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Published on Web 08/08/2007

Resolution of the Spectroscopy versus Crystallography Issue for NO Intermediates of Nitrite Reductase from *Rhodobacter sphaeroides*

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Copper nitrite reductase (NiR) is a homotrimeric enzyme, containing a T1 copper site, which transfers electrons to the T2 catalytic site, where nitrite is reduced by one electron to nitric oxide ($NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$).[1−3] Recently a side-on bound copper nitrosyl complex of NiR has been crystallized by reacting the reduced enzyme with excess NO.[4] On the basis of the EPR spectrum of the species generated by the reaction of reduced NiR with saturated NO in solution, it has been assigned as an η^2-NO^--Cu^{2+} species. However, spectroscopic data on several Cu-nitrosyl model complexes[5] and an NO adduct formed by reacting reduced NiR with nitrite[6] and density functional theory (DFT) calculations on an η^2-NOCu species[7] describe these complexes as $Cu^+NO^\bullet$. In this study, we define the species generated in solution by reacting reduced NiR with NO, using EPR, MCD, and ENDOR spectroscopy and correlate these with that observed by crystallography.

Reduced NiR reacted with saturated NO solution generates the EPR spectrum in Figure 1A, red, which is equivalent to the spectrum reported by Tocheva et al.[4] This species has an EPR signal characteristic of an oxidized T2 Cu ($g_{||} = 2.30$ and $A_{||} = 117$ G). The MCD spectrum (Figure 1B, red) has low-energy d−d bands, also indicative of a T2 cupric complex. Both EPR and MCD spectra show a negligible T1 contribution indicating that this site is reduced (Figure S1, Supporting Information).

Nitrite binds to the T2 site of the resting WT enzyme,[8,9] but the EPR and, in particular, the MCD features of the nitrite bound T2 site are obscured by the dominant T1 Cu signals. However under these conditions (nitrite bound), the T1 Cu could be selectively reduced with acsorbate. The EPR spectrum of the nitrite bound T2 species (Figure 1A, blue) is identical to that produced above by reacting reduced NiR with excess NO. The MCD spectrum (Figure 1B, blue) of the T1-reduced, T2-nitrite-bound form is also identical to that generated by the reduced enzyme with NO. The T2 EPR spectrum and the MCD d−d bands of the resting WT enzyme are very sensitive to nitrite binding (Figure S1C,D). Thus the EPR and MCD data strongly indicate that the species generated by the reaction of excess NO with reduced NiR has T1 reduced and T2 oxidized with nitrite bound.

Further N^{14} and H^1 ENDOR data for this species generated by the reaction of reduced NiR with an NO solution (Figure 1C,D, red) and those for a form with T1 + T2 oxidized and nitrite bound (collected at $g = 2.266$ where there is no contribution from T1, Figure 1C,D, blue) have the same features. The nitrogen features (Figure 1C) from $Cu^{2+}NO_2^-$ bound T2 are from its histidines, and these features all show lower ENDOR frequencies and smaller hyperfine couplings owing to the change of the T2 axial ligand from aquo to nitrite.[10]

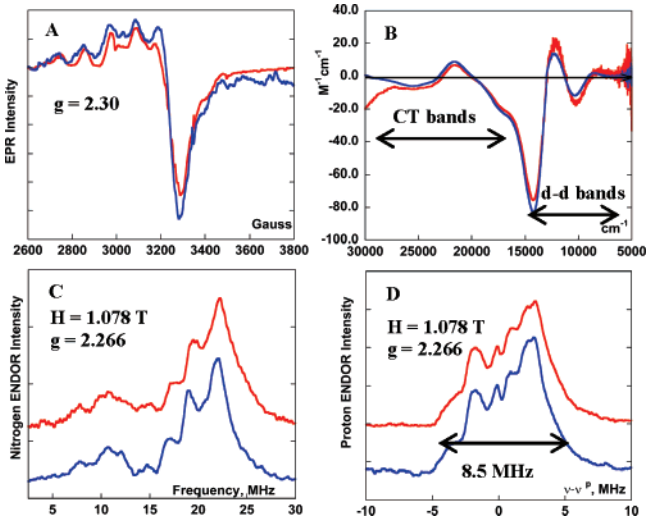

Figure 1. Overlay of reduced NiR with excess NO (red) and nitrite bound NiR (blue) (A) EPR, (B) MCD, (C) N^{14} ENDOR, (D) H^1 ENDOR, with exchangeable features with 8.5 MHz coupling. Parts A and B have nitrite bound to T2 site with the T1 Cu reduced to remove intense overlaying T1 features. ENDOR data are collected at 1.078 T where there is no contribution from T1 Cu.

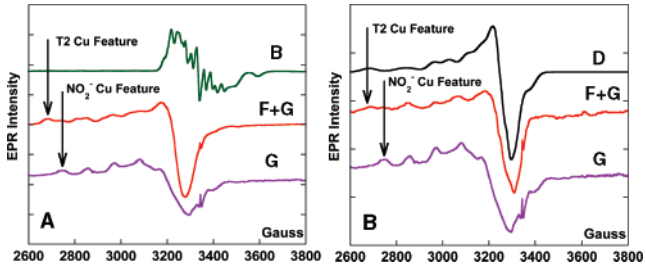

Figure 2. EPR spectra (A) following the reaction of reduced NiR with substoichiometric NO solution (10:1 enzyme/NO) trapped at 1 s (green), 4 s (red), and 18 s (purple); (B) following the reaction of the resting oxidized enzyme (black) with NO gas at 4 s (red) and 90 s (purple). Letters correspond to Scheme 1.

From the above results the reaction of reduced NiR with excess NO gives T1 reduced T2 Cu^{2+}-NO_2^-. However, the reaction of the reduced enzyme with limited NO has an EPR signal (Figure 2A, green), characteristic of a $Cu^+NO^\bullet$ species.[6] This species lacks d−d bands in the MCD spectrum (Figure S3), further confirming that the Cu is reduced. Therefore, reduced NiR forms a $Cu^+NO^\bullet$ species at low concentrations of NO.

The reaction of reduced NiR with excess NO involves two one-electron oxidations; reduced T2 → oxidized T2, and NO → NO_2^-. A similar reaction has been observed for the coupled binuclear Cu active sites in hemocyanins[11] (Scheme S1). Deoxyhemocyanin

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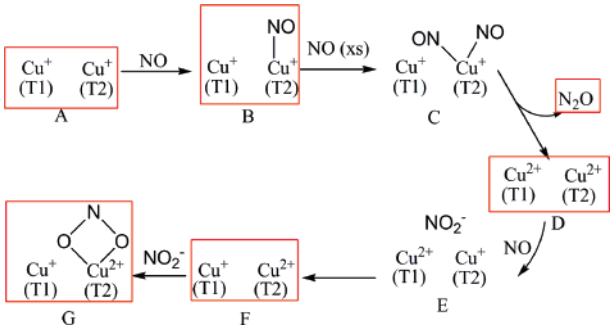
10.1021/ja072841c CCC: $37.00

Scheme 1. Proposed Mechanism for the Formation of Nitrite Bound T2 NiR from Reduced Enzyme with Excess NO[a]

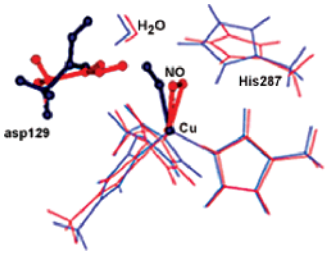


[a] Red boxes = species observed.

($2Cu^+$) reacts with NO generating N_2O, and a met ($2Cu^{2+}$) form, which further reacts with NO to generate the half-met ($Cu^{2+}Cu^+$) nitrite-bound form. N_2O evolution is in fact detected during the reaction of reduced NiR with excess NO, using nitrous oxide reductase (35 μmols N_2O reduced min^{-1} mg^{-1} of enzyme), which requires N_2O as its substrate.[12] Reaction of the resting WT NiR (analogous to the met form of hemocyanin) with NO generates the T1 reduced, T2 oxidized form of the enzyme ($g_{||} = 2.30$, $A_{||} = \sim 130$ G), which converts to the nitrite-bound T2 form (Figure 2B,D → F → G, Scheme 1), indicating NO is oxidized to nitrite and nitrite binding is slow.

To evaluate the entire reaction mechanism, reduced NiR was reacted with limited NO solutions and intermediates were trapped at different times. We were able to trap the $Cu^+NO^\bullet$ form at shorter times, leading to the formation of the T1 reduced, T2 oxidized form, and ultimately generating the T1 reduced, T2 oxidized nitrite-bound form (Figure 2A, Scheme 1, A → B → F → G) at longer times. A reaction mechanism consistent with these results is presented in Scheme 1. Note that in the scheme, intermediate E is not observed because of rapid ET from T2 → T1.[13] The experimental details and kinetic rates are provided in the Supporting Information.

There is still one issue to resolve on correlating the spectroscopy with crystallography. The Cu−NO species trapped in the crystal structure has a side-on conformation.[4] DFT calculations indicate that this species is best described as a $Cu^+-NO^\bullet$ species.[7] However an end-on Cu−NO species is also trapped after limited exposure to NO gas (B, in Scheme 1) which is equivalent to the species generated by reacting reduced NiR with nitrite (η^1-CuNO).[6] Geometry optimized DFT calculations[14] of both side-on (Figure 3, red) and end-on (Figure 3, blue) Cu−NO adducts indicate that both forms have a $Cu^+NO^\bullet$ electronic configuration and that the end-on structure is more stable by 7 kcal/mol. However the orientation of the second-sphere aspartic acid residue (Asp129)[9,15] appears to play a key role in determining the geometry of the Cu−NO species. In the side-on adduct this residue is perpendicular to the Cu−N−O plane and H-bonds to the O of N−O (Figure 3, red) while in the end-on structure the Asp129 residue H-bonds to the O of NO and is parallel to the Cu−N−O plane (Figure 3, blue). Both orientations of the Asp129 residue have been observed in crystal structures.[15] The Asp129 residue exists in the perpendicular orientation in the crystal structures of the oxidized and reduced NiR and H-bonds to



	side-on	end-on
Cu-N (NO)	1.91	1.83
Cu-O (NO)	2.04	2.75
Cu-N	1.95	1.98
Cu-N	2.01	2.07
Cu-N	2.21	2.11
N-O	1.22	1.18
Cu-N-O	75	131

Figure 3. Overlay of the DFT optimized side-on bound NO (red) and end-on bound NO (blue) structures indicating the different orientations of the aspartate residue.

the axial ligand. However, to form the more stable end-on Cu^+-$NO^\bullet$ adduct this aspartate residue has to rotate 90° which can occur in solution but may not be feasible in a crystal.

In summary, the species generated in solution during the reaction of reduced NiR with excess NO is the T1 reduced, T2 oxidized nitrite-bound form and not the side-on Cu−NO species observed in the crystal structure. The latter is best described as $Cu^+NO^\bullet$ rather than $Cu^{2+}NO^-$. The mechanism for the reaction of reduced NiR with excess NO involves its two electron oxidation, generating N_2O. This oxidized form of the enzyme oxidizes NO generating nitrite bound to the oxidized T2 site generated by ET from T2 → T1. DFT calculations provide insight into factors which favor the η^2-$NO{\cdot}Cu^+$ complex in the crystal structure.

Acknowledgment. This research is supported by NIH Grant DK-31450 (E.I.S) and NIH Grant EB00326929 (C.P.S).

Supporting Information Available: Complete ref 14, the experimental and computational details, H^1, N^{14} ENDOR at high field, EPR, MCD of WT, $Cu^+NO^\bullet$ species, and optimized geometries. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA072841C