cytochrome c' R124F	1662	529
Cytochrome c' R124Q	1662	532
Cytochrome c' R124E	1661	533
FixL	1675	525
H93YMb	1672	524
CooA	1672	523
sGC	1680	522
NosP from <i>Vc</i>	1669	527
NosP from <i>Pa</i>	1671	525
NosP from <i>Lpg</i>	1665	530