Supplementary Information

Regulation of nitric oxide signaling through selective formation of a distal NO receptor-ligand complex

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Supplementary Results

Supplementary Notes

Discussion of computational analysis of MTSL rotamers

The conformational dynamics of the MTSL spin label were analyzed using the crystal structure of So H-NOX with NO bound on the proximal face of the heme (PDB code 4U9B).\(^1\) This structure is an appropriate starting point for modeling the spectroscopically detected species because the NO-bound forms of So H-NOX (whether proximal or distal, five-coordinate or six-coordinate) are expected to adopt similar global conformations, with His 103 not ligated to the Fe center. Moreover, the site to which the spin label is attached (Cys 17) is far removed from the heme and its dynamics are expected to be largely uncoupled to conformational changes associated with ligand binding at the heme Fe center. In order to account for small structural differences between five- and six-coordinate Fe(II)–NO species, we constructed a distal, five-coordinate Fe(II)–NO model by inverting the Fe, N, and O atomic coordinates of the proximal, five-coordinate Fe(II)–NO structure about the center of the heme. A six-coordinate, proximal Fe(II)–NO model was built by translating the Fe(II)–NO unit so that the Fe atom is positioned in the center of the heme (a very minor perturbation that was performed for the sake of completeness). The relevant coordinates are given in Supplementary Table 2. The effects of the Fe–N–O angle and the heme–Fe–N–O dihedral angle were ignored because these values are unknown for the solution-state species. Note that both models have identical
ensembles of spin label rotamers because the models differ only in the atomic coordinates of the Fe(II)–NO unit.

For both models, the resulting ensemble of distances can be divided into three distinct groups (Supplementary Fig. 6 and 7): a shorter set (MTSL–Fe ≈ 2.1 nm), an intermediate set (MTSL–Fe ≈ 2.5 nm), and a minor, longer set (MTSL–Fe ≈ 3.0 nm). The distribution of conformers was highly dependent on the freezing temperature specified in the calculation; as such, we chose the default value of 175 K to approximate the water/glycerol glassing temperature and to make the analysis as unbiased as possible. Neither the shorter nor the longer ensemble of conformations corresponds to the experimentally observed distances, so we focus our discussion on the intermediate set of distances. Regardless, all sets of distances show the same changes upon NO coordination to the distal vs proximal faces (*vide infra*).

For the distal, five-coordinate Fe(II)–NO structure, we obtain the following MTSL–atom distances: MTSL–Fe = 2.5 nm, MTSL–N = 2.35 nm, and MTSL–O = 2.3 nm. Assuming a spin distribution$^{2,3}$ of 0.47 spins on Fe and 0.53 spins on NO, and equal spin distribution on N and O (that is, 0.265 spins on each atom), we obtain a weighted distance distribution centered at 2.4 nm. This compares well with the experimentally determined distance of 2.3 nm. For the proximal, six-coordinate Fe(II)–NO structure, we obtain the following MTSL–atom distances: MTSL–Fe = 2.5 nm, MTSL–N = 2.7 nm, and MTSL–O = 2.75 nm. Assuming a spin distribution$^{2,3}$ of 0.21 spins on Fe and 0.78 spins on NO, and equal spin distribution on N and O (that
is, 0.39 spins on each atom), we obtain a weighted distance distribution for the MTSL–Fe(II)–NO spin pair centered at 2.7 nm, which is also similar to the experimentally derived distance of 2.8 nm. Thus, the computational results show that the effective MTSL–Fe(II)–NO distance should differ by approximately 0.3 nm between NO bound on the distal and proximal faces of the heme. Finally, we note that the computed distance distributions based on the entire ensemble of computed MTSL rotamers shift by ~0.3 nm between distal- and proximal-NO complexes.

**Discussion of factors that affect the analysis of DEER spectra**

The aim of the EPR/DEER component of our study is to observe how the Fe(II)–NO speciation changes with increasing NO concentration and to track differences in effective distances between the MTSL spin label and the Fe(II)–NO units. We approximate the Fe(II)–NO spins as if they are points, and this approximation is valid only if (i) there is little difference in spin distribution across the Fe(II)–NO units in the two observed Fe(II)–NO species and (ii) the effects of orientation selection on the DEER experiment are either minimal, or the DEER spectra of the two species sample similar orientations of the Fe(II)–NO unit. We address these two matters below.

*Spin distribution in the Fe(II)–NO species*: The two Fe(II)-NO species of interest in our study are a five-coordinate species that forms under relatively low NO concentrations and a six-coordinate species that forms under very high NO concentrations. The spin distribution in five- and six-coordinate Fe(II)–NO
complexes has been extensively studied,\textsuperscript{2,3} and it is estimated that five-coordinate Fe(II)–NO species have somewhat higher spin density on the (heme)Fe unit (0.47 vs. 0.21 spins) and lower spin density on the NO ligand (0.53 vs. 0.78 spins). Given the somewhat different spin distributions, we sought to estimate the maximum change in effective distance that might be expected as result of their different electronic structures (but not due to other factors such as the face to which NO binds). If we adopt reasonable geometrical parameters and spin values from the literature\textsuperscript{2-4} (Supplementary Fig. 8), fix the Fe–N–O angle to 180° (resulting in a maximal shift in spin distribution along the Fe–MTSL vector), and assume that the spin distribution across the NO unit is equally distributed between N and O, we obtain weighted centers for the Fe(II)–NO spin density that differ by ~0.6 Å (0.06 nm) (Supplementary Fig. 8).

Given that the Fe, N, and O atoms and the nitroxide spin are not perfectly aligned in the real system, the effect of the different electronic structures on the distance distribution should be significantly lower that what is estimated here. The experimentally observed distance difference is much greater—~0.4 nm (4 Å)—and we therefore conclude that this cannot be attributed to the generation of five- and six-coordinate species with NO bound on the same face of the heme.

\textit{Orientation selection:} The EPR spectrum of the Fe(II)–NO species is sufficiently broad that the probe pulses excite only certain orientations. Therefore, the effective distances between the five- and six-coordinate species are most reliably compared
when the DEER experiments probe similar orientations with respect to the molecular frame. DEER spectra were recorded with the probe pulses positioned at \( g = 2.0165 \). This corresponds to orientations along \( g_{\text{min}} \) of the five-coordinate species \((g = [2.111, 2.024, 2.013])\) and \( g_{\text{mid}} \) of the six-coordinate species \((g = [2.094, 2.014, 1.977])\). As has been noted elsewhere,\(^2\) the \( g_{\text{min}} \) vector in five-coordinate Fe(II)–NO species is most closely aligned with the Fe–N(O) vector, and the \( g_{\text{mid}} \) vector in six-coordinate Fe(II)–NO species is also most closely aligned with the Fe–N(O) vector (Supplementary Fig. 9). In addition, the MTSL spin label is nearly perpendicular to the plane of the heme (Supplementary Fig. 6), so the interspin vector is also closely aligned with the Fe–N(O) vector. Thus, to a reasonable approximation, the orientations that are probed in the DEER experiments are similarly aligned along the interspin vector for both the five- and the six-coordinate Fe(II)–NO species. Because the DEER experiments probe similar orientations with respect to the molecular frame, comparison of the two MTSL–Fe(II)–NO distances is appropriate.
References

1. Herzik Jr., M.A., Jonnalagadda, R., Kuriyan, J. & Marletta, M.A. Structural insights into the role of iron–histidine bond cleavage in nitric oxide-induced activation of H-NOX gas sensor proteins. *Proc. Natl. Acad. Sci. U.S.A.* **111**, E4156-E4164 (2014).

2. Goodrich, L.E., Paulat, F., Praneeth, V.K.K. & Lehnert, N. Electronic Structure of Heme-Nitrosyls and Its Significance for Nitric Oxide Reactivity, Sensing, Transport, and Toxicity in Biological Systems. *Inorganic Chemistry* **49**, 6293-6316 (2010).

3. Lehnert, N., Scheidt, W.R. & Wolf, M.W. Structure and Bonding in Heme–Nitrosyl Complexes and Implications for Biology. Vol. 154 155-223 (Springer Berlin Heidelberg, Berlin, Heidelberg, 2013).

4. Lehnert, N. EPR and Low-temperature MCD Spectroscopy of Ferrous Heme Nitrosyls. in *The Smallest Biomolecules: Diatomics and their Interactions with Heme Proteins* (ed. Ghosh, A.) 147–171 (Elsevier, Amsterdam, The Netherlands, 2008).

5. Martin, E., Berka, V., Sharina, I. & Tsai, A.L. Mechanism of binding of NO to soluble guanylyl cyclase: implication for the second NO binding to the heme proximal site. *Biochemistry* **51**, 2737-46 (2012).

6. Moore, E.G. & Gibson, Q.H. Cooperativity in the dissociation of nitric oxide from hemoglobin. *J Biol Chem* **251**, 2788-94 (1976).
Supplementary Tables

Supplementary Table 1. Comparison of NO dissociation rate constants from SoH-NOX treated with substoichiometric and excess NO.

CO/DT corresponds to the CO_sat/dithionite NO trap and DTCS corresponds to the ferro-di(N-(dithiocarboxy)sarcosine [Fe2+(DTCS)]2) NO trap. Values reported for SoH-NOX were averaged from multiple replicates. Error is reported in standard deviation. All values are from this work unless otherwise annotated. The amplitudes of each corresponding phase are shown as a percent of the calculated total.

| Protein          | NO treatment | \(k_1\) (s\(^{-1}\)) | \(k_2\) (s\(^{-1}\)) | \(\Delta A_1\) (%) | \(\Delta A_2\) (%) | Temp (°C) | Trap   |
|------------------|--------------|----------------------|----------------------|---------------------|---------------------|-----------|--------|
| S. oneidensis    | Excess       | 0.00035 ±0.00003     | 0.00007 ±0.00001     | 29                  | 71 ±5               | 10        | CO/DT  |
|                  |              | 0.00079 ±0.00008     | 0.00014 ±0.00001     | 21                  | 79 ±1               | 15        | CO/DT  |
|                  |              | 0.00059 ±0.00007     | 0.00016 ±0.00001     | 35                  | 65 ±4               | 15        | DTCS   |
|                  | Sub          | 0.00135 ±0.00010     | 0.00028 ±0.00004     | 29                  | 71 ±6               | 20        | CO/DT  |
|                  | Excess       | 0.00109 ±0.00005     | 0.00010 ±0.00001     | 19                  | 81 ±5               | 10        | CO/DT  |
|                  |              | 0.00087 ±0.00009     | 0.00014 ±0.00002     | 25                  | 75 ±2               | 15        | CO/DT  |
|                  |              | 0.00066 ±0.00005     | 0.00018 ±0.00001     | 33                  | 67 ±3               | 15        | DTCS   |
|                  |              | 0.00133 ±0.00006     | 0.00025 ±0.00001     | 32                  | 68 ±2               | 20        | CO/DT  |
| V. cholera       | Excess       | 0.01 N.R.            | 0.001 N.R.           | 29                  | 71 N.R.             | 24        | CO/DT *|
|                  | Sub          | 0.01 N.R.            | 0.001 N.R.           | 16                  | 84 N.R.             | 24        | CO/DT *|
| β1 (1-194)       | Excess       | 0.0030 0.0002        | 0.00013 ±0.00001     | 6                   | 94 ±1               | 10        | CO/DT **|
| β1 (1-385)       | Excess       | 0.0083 0.0022        | 0.00018 ±0.00004     | 7                   | 93 ±1               | 10        | CO/DT **|
| sGC              | Excess       | 0.0118 0.0071        | 0.00012 ±0.00002     | 5                   | 95 ±1               | 10        | CO/DT **|

* Values obtained from work done by Martin, E. *et al* \(^5\)

** Values obtained from work done by Moore, E. G. and Gibson, Q. H. \(^6\)

N.R. Values were not reported
**Supplementary Table 2. Coordinates of the Fe(II)-NO atoms used for DEER simulations.**

|        | Proximal, five-coordinate structure (4U9B)¹ | Distal, five-coordinate model | Proximal, six-coordinate model |
|--------|-------------------------------------------|-----------------------------|-----------------------------|
| Fe (Å) | [23.540, 103.239, 5.974]                  | [23.406, 102.887, 6.138]    | [23.473, 103.063, 6.056]    |
| N (Å)  | [24.149, 104.745, 5.205]                  | [22.797, 101.381, 6.907]    | [24.082, 104.569, 5.287]    |
| O (Å)  | [24.919, 105.748, 5.902]                  | [22.027, 100.378, 6.210]    | [24.852, 105.572, 5.984]    |
Supplementary figures

Supplementary figure 1. NO dissociation rate measured by two traps.
Top and middle panel: time courses for So H-NOX NO dissociation at 15 °C determined using a CO$_{sat}$/dithionite trap; bottom panel: time courses for So H-NOX NO dissociation at 15 °C determined using ferro-di(N(dithiocarboxy) sarcosine/dithionite trap. NO dissociation was monitored by electronic absorption spectroscopy (left). Raw data were extracted from the difference spectra (inset) and plotted against the acquisition time course (right) (black circles). Rates were determined by single (gray line) or double (red line) exponential fits (right) with the corresponding residuals shown above each plot. The time courses shown are representative of dissociation experiments repeated ≥ 5 times.
Supplementary figure 2. Q-band EPR spectra of So H-NOX treated with different NO concentrations.

Data is shown in black and total simulation is shown in magenta. Simulation parameters for five-coordinate distal Fe(II)–NO (green): \( g = [2.111, 2.024, 2.013], A^{(14)N} = [20, 20, 51] \text{ MHz}, g\text{-strain} = [0.019, 0.014, 0.001], A\text{-strain} = [40, 80, 0.1] \text{ MHz} \). Simulation parameters for six-coordinate proximal Fe(II)–NO (blue): \( g = [2.094, 2.014, 1.977], A^{(14)N} = [20, 30, 30] \text{ MHz}, g\text{-strain} = [0.011, 0.012, 0.008], A\text{-strain} = [40, 40, 40] \text{ MHz} \). Simulation parameters for minor Fe(II)–NO species (gray): \( g = [2.08, 2.05, 1.977], A^{(14)N} = [20, 30, 30] \text{ MHz}, g\text{-strain} = [0.03, 0.03, 0.01], A\text{-strain} = [40, 40, 40] \text{ MHz} \). See Experimental section for further details on data acquisition and simulation.
Supplementary figure 3. Optimization of site-specific MTSL labeling on So H-NOX C17.

a) So H-NOX titrated with different concentration of MTSL. MTSL was titrated into 10 μM So H-NOX with a protein : MTSL ratio from 1:1 up to 1:500. Protein : MTSL = 1:10 resulted in the best yield for +1 MTSL product (highlighted in green).

b) Time course of the MTSL reaction. 10 μM So H-NOX and 100 μM MTSL were incubated from 30 min to 5 hours. 30 min incubation resulted in the best yield for +1 MTSL product (highlighted in green).
Supplementary figure 4. Mass spectrometry characterization of So H-NOX-MTSL.
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of trypsin-digested So H-NOX-MTSL validated that C17 was the main MTSL labeling site. Main panel: intact protein mass spectrometry showed a mass increase of 184 Da after the MTSL labeling reaction, indicating only one molecule of MTSL is attached to the protein; inset table: LC-MS/MS analysis of trypsin-digested So H-NOX-MTSL demonstrated that C17 was the main MTSL modified cysteine residue based on relative ion abundances of MTSL-labeled and unlabeled peptides with batch-to-batch labeling efficiency of 60%-90%.

| Digest sample | Cys17 occupancy (%) * |
|---------------|------------------------|
| 1             | 56                     |
| 2             | 63                     |
| 3             | 61                     |
| 4             | 62                     |

* Based on relative ion abundances of MTSL-labeled and unlabeled peptides
Supplementary figure 5. Background subtraction of the DEER data.
a) DEER data (black) and background (blue). b) Corresponding background-subtracted DEER data (black) and fit (red) reproduced from Figure 2. The lower modulation depth for this sample may be attributed to the faster relaxation of the nitroxide spin label due to the presence of excess NO in solution. b) Corresponding distance distributions reproduced from Figure 2.
Supplementary figure 6. Computational analysis of MTSL rotamers.

a) Computationally generated C17–MTSL rotamers based on So H-NOX crystal structure (4U9B). MTSL occupancy is shown in gradient (white: low occupancy; red: high occupancy). Two main sets of rotamers are highlighted in dashed line circle with average distances labeled next to it. b) Relative location between distal-bound Fe(II)–NO to C17–MTSL rotamers. c) Relative location between proximal-bound Fe(II)–NO to C17–MTSL rotamers. Fe(II)–NO is highlighted in thicker sticks.
Supporting figure 7. Computed MTSL–atom distance distributions for Fe(II)-NO species.
a) Distal, five-coordinate Fe(II)–NO species. b) Proximal, six-coordinate Fe(II)–NO species. Each distribution corresponds to the distance between MTSL and the colored atom. c) The computed MTSL–Fe(II)–NO distance distribution generated as the sum of the individual MTSL–atom traces weighted by the spin distribution (distal, red; proximal, blue).
Supplementary figure 8. Determination of the largest anticipated difference in effective MTSL–Fe(II)–NO distance for five- versus six-coordinate Fe(II)–NO species.

Left: Representations of five- and six-coordinate Fe(II)–NO species with bond lengths indicated in red and spin distributions indicated in blue, both taken from Lehnert et al. Right: Representations of hypothetical five- and six-coordinate Fe(II)–NO species with the same bond metrics and spin distributions but with linearized Fe–N–O moieties, which should give the largest change in effective MTSL–Fe(II)–NO distance. The weighted centers of the spin distributions are indicated by blue dots.
Supplementary figure 9. Orientation of the $g$-tensor in Fe(II)-NO complexes. Adapted from Lehnert et al.$^2$
Supplementary figure 10. Intact protein mass spectrometry showed no mass increase for sample after NO treatment.

a) Intact protein mass spectrum of unlabeled So H-NOX. Mass determined was 22,640 Da.  
b) Intact protein mass spectrum of So H-NOX-MTSL before NO treatment. The main species showed a molecular mass of 22,825 Da, with a 185 Da mass increase from the unlabeled protein, corresponding to +1 MTSL product.  
c) Intact protein mass spectrum of So H-NOX-MTSL after NO treatment. The main mass detected in this spectrum was 22,828 Da. This 3 Da mass increase was due to hydrogen/deuterium exchange between the protein and D₂O-based buffer.
Supplementary figure 11. Q-band EPR spectra of So H-NOX treated with NO\(_{(g)}\).

So H-NOX (200 μM) was treated with different concentrations of NO\(_{(g)}\). Data is shown in black and total simulation is shown in magenta. Simulation parameters are nearly identical to those used for Figure 2 and Supplementary figure 2: five-coordinate distal Fe(II)–NO (green): \(g = [2.111, 2.024, 2.0133]\), \(A^{(14N)} = [20, 20, 51]\) MHz, \(g\)-strain = \([0.018, 0.014, 0.001]\), \(A\)-strain = \([40, 80, 0.1]\) MHz. Simulation parameters for six-coordinate proximal Fe(II)–NO (blue): \(g = [2.092, 2.012, 1.972]\), \(A^{(14N)} = [20, 30, 30]\) MHz, \(g\)-strain = \([0.010, 0.012, 0.010]\), \(A\)-strain = \([40, 40, 40]\) MHz. Simulation parameters for minor Fe(II)–NO species (gray): \(g = [2.08, 2.05, 1.972]\), \(A^{(14N)} = [20, 30, 30]\) MHz, \(g\)-strain = \([0.03, 0.03, 0.01]\), \(A\)-strain = \([40, 40, 40]\) MHz.
MHz.
Supplementary figure 12. So H-NOX signaling assay with different NO concentrations.