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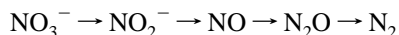
Direct Observation by FTIR Spectroscopy of the Ferrous Heme–NO⁺ Intermediate in Reduction of Nitrite by a Dissimilatory Heme *cd*₁ Nitrite Reductase

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Denitrification is the dissimilatory reduction of nitrogen–oxygen species by certain bacteria.^{1–3} It constitutes a key part of the global nitrogen cycle, in that denitrification is responsible for evolution of N₂(g) from the biosphere and geosphere to replenish the atmosphere. In addition, denitrification causes a substantial reduction in crop yields, since up to 25–30% of added nitrogen fertilizer can be transformed to N₂ and N₂O by soil microorganisms. One of the products of denitrification, N₂O, is a “greenhouse gas” that has also been linked to ozone destruction in the stratosphere.⁴ In most, if not all, organisms, denitrification occurs in four steps via the sequence:



The step involving the reduction of nitrite has been the focus of substantial attention and considerable controversy,^{2,3} because it represents the branch point from assimilatory nitrate reduction and a possible point of attack for development of agricultural chemicals that might selectively inhibit denitrification.

Two types of nitrite reductase are known from denitrifying bacteria.⁵ One is a copper-containing enzyme that exists in most cases as a trimer; each monomer contains both an unusual type 1 Cu site and a type 2 Cu site that is the site of NO₂[−] reduction.^{6,7} The second and more commonly encountered in nature is heme-containing enzymes, the *cd*₁ NiR's.⁵ All examples characterized to date are dimers, with each monomer containing both a heme *c* and a heme *d*₁ (dioxoisobacteriochlorin) chromophore.⁹ The roles of the two heme centers in catalysis remain unresolved, although the heme *d*₁ is presumed to be the site at which nitrite is reduced. Substantial evidence has been adduced for the existence of an electrophilic heme–nitrosyl intermediate in the reduction of nitrite (formulated as either Fe²⁺–NO⁺ or Fe³⁺–NO), based primarily on isotope exchange and trapping experiments.^{10,11} Its formation from nitrite is, however, too fast to be detected even by stopped-flow studies.¹² We report herein the results of FTIR studies of the heme *cd*₁ nitrite reductase from *Pseudomonas stutzeri* JM300 in which we have been able to detect an Fe²⁺–NO⁺ species formed by reaction of NO product with the oxidized enzyme;

in addition, we report FTIR spectra of the NO complex of methemoglobin.

Infrared spectroscopy has proven to be a sensitive technique for the direct observation of certain ligands bound to metallo-proteins and for probing the local environment of the ligand binding site.^{13,14} The ligands most commonly used in such studies are CO, CN[−], and N₃[−].^{15–18} In contrast, NO has been seldom studied in this regard, because the N–O stretch for NO itself and NO bound to most metal centers lies in the same spectral region as the strong protein amide I band. Complexes in which NO is bound to more oxidized metal centers should have appreciable NO⁺ character and should exhibit N–O stretches at higher frequencies, where there is less interference from the protein background. Unfortunately, such complexes tend to be less stable. The only such reports of which we are aware are studies of nitrosylhemoglobin, Hb–NO, for which ν_{NO} values of 1587 and 1615 cm^{−1} were reported for ¹⁵NO and ¹⁴NO, respectively.¹⁹ In these studies, NO complex of ferric horseradish peroxidase (HRP) was reported to give a ν_{NO} at 1865 cm^{−1} for ¹⁵NO^{19a} and the NO complex of methHb was reported to give a ν_{NO} at 1925 cm^{−1} for ¹⁴NO,^{19b} but no spectra were shown.

Consequently, we began by examining the FTIR spectra of concentrated samples of methHb.²⁰ Optical spectra of methHb treated with 1 atm NO (not shown) show that a new species with λ_{max} at 533 and 566 nm is formed rapidly (<1 min) and decays over 15–30 min to the characteristic shallow double-maximum spectrum of Hb–NO (λ_{max} = 540 and 570 nm²²). Infrared spectra taken over the same time period are shown in Figure 1 as ¹⁴NO minus ¹⁵NO difference spectra. The features at low energy that increase in intensity with time are clearly due to Hb–NO, with $\Delta\nu_{1/2} \approx 10$ cm^{−1} and ν_{NO} = 1615 and 1587 cm^{−1} for ¹⁴NO and ¹⁵NO, respectively, in good agreement with earlier work.¹⁹ At higher energies, however, a new feature is observed that decays with time. This feature is remarkably sharp ($\Delta\nu_{1/2}$ = 8 cm^{−1}) and appears at 1925 and 1889 cm^{−1} for ¹⁴NO and ¹⁵NO, respectively. This feature is most reasonably assigned to the methHb–NO species, which can be formulated as containing either a heme Fe³⁺–NO unit or a heme Fe²⁺–

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(20) The *cd*₁ NiR from *P. stutzeri* jm300 was purified as described;²¹ preparations had A₄₁₂/A₂₈₀ ratios of ca. 1.2 and gave a single band with *M_r* ≈ 59 kDa upon SDS–PAGE. Protein concentrations were determined using a value of 300 mM^{−1} cm^{−1} for ϵ_{412} . Human hemoglobin (Hb) was obtained from Sigma; it typically contained ca. 75% methemoglobin, with the balance primarily oxyhemoglobin. All samples were prepared in 100 mM potassium phosphate buffer, pH 6.0, in D₂O by repeated buffer exchange with Amicon Centricon microconcentrators; typical protein concentrations were ≈5 mM for Hb and 2 mM for *cd*₁ NiR. ¹⁴NO was obtained from Matheson, and ¹⁵NO was prepared from Na¹⁵NO₂ by reaction of 1 mL of 200 mM H₂SO₄, 1 mL of 100 mM KI, and 1 mL of 290 mM Na¹⁵NO₂ in a 5 mL argon-flushed vial. Samples were flushed repeatedly with argon before being mixed with NO gas in a gas-tight Hamilton syringe and anaerobic transfer to a CaF₂ transmission IR cell with a Teflon spacer, path length 0.024 mm. IR spectra were recorded within ≈1 min of mixing with NO on a Mattson Cygnus 100 spectrophotometer operating at room temperature, using a scan time of 1 min and a resolution of 2 cm^{−1}. Data were accumulated on a PC using Mattson's FIRST software for collection and analysis; background spectra were measured with a D₂O blank. Optical spectra were recorded on an HP-8452 diode array spectrophotometer.

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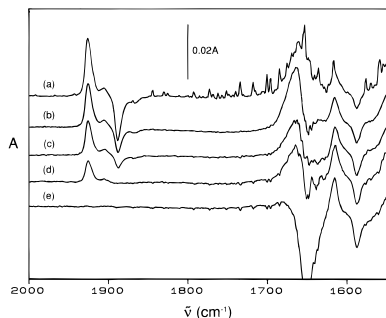


Figure 1. FTIR spectra of D₂O solutions of metHb at (a) 1 min, (b) 3 min, (c) 5 min, (d) 10 min, and (e) 30 min after mixing with NO, presented as ¹⁴NO minus ¹⁵NO difference spectra. The absorbance scale is given by the vertical bar. Experimental details are given in ref 20.

NO⁺ unit resulting from electron transfer from NO to the ferric heme. The isotopic shift is in good agreement with that calculated for a simple diatomic model,¹⁴ suggesting the existence of significant NO⁺ character. (In addition, a small amount of a second oxidized species is observed at 1905 and 1870 cm⁻¹ for ¹⁴NO and ¹⁵NO, respectively, and appears to originate from a minor NO-bound species.) The peaks due to metHb-NO decay smoothly with time, while those due to Hb-NO increase, consistent with net reduction of the former to the latter by NO; the instability of the former is well-documented. The total intensity due to the two features decreases by ca. 50% with time, suggesting that the N-O stretch in the oxidized species has a significantly greater extinction coefficient than that in the reduced species.

Optical spectra of the oxidized *cd*₁ NiR in the αβ region show peaks at 524 and 558 nm, attributed to the heme *c*, and a peak at 640 nm, attributed to the heme *d*₁.²³ Upon reaction with NO at pH 6.0 for 1 min (data not shown), the peak at 524 nm shifted only slightly (to 529 nm), that at 558 nm doubled in intensity and shifted to 556 nm, and that at 640 nm decreased in intensity by ≈20%, with a shift to 637 nm. Upon standing for 30 min, the two higher energy peaks shifted in energy (to 530 and 563 nm) and intensity, to give a spectrum essentially identical to that observed upon reaction of NO with the reduced *cd*₁ NiR.²⁴ In contrast, the heme *d*₁ feature recovered 95% of its original intensity and shifted back to 640 nm upon standing. These results are consistent with the initial formation of an unstable oxidized NiR-NO complex, followed by reduction by NO.

FTIR spectra of solutions of the oxidized *cd*₁ NiR in the presence of NO are shown in Figure 2 as ¹⁴NO minus ¹⁵NO difference spectra. Absorptions at 1910 and 1874 cm⁻¹ for ¹⁴NO and ¹⁵NO, respectively, are readily identified as originating from an oxidized heme-NO complex that decays with time. Both the narrowness of the absorption (Δν_{1/2} = 6–7 cm⁻¹) and the lower intensity of the peaks vs those observed for metHb-NO⁺ are consistent with our results on the CO complex of the reduced *cd*₁ NiR with CO, for which narrow lines and an extinction coefficient ca. 5 times lower than that of deoxyHb-CO were observed.²⁵ The most reasonable assignment of the 1910 (1874) cm⁻¹ band is to a ferric heme-NO complex formed by reaction of the oxidized heme *d*₁ with NO; this is the reverse of the reaction normally used to evolve NO from NO₂⁻ in the mechanism proposed for the enzyme,²⁶ shown in Scheme 1. The lower signal-to-noise ratio in the NiR spectra and the

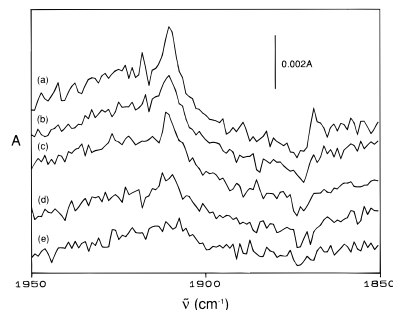


Figure 2. FTIR spectra of D₂O solutions of *P. stutzeri cd*₁ NiR at (a) 1 min, (b) 3 min, (c) 5 min, (d) 10 min, and (e) 30 min after mixing with NO, presented as ¹⁴NO minus ¹⁵NO difference spectra. Other details are same as in Figure 1.

Scheme 1. Proposed Mechanism for Formation of NO from Nitrite at the Heme *d*₁ Site of Heme *cd*₁ Nitrite Reductase²⁶

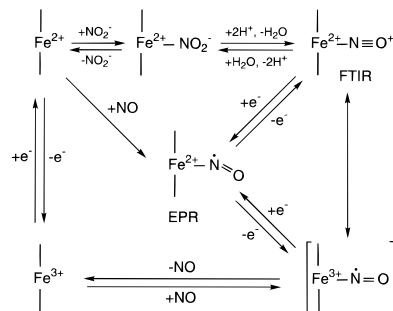


Table 1. Reported Values for N-O Stretching Frequencies in Heme Protein-NO Adducts

species	¹⁴ N ¹⁶ O frequency (cm ⁻¹)	¹⁵ N shift (obs) (cm ⁻¹)	¹⁵ N shift (calc) (cm ⁻¹)
ox <i>cd</i> ₁ NiR-NO	1910	36	34.3
metHb-NO	1925	36	34.5
ox HRP-NO	1865 ^{a,b}		
deoxyHb-NO	1616.5 ^c	28	29
metHb-NO	1925 ^c		

^a HRP = horseradish peroxidase. Data from ref 19a. ^b Frequency for ¹⁵N¹⁶O complex. ^c Data from ref 19b.

overlap with the intense amide I band around 1600 cm⁻¹ precluded direct observation by FTIR of the reduced heme-NO complex(es) that presumably form at longer times. It is known, however, that in the reduced state both the hemes *c* and *d*₁ of the analogous heme *cd*₁ NiR from *Pseudomonas aeruginosa* form complexes with NO,²⁴ and our own results suggest that this is also true for the *P. stutzeri* enzyme. Whether the unstable oxidized *d*₁-NO species that is formed is best formulated as an Fe²⁺-NO⁺ or Fe³⁺-NO complex is, to some extent, a matter of semantics. The value of ν_{NO} and the isotopic shift are both consistent with a linear Fe-N-O unit in which substantial donation of charge from NO to Fe³⁺ has occurred, resulting in a species with considerable NO⁺ character.²⁷ The value of ν_{NO} observed for the oxidized *cd*₁ NiR lies in the middle of the admittedly limited range reported for such species in proteins (Table 1) and does not provide direct evidence for the highly electrophilic behavior manifested in H₂¹⁸O exchange and nucleophile trapping experiments. Further studies using FTIR, resonance Raman, and other spectroscopies are in progress to fully characterize the oxidized heme-NO species and its interaction with the protein environment.

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