Nitric Oxide Reductase from *Paracoccus denitrificans* Contains an Oxo-Bridged Heme/Non-Heme Diiron Center

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In this report, we describe direct spectroscopic evidence supporting the presence of a μ-oxo bridged dinuclear active site in the bacterial enzyme nitric oxide reductase (NOR). As part of the denitrification process, NOR, a cytochrome *bc* complex, reduces NO to N_2O.[1] The enzyme is an integral membrane protein composed of two subunits, norC (16 kDa) and norB (54 kDa), that bind *c* and *b* hemes, respectively. Hydropathy plots of norB indicate the presence of 12 trans-membrane α-helices, and sequence alignment with subunit I of cytochrome *c* oxidase (C*c*O) predicts that the His ligands of heme *a*, heme a_3, and Cu_B in C*c*O are conserved in norB.[2] Because copper is absent in NOR, the Cu_B site has been proposed to bind iron. Detergent solubilization and purification of NOR from *Paracoccus denitrificans* using lauryl maltoside yield a highly active enzyme with the stoichiometry of one heme *c*, two heme *b*, and one non-heme iron.[3]

The EPR-silent character of one heme *b* and the non-heme iron was proposed to arise from antiferromagnetic coupling of these two centers through a bridging ligand.[3,4] In this model, NOR utilizes this novel heme/non-heme diiron site to reduce two NO molecules, while the two hexacoordinate low-spin (6cLS) hemes mediate electron transfer to the active site (Figure 1). In addition to the two 6cLS hemes contained in oxidized NOR, resonance Raman (RR) spectra have identified a pentacoordinate high-spin heme (5cHS) with an axial ligand predicted to be an oxo or hydroxo group bridging the two irons.[5]

In an attempt to detect vibration(s) involving a putative oxo or hydroxo bridging ligand to the 5cHS heme iron, we incubated fully oxidized NOR in ^{18}O-labeled water. Indeed, such bridging ligands are known to be readily exchangeable with solvent.[6] Using 442-nm excitation, we observed a RR band at ∼810 cm^{-1} that displays an ^{18}O-downshift of ∼30 cm^{-1} (Figure 2). The RR difference spectrum (^{16}O-water minus ^{18}O-water) also shows that a vibration at ∼830 cm^{-1} decreases in intensity (Figure 2). The latter change can be interpreted in terms of interacting vibrations.

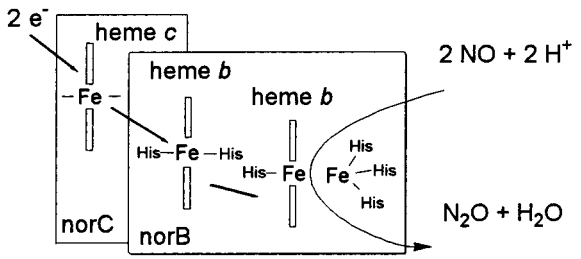

Figure 1. Schematic representation of the structure and function of NOR deduced from sequence similarities with C*c*O.

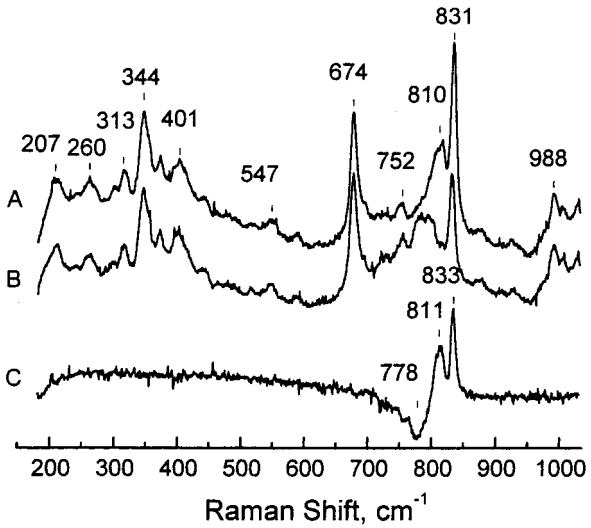

Figure 2. Low-frequency RR spectra of oxidized NOR in $H_2{}^{16}O$ (A) and $H_2{}^{18}O$ (B). The Raman band from pH 7.0 phosphate buffer at 988 cm^{-1} was used to normalize spectra A and B to obtain the difference spectrum C. The data were obtained at room temperature with 442-nm excitation.

As the 810-cm^{-1} band downshifts to ∼780 cm^{-1}, the frequency match with the 830-cm^{-1} band decreases and results in a loss of intensity. The observed $t_{1/2}$ for the $H_2{}^{18}O$ exchange reaction was ∼5 min. No significant RR changes were observed after incubation of oxidized NOR in deuterated water or between pH 6.0 and 9.0 (data not shown).

The ∼810-cm^{-1} signal could conceivably be assigned to a ν(Fe═O) from a heme ferryl-oxo species, but further experiments did not support such conclusion (see below). In peroxidases, such a species is an intermediate of the catalytic cycle and is known as Compound II. Under certain experimental conditions, the Fe^{IV}═O group is exchanged with bulk water, and in horseradish peroxidase the ν(Fe═O) at ∼780 cm^{-1} is downshifted ∼30 cm^{-1} in $H_2{}^{18}O$.[7] In "as isolated" cytochrome *bd* oxidase, a terminal oxidase of *Escherichia coli*, the heme *d* is in a mixture of oxygenated forms and a ferryl-oxo state with a ν(Fe═O) at 815 cm^{-1}.[8] In NOR, we can rule out the assignment of the ∼810 cm^{-1} signal to a ferryl-oxo species. Indeed, the ^{18}O-sensitive RR signal shown in Figure 2 is also observed after an anaerobic redox cycle of NOR through sequential treatment with dithionite and ferricyanide (data not shown).

In linear oxo-bridged diferric compounds, the symmetric stretch, ν_s(Fe−O−Fe), at ∼400 cm^{-1} is Raman-active only, while the asymmetric stretch, ν_{as}(Fe−O−Fe), at ∼850 cm^{-1} is IR-active only, but if the Fe−O−Fe unit adopts a bent geometry, both

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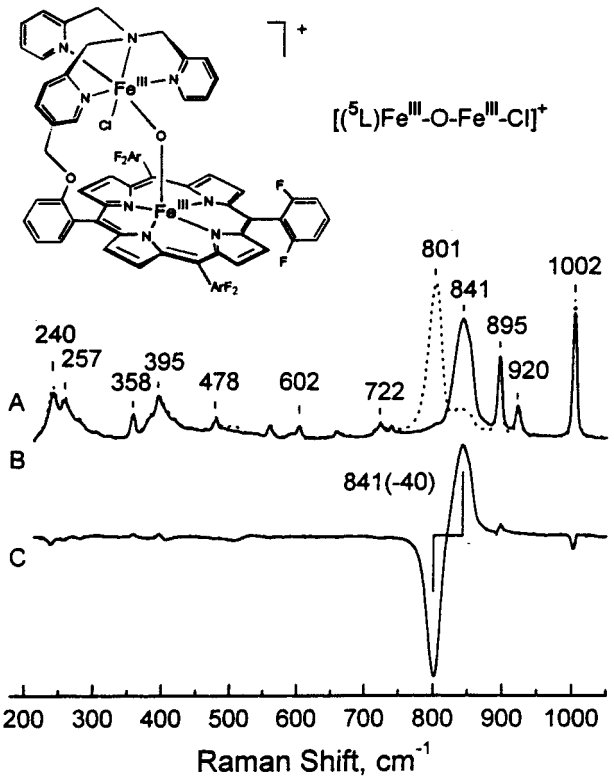

Figure 3. Low-frequency RR spectra of $[(^5L)Fe^{III}-O-Fe^{III}-Cl]^+$ dissolved in acetonitrile with 20% $H_2{}^{16}O$ (solid line A) or 20% $H_2{}^{18}O$ (dashed line B). The Raman band of acetonitrile at 920 cm^{-1} was used to normalize spectra A and B to obtain the difference spectrum C. The data were obtained at room temperature with 442-nm excitation.

vibrational modes become IR and Raman active.[9] Exhaustive RR studies on non-heme oxo-bridged diiron models have shown that the intensity ratio between ν_{as} and ν_s is usually small. In contrast, complexes with nonequivalent sets of ligands give rise to RR spectra with large I_{as}/I_s ratio.[10]

Oxo-bridged heme/non-heme dinuclear complexes have been synthesized to mimic the active sites of CcO and NOR.[11] In particular, a heme/non-heme diiron model composed of a tris(2-pyridylmethyl)amine moiety tethered to a synthetic porphyrin $[(^5L)Fe^{III}-O-Fe^{III}-Cl]^+$ was characterized by X-ray crystallography.[11d] This compound was dissolved in an acetonitrile/water mixture and examined by RR spectroscopy (Figure 3).

Comparing the spectra recorded in ^{16}O- and ^{18}O-water identifies the $\nu_{as}(Fe-O-Fe)$ at 841 cm^{-1}. As in NOR the asymmetric vibration is strongly resonance enhanced while the symmetric vibration is not observed. We are currently undertaking a detailed characterization of this family of compounds,[12] but the similarity of the RR signatures in NOR and in $[(^5L)Fe^{III}-O-Fe^{III}-Cl]^+$ is striking, and further supports the presence of an oxo-bridged heme/non-heme diiron center in the enzyme.

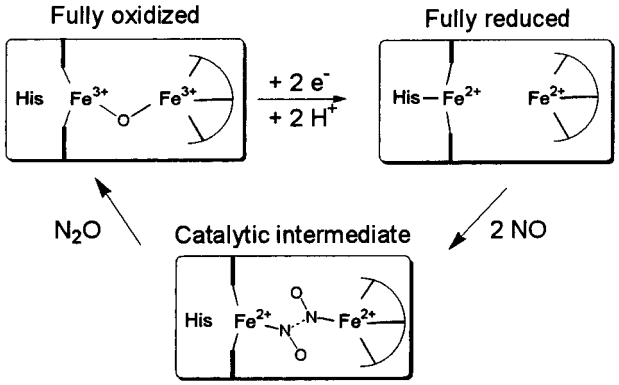

Figure 4. Proposed structures of the catalytic site of NOR during enzymatic turnover.

The present findings are entirely consistent with our previous proposal for the NOR catalytic cycle.[5] In the fully reduced active site, the ferrous HS heme *b* is ligated to a histidine. After binding of two molecules of NO, the iron−histidine bond is broken. As the product is formed, the two irons are oxidized and become bridged through an oxo group. Reduction with concomitant protonation of the active site to the diferrous state results in the removal of the oxo bridge and ligation of the histidine to the heme iron (Figure 4).

From spectroscopic studies of non-heme diiron models,[10a] a $\nu_{as}(Fe-O-Fe)$ at ~810 cm^{-1} corresponds to an Fe−O−Fe angle of ~145° and an Fe···Fe distance ≤3.5 Å. The crystal structure of $[(^5L)Fe^{III}-O-Fe^{III}-Cl]^+$ unveiled an Fe−O−Fe angle of 157° and an Fe···Fe distance of 3.47 Å.[11d] In contrast, crystal structures of CcO have shown a ~5 Å separation between Fe and Cu that may preclude the formation of an oxo bridge.[13] Consequently, the dinuclear site of CcO has been proposed to be bridged through a peroxo group,[13d] or interacting through hydroxy and aqua ligands on the heme a_3 and the Cu_B.[14] Very recently, the crystal structure of the *ba*$_3$-cytochrome *c* oxidase from *Thermus thermophilus* was solved at 2.4 Å resolution.[15] In this structure, the spherical electron density between the heme a_3 and the Cu_B is consistent with a single oxygen atom (oxo, hydroxo, or aqua) but the Fe···Cu distance of 4.4 Å is still large. Despite the difference in metal−metal distance, NOR and CcO are both capable of catalyzing NO and O_2 reduction to N_2O and H_2O.[16] Instead, structural differences may relate to the proton-translocation process, since NOR activity is not associated with proton pumping.

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