

## Supporting Information

### Resolution of the Spectroscopy vs. Crystallography issue for NO intermediates of Nitrite Reductase from *Rhodobacter Sphaeroides*

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All reagents were of the highest grade commercially available and were used without further purification. *R<sub>s</sub>NiR* was isolated and purified (pH~7.2) as previously reported in Zhao, Y.; Lukoyanov, D. A.; Toropov, Y. V.; Wu, K.; Shapleigh, J. P.; Scholes, C. P. *Biochem.* **2002**, *41*, 7464; Olesen, K.; Veselov, A.; Zhao, Y.; Wang, Y.; Danner, B.; Scholes, C. P.; Shapleigh, J. P. *Biochem.* **1998**, *37*, 6086.

For the production of reduced NiR samples for the NO reaction, NiR having a subunit concentration of 0.5 mM, 50 mM pH 7.2 phosphate buffer, and 10  $\mu$ M PMS redox mediator (phenazine methosulfate, Sigma), was deaerated by Argon exchange for 5 min with gentle agitation under a continuously flowing, scrubbed Argon atmosphere. A 2-fold molar excess of deaerated, Argon-exchanged NADH (Sigma) reductant was added. Scrubbed NO (made by passing highest grade NO gas from Praxair through thoroughly degassed 1M NaOH and H<sub>2</sub>O solutions) saturated buffer solutions (~ 2mM) was added to the reduced enzyme in a thoroughly degassed glove bag and then the sample was transferred to an EPR tube where the sample was frozen. The same results were obtained when ascorbate was used as the reductant. For intermediate trapping, sub-stoichiometric amounts of NO were required. For enzyme: NO concentrations of 1:10, 1:2, the final product i.e. the nitrite bound T2 site was formed and no intermediates were observed. For enzyme: NO concentrations of 10:1, all the intermediates were observed at different times. The reduced enzyme and NO solution were syringed and immediately frozen in an EPR tube with isopentane immersed in a methanol/dry ice bath, which was then placed in liquid nitrogen. In the sub-stoichiometric (10:1 enzyme:NO) amounts of NO, the first intermediate (Cu<sup>+</sup>NO<sup>-</sup>) was observed in 1-2 seconds, followed by a ~ 1:1 mixture of the T2 oxidized and nitrite bound to T2 site which was trapped in 4-6 seconds, and this generated the nitrite bound form at longer time scales (18-20 seconds). The reaction scheme with kinetic rates is given in Scheme S2, Figure S7.

For the nitrite bound samples, one equivalent nitrite was added to the enzyme, followed by reduction with ascorbate which reduces the T1 Cu (pH ~7.2).

In evaluating for the possibility of generation of N<sub>2</sub>O in the reaction mixture, the above reaction was performed and an outlet syringe from the reaction vial was used to saturate the buffer solution used for the activity assay. Excess reductant was added for multiple turn-overs to generate enough N<sub>2</sub>O required for observable N<sub>2</sub>OR activity. Simultaneously, *PnN<sub>2</sub>OR* enzyme was incubated in excess of degassed solution of methyl viologen and dithionite in Tris buffer (pH~7.3), in the glove box (required to activate the enzyme, Ghosh, S.; Gorelsky, S. I.; Chen, P.; Cabrito, I.; Moura, J. J. G.; Moura, I.; Solomon, E. I. *J. Am. Chem. Soc.* **2003**, *125*, 15708). Activity of the enzyme was determined spectrophotometrically, following the oxidation of

dithionite reduced methyl viologen at 600 nm using standard protocol (Prudêncio, M.; Pereira, A. S.; Tavares, P.; Besson, S.; Cabrito, I.; Brown, K.; Samyn, B.; Devreese, B.; VanBeeumen, J.; Rusnak, F.; Fauque, G.; Moura, J. J. G.; Tegoni, M.; Cambillau, C.; Moura, I. *Biochem.* **2000**, *39*, 3899, Kristjansson, J. K.; Hollocher, T. C. *J. Biol. Chem.* **1980**, *255*, 704). The activity initiated by adding the saturated buffer solution was  $35 \pm 5$   $\mu\text{mol}$ s  $\text{N}_2\text{O}$  reduced  $\text{min}^{-1}\text{mg}^{-1}$  of  $\text{N}_2\text{OR}$ . As a control, a similar set up was arranged, but without NiR and activity was measured to be  $12 \pm 2$  units. The same background was observed in an assay without  $\text{N}_2\text{OR}$ . This indicates that the background of  $12 \pm 2$  units is due to the reduction of NO by excess dithionite/methyl viologen solution.

MCD data were collected on CD spectro-polarimeters (JASCO J810 with a S20 PM tube for the UV/Vis region, and J200 with an InSb detector for the near-IR region) with sample compartments modified to accommodate magnetocryostats (Oxford Instruments, SM4-7T). The enzymes were exchanged to deuterated buffers for preparing MCD samples, and 50% glycerol was used as a glassing agent. Data was collected at 7T, 5K. EPR spectra were obtained using a Bruker EMX spectrometer, ER 041 XG microwave bridge, and ER 4102ST cavity. All X band EPR samples were run at 77 K in a liquid nitrogen finger dewar. A Cu standard (1.0 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  with 2 mM HCl and 2 M  $\text{NaClO}_4$ ) was used for spin quantitation of the EPR spectra. EPR data of Intermediate B was obtained at liquid He temperature. *Q-band ENDOR* measurements were performed under dispersion ( $\chi'$ ), rapid passage field-modulated conditions at 2 K with the cryogenically tunable TE<sub>011</sub> Q-band resonator (Sienkiewicz, A., Smith, B. G., Veselov, A., and Scholes, C. P. *Rev. of Scientific Instruments*, **1996**, *67*, 2134.), as previously reported (Veselov, A., Olesen, K., Sienkiewicz, A., Shapleigh, J. P., Scholes, C. P. *Biochem.* **1998**, *37*, 6095 and Zhao, Y., Lukoyanov, D. A., Toropov, Y. V., Wu, K., Shapleigh, J. P., Scholes, C. P. *Biochem.* **2002**, *41*, 7464). In doing ENDOR, we monitor the radio-frequency RF-induced change in the rapid-passage, 100 KHz field modulated dispersion EPR signal as we sweep the frequency of the RF field.

*ENDOR Theory – Protons.* The frequencies of proton ENDOR features,  $\nu_{\text{ENDOR}}^{\text{P}}$ , center to first order at the free proton nuclear Zeeman frequency,  $\nu^{\text{P}}$ . The proton frequencies are split away from  $\nu^{\text{P}}$  by  $\pm 1/2A$ , where A is the electron-proton hyperfine coupling (Usov, O. M., Choi, P. S.T., Shapleigh, J. P., Scholes, C. P. *J. Am. Chem. Soc.* **2005** *127*, 9485; Usov, O. M., Sun, Y., Grigoryants, V. M., Shapleigh, J. P., Scholes, C. P. *J. Am. Chem. Soc.* **2006**, *128*, 13102; Zhao, Y., Lukoyanov, D. A., Toropov, Y. V., Wu, K., Shapleigh, J. P., Scholes, C. P. *Biochem.*

**2002**, 41, 7464 ). Proton ENDOR frequencies, occurring as “+” or as “-” Zeeman branches, are ( Hoffman, B. M., DeRose, V. J., Doan, P. E., Gurbiel, R. J., Houseman, A. L. P., and Telser, J. *In Biological Magnetic Resonance*, **1993**, Vol. 13: EMR of Paramagnetic Molecules, L. J. Berliner, and J. Reuben, eds. (New York, Plenum):

$$P v_{\text{ENDOR}}^{\pm} = |v^{\text{P}} \pm A/2|. \quad (1)$$

First-order expressions hold when  $v^{\text{P}} > A/2$ , as is the case here.

*ENDOR Theory – Nitrogens.* The first-order expressions for spin 1  $^{14}\text{N}$  ENDOR frequencies, notably relevant to histidine nitrogen ligands of Type 2 copper, are:

$${}^{14}v_{\text{ENDOR}}^{+} = |{}^{14}A/2 \pm 3/2P + {}^{14}v| \text{ and } {}^{14}v_{\text{ENDOR}}^{-} = |{}^{14}A/2 \pm 3/2P - {}^{14}v| \quad (2)$$

where  ${}^{14}A$  is the hyperfine coupling,  $P$  the quadrupolar coupling, and  ${}^{14}v$  ( $\approx 3.32$  MHz at 1.078 T) is the  $^{14}\text{N}$  nuclear Zeeman frequency. (If quadrupolar splitting is resolved, the quadrupolar splitting will be  $3|P|$ .) The  ${}^{14}v_{\text{ENDOR}}^{+}$  branch is often the only branch observable with rapid passage Q-band ENDOR for nitrogen features from histidine Usov, O. M., Choi, P. S.-T., Shapleigh, J. P., Scholes, C. P. *J. Am. Chem. Soc.* **2005** 127, 9485; Veselov, A. V., Osborne, J. P., Gennis, R. B., Scholes, C. P. *J. Am. Chem. Soc.* **2000**, 122, 8712.

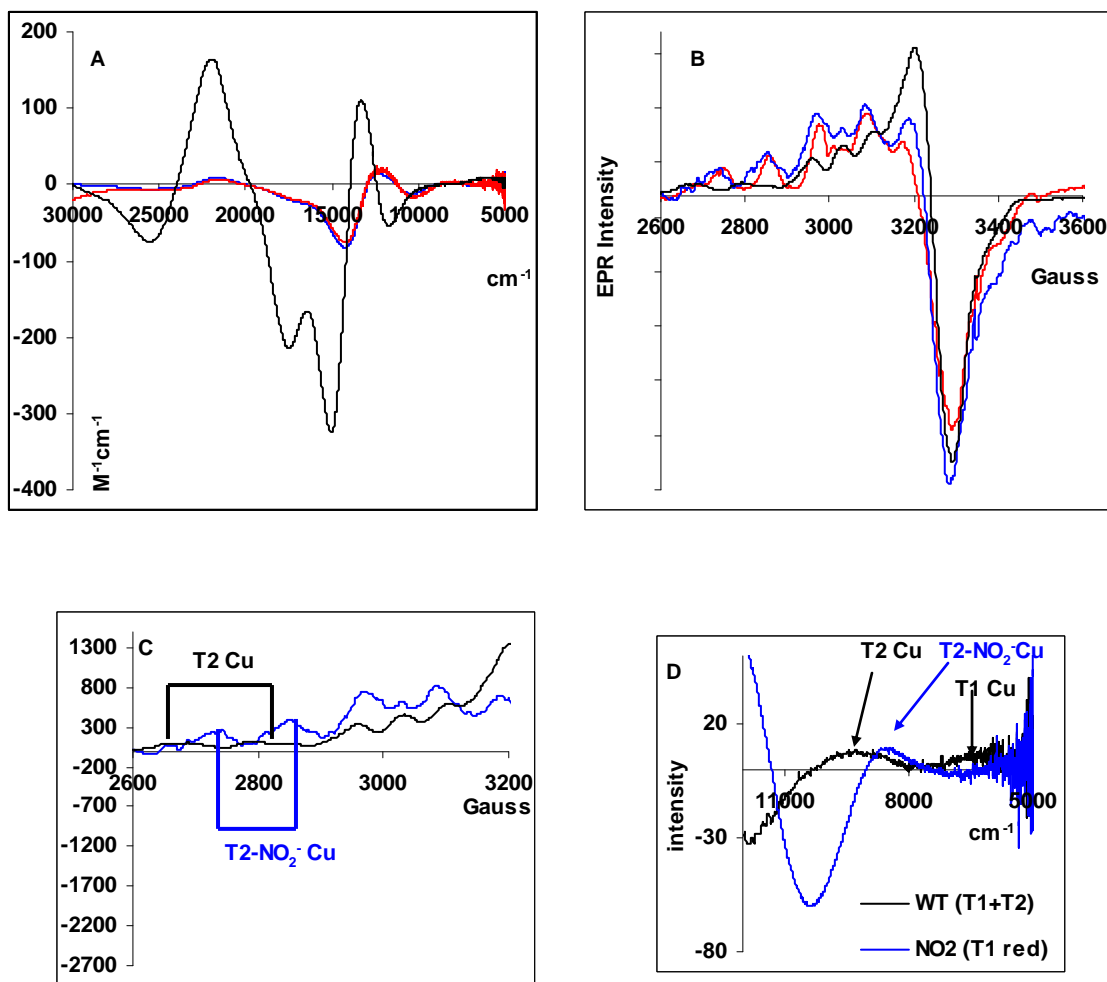
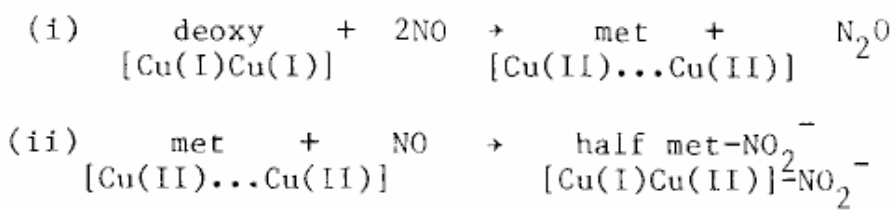


Figure S1. Overlay of WT NiR (black) reduced NiR with excess NO (red) and nitrite bound NiR (blue): (A) MCD spectra collected at 7T, 5K, (B) EPR spectra, 10 mW power, (C) EPR spectra of the T2 hyperfine perturbed by nitrite binding. The T2 hyperfine ( $A_{||} = 150G$ , black) splitting decreases to 117G, and shifts to higher field (blue) on nitrite binding. (D) MCD spectra for near IR region showing that the dd bands are perturbed on nitrite binding. The T2 dd band in the resting WT NiR (black) shifts from  $9500\text{ cm}^{-1}$  to  $8600\text{ cm}^{-1}$  in the T1 reduced T2-nitrite bound form (blue).



Scheme S1. Reaction scheme for hemocyanin (adapted from Solomon, E. I. *Copper Proteins*, Spiro, T. J. Ed. Acad. Press, NY 1981, Ch. 2)

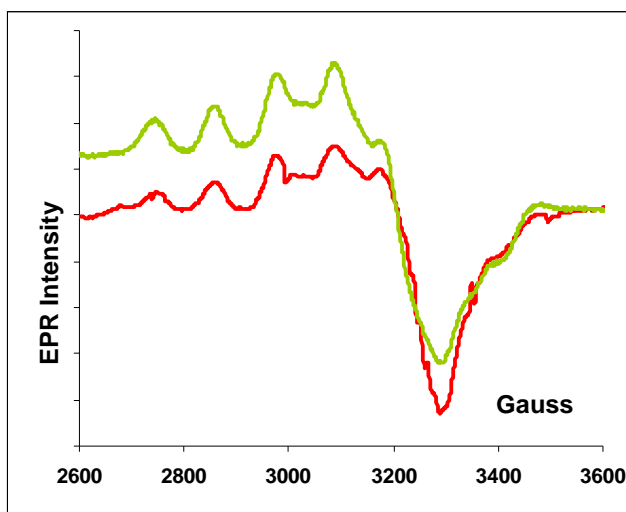


Figure S2. EPR spectra of A) reduced NIR with excess NO (red); B) resting oxidized NIR with excess NO (green).

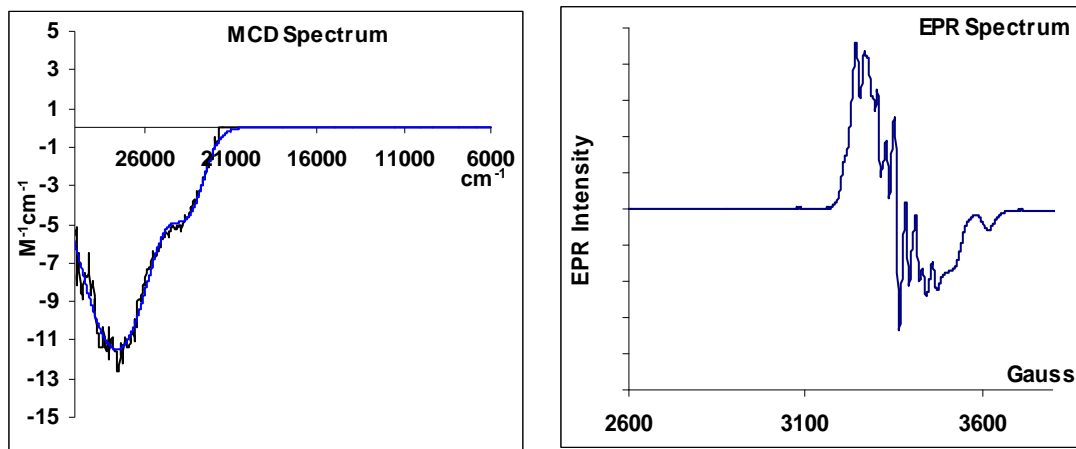


Figure S3. A) MCD spectrum, 7T, 5K; B) EPR spectrum at 15K, 2mW of  $Cu^{+}NO^{-}$  species, generated by reacting reduced Nir in excess NADH and two equivalents of nitrite. It is the same species as generated by Usov *et. al.* in Usov, O. M., Sun, Y., Grigoryants, V. M., Shapleigh, J. P., Scholes, C. P. *J. Am. Chem. Soc.* **2006**, *128*, 13102. Note that Fig. S3B is Fig 2A, green, generated by the reaction of low amount of NO with reduced NiR.

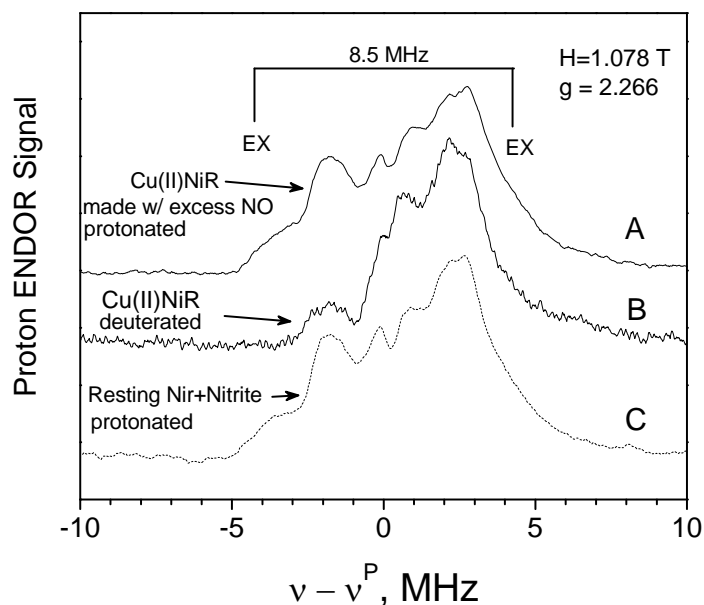


Figure S4. Proton ENDOR spectra A (protonated solvent) and B (deuterated solvent) from Cu(II)NiR made from reduced NiR and excess exogenous NO are provided for comparison to Spectrum C from the nitrite-bound Type 2 cupric center in oxidized resting NiR in protonated solvent. The features labeled EX are exchangeable features with a coupling of about 8.5 MHz. Spectrum C is essentially identical to spectrum A. These spectra were obtained at a field of 1.078 T ( $g = 2.266$ ) where there is no contribution from Type 1 copper. The conditions for data collection for each spectrum were as follows: adiabatic rapid passage;  $T = 2\text{K}$ ; microwave power = 80 nW; 100 kHz field modulation = 2 G ptp; a system time constant = 80 ms; radio frequency power  $\approx 20$  W, radio frequency sweep rate = 2 MHz/s; overall signal averaging time = 500s;  $\nu_{\text{EPR}} = 34.10$  MHz; Radio frequency was pulsed with a 100/900  $\mu\text{s}$  duty cycle.



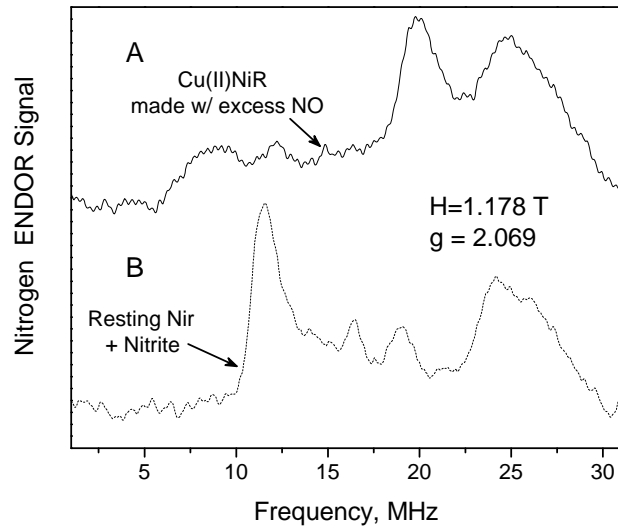


Figure S5. We provide a comparison of Spectrum A from Cu(II)NiR made from reduced NiR and excess exogenous NO and Spectrum B from nitrite-bound Type 2 cupric center in oxidized resting NiR at a field of 1.178 T ( $g = 2.069$ ). The features from spectrum A are from the histidine ligands of Type 2 copper. The features from spectrum B indicate a major additional contribution of Type 1 features. The conditions for data collection for each spectrum were as follows: adiabatic rapid passage;  $T = 2\text{ K}$ ; microwave power = 80 nW; 100 kHz field modulation = 2 G ptp; a system time constant = 81 ms; radiofrequency power  $\approx 20\text{ W}$ ; radiofrequency sweep rate = 3 MHz/s; overall signal averaging time = 1000 s;  $\nu_{\text{EPR}} = 34.10\text{ GHz}$ . Radio frequency power was pulsed with a 100/900  $\mu\text{s}$  duty cycle.

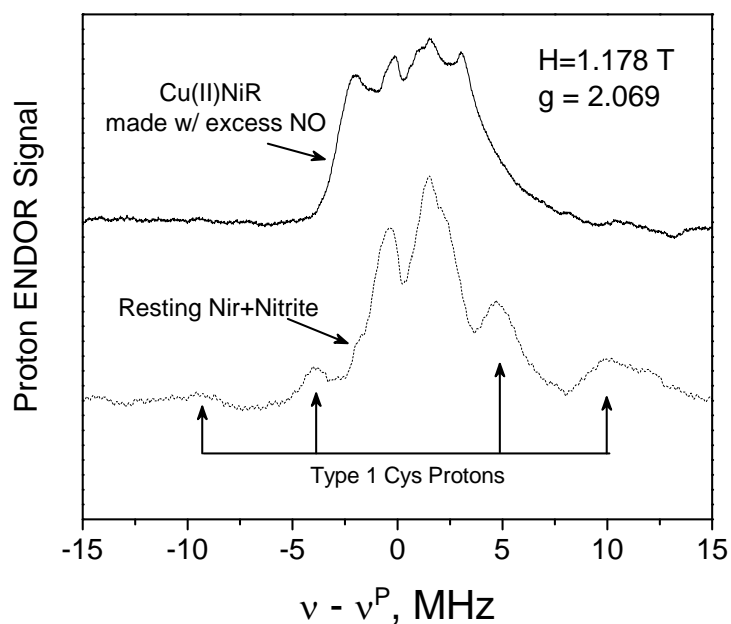
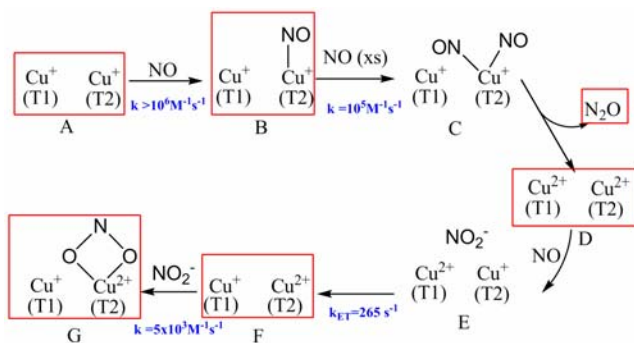


Figure S6. We provide a comparison of Spectrum A from Cu(II)NiR made from reduced NiR and excess exogenous NO and Spectrum B from nitrite-bound oxidized resting NiR at a field of 1.178 T ( $g = 2.069$ ). The features from spectrum B indicate a major additional contribution of Type 1 features which are indicated by arrows and have previously been assigned as methylene protons of the Type 1 cysteine ligand. The conditions for data collection for each spectrum were as follows: adiabatic rapid passage;  $T = 2\text{K}$ ; microwave power = 80 nW; 100 kHz field modulation = 2 G ptp; a system time constant = 80 ms; radio frequency power  $\approx 20$  W, radio frequency sweep rate = 2 MHz/s; overall signal averaging time = 500s;  $\nu_{\text{EPR}} = 34.10$  MHz; Radio frequency was pulsed with a 100/900  $\mu\text{s}$  duty cycle; averaging time = 1000 s;  $\nu_{\text{EPR}} = 34.10$  GHz. Radio frequency power was pulsed with a 100/900  $\mu\text{s}$  duty cycle.



Scheme S2. Reaction mechanism with kinetic rates

$k_{\text{ET}}$  rate obtained from Farver, O.; Eady, R. R.; Abraham, Z. H. L.; Pecht, I. *Febs. Lett.* **1998**, 436, 239.

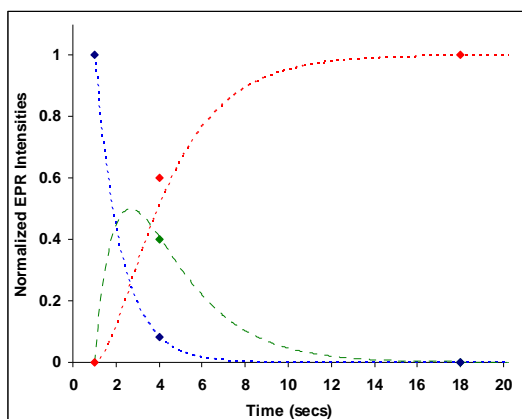


Figure S7. Rate of  $\text{Cu}^+\text{NO}$  species decay (blue), rate of formation and decay of the resting T2 species (green), rate of formation of the nitrite bound T2 species (red).

Gaussian Reference:

Gaussian 03, Revision C.02, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, Jr., J. A.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.;

Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; and Pople, J. A.; Gaussian, Inc., Wallingford CT, 2004.

The computational model chosen included the three co-ordinated Histidine residues and two second sphere residues, aspartate 129 and Histidine 287. Additionally a H<sub>2</sub>O molecule that H-bonds to both asp129 and his287 was included.

Geometries were optimized using G03 ver. C01. For Cu, N, O atoms 6-311g\* basis set was used and a 6-31g\* basis set was used for C and H atoms. Single point calculations were performed using a 6-311+g\* basis set on all atoms. Both the structures have the single unpaired electron localized on the NO suggesting that both of these can be described as a Cu<sup>+</sup>NO<sup>-</sup> species (as has been suggested in Wasbotten, I. H.; Ghosh, A. *J. Am. Chem. Soc.* **2005**, *127*, 15384.).

#### Optimized Co-ordinates for side-on CuNO

H	1.252487	-5.180570	-0.643846
H	0.318923	-3.937233	-0.759046
O	1.191156	-4.236974	-0.444500
C	3.508388	4.517107	-0.336115
C	2.181981	3.849058	-0.183281
N	1.170334	4.345839	0.618553
C	1.662786	2.689765	-0.702840
C	0.111543	3.499350	0.552022
N	0.381408	2.474522	-0.237040
H	1.209274	5.210024	1.145407
H	4.103799	4.002681	-1.094097
H	4.076956	4.517173	0.602072
H	3.403546	5.559407	-0.659152
H	2.119923	2.010461	-1.406401
H	-0.820370	3.673560	1.072141
C	5.150774	0.977644	-1.003466
C	4.641927	-0.422059	-0.773282
N	5.406151	-1.445067	-0.216105
C	3.414346	-0.988434	-0.994379
C	4.678163	-2.565361	-0.103673
N	3.459606	-2.305067	-0.569710

H	2.664298	-2.978932	-0.581280
H	4.450710	1.518533	-1.641634
H	6.123718	0.968776	-1.506108
H	5.256372	1.521633	-0.058504
H	2.515611	-0.568082	-1.413542
H	5.025228	-3.508677	0.290776
N	0.432688	-0.284979	-1.609098
O	-0.468792	0.121655	-2.315083
Cu	-0.708677	0.569719	-0.348795
C	-5.919679	1.277562	1.440258
C	-4.676733	1.295518	0.629504
N	-4.619632	1.744440	-0.678400
C	-3.394968	0.920044	0.925659
C	-3.350461	1.634327	-1.123552
N	-2.579043	1.137190	-0.167918
H	-5.406777	2.081428	-1.220520
H	-5.713487	0.865124	2.430945
H	-6.700322	0.660872	0.978953
H	-6.331218	2.284642	1.578208
H	-3.011366	0.508393	1.847042
H	-3.033983	1.914539	-2.117555
C	0.579261	-3.046650	3.762263
C	0.350632	-1.820750	2.937362
N	0.608904	-0.539197	3.398420
C	-0.117778	-1.638645	1.660211
C	0.303819	0.348357	2.427001
N	-0.139171	-0.286805	1.353896
H	0.951392	-0.300280	4.320549
H	0.413196	-3.937329	3.151607
H	-0.103238	-3.093797	4.619171
H	1.604184	-3.088874	4.149181
H	-0.447867	-2.384719	0.952950
H	0.423893	1.416686	2.526746
C	-3.992556	-3.406381	-1.248738
C	-2.653869	-2.801484	-1.590681
O	-2.773662	-1.832901	-2.510838
O	-1.575912	-3.120185	-1.119465

H	-3.890053	-4.092814	-0.407984
H	-4.374486	-3.949887	-2.119926
H	-4.713819	-2.618441	-1.015194
H	6.381577	-1.371591	0.051773
H	-1.892986	-1.477259	-2.732423

End-on

O	0.527796	-4.124068	-0.123937
O	-0.984487	-1.100177	-2.490911
N	-0.406142	-0.680056	-1.560210
Cu	-0.492641	0.764895	-0.428651
C	3.954191	4.080143	-0.886273
C	2.561756	3.621089	-0.636780
N	1.636772	4.317055	0.119870
C	1.912984	2.470442	-0.996084
C	0.496427	3.586468	0.192650
N	0.638704	2.448954	-0.465565
H	4.431718	3.444428	-1.636183
H	4.561093	4.050974	0.026900
H	3.985694	5.106637	-1.270739
H	2.271238	1.683653	-1.643711
H	-0.393646	3.910601	0.713337
H	1.765622	5.247448	0.502291
C	5.364915	0.352054	-1.049857
C	4.520014	-0.873223	-0.759252
N	5.076824	-2.138940	-0.592365
C	3.165121	-1.069112	-0.605394
C	4.120077	-3.046643	-0.355059
N	2.950196	-2.415669	-0.360835
H	4.717197	1.213387	-1.216464
H	5.974297	0.200176	-1.948129
H	6.032425	0.579817	-0.211437
H	2.340245	-0.375059	-0.609036
H	4.279418	-4.096168	-0.145995
H	6.067472	-2.356975	-0.642363
C	-5.483174	2.515892	1.395031
C	-4.344487	2.076378	0.536554

N	-4.384660	2.106798	-0.847212
C	-3.097000	1.587385	0.830216
C	-3.205819	1.655632	-1.332015
N	-2.397224	1.331508	-0.337140
H	-5.222491	2.398252	2.449975
H	-6.386835	1.924916	1.206178
H	-5.731704	3.570490	1.227608
H	-2.666549	1.390668	1.801257
H	-2.981423	1.582445	-2.386440
H	-5.167919	2.420953	-1.407251
C	0.439934	-2.468537	4.241657
C	0.437869	-1.369495	3.233200
N	0.990086	-0.125851	3.483097
C	-0.054096	-1.280159	1.954463
C	0.813263	0.652439	2.388851
N	0.185402	-0.016347	1.436929
H	-0.019053	-3.364728	3.815267
H	-0.130665	-2.197734	5.136590
H	1.455971	-2.730052	4.560629
H	-0.586350	-2.033722	1.394837
H	1.158331	1.674040	2.327332
H	1.422369	0.157095	4.353434
C	-4.512007	-2.655204	-0.725514
C	-3.145042	-3.161543	-1.107132
O	-3.106489	-3.556811	-2.383557
O	-2.180299	-3.231493	-0.363427
H	-4.469849	-2.166445	0.247386
H	-5.206643	-3.500901	-0.676802
H	-4.887840	-1.970237	-1.491635
H	0.501186	-5.092928	-0.161925
H	-0.399616	-3.818449	-0.205934
H	-2.212395	-3.872646	-2.602633
H	2.033851	-2.897685	-0.232358