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 N_2(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 N_2 and N_2O by soil microorganisms. One of the products of denitrification, N_2O, 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:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

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 NO_2^- 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 Fe^{2+}−NO^+ or Fe^{3+}−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 Fe^{2+}−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 metalloproteins 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 N_3^-.[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 ^{15}NO and ^{14}NO, respectively.[19] In these studies, NO complex of ferric horseradish peroxidase (HRP) was reported to give a ν_{NO} at 1865 cm^{-1} for ^{15}NO[19a] and the NO complex of metHb was reported to give a ν_{NO} at 1925 cm^{-1} for ^{14}NO,[19b] but no spectra were shown.

Consequently, we began by examining the FTIR spectra of concentrated samples of metHb.[20] Optical spectra of metHb 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 nm[22]). Infrared spectra taken over the same time period are shown in Figure 1 as ^{14}NO minus ^{15}NO 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 ^{14}NO and ^{15}NO, 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 ^{14}NO and ^{15}NO, respectively. This feature is most reasonably assigned to the metHb−NO species, which can be formulated as containing either a heme Fe^{3+}−NO unit or a heme Fe^{2+}−

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(20) The cd_1 NiR from *P. stutzeri* jm300 was purified as described;[21] preparations had A_{412}/A_{280} ratios of ca. 1.2 and gave a single band with $M_r \approx 59$ 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, pD 6.0, in D_2O by repeated buffer exchange with Amicon Centricon microconcentrators; typical protein concentrations were ≈5 mM for Hb and 2 mM for cd_1 NiR. ^{14}NO was obtained from Matheson, and ^{15}NO was prepared from $Na^{15}NO_2$ by reaction of 1 mL of 200 mM H_2SO_4, 1 mL of 100 mM KI, and 1 mL of 290 mM $Na^{15}NO_2$ 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 CaF_2 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 D_2O blank. Optical spectra were recorded on an HP-8452 diode array spectrophotometer.

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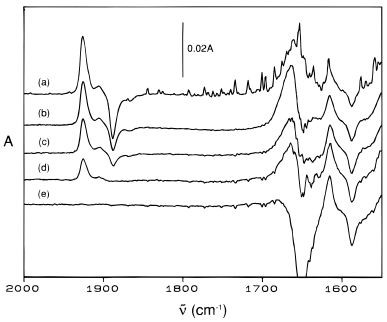

Figure 1. FTIR spectra of D_2O solutions of metHb at (a) 1 min, (b) 3 min, (c) 5 min, (d) 10 min, and (e) 30 min after mixing with NO, presented as ^{14}NO minus ^{15}NO difference spectra. The absorbance scale is given by the vertical bar. Experimental details are given in ref 20.

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 ^{14}NO and ^{15}NO, 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 $\alpha\beta$ 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 ^{14}NO minus ^{15}NO difference spectra. Absorptions at 1910 and 1874 cm^{-1} for ^{14}NO and ^{15}NO, respectively, are readily identified as originating from an oxidized heme−NO complex that decays with time. Both the narrowness of the absorption ($\Delta\nu_{1/2} = 6-7$ 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 NO_2^- in the mechanism proposed for the enzyme,[26] shown in Scheme 1. The lower signal-to-noise ratio in the NiR spectra and the 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 $Fe^{2+}-NO^+$ or $Fe^{3+}-NO$ 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 Fe^{3+} 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 $H_2{}^{18}O$ 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.

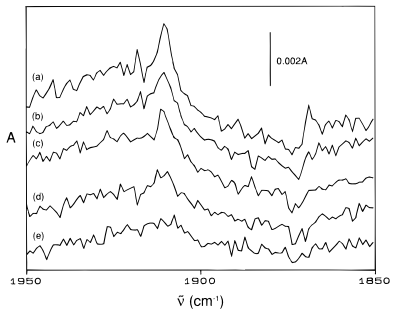

Figure 2. FTIR spectra of D_2O solutions of *P. stutzeri* cd_1 NiR at (a) 1 min, (b) 3 min, (c) 5 min, (d) 10 min, and (e) 30 min after mixing with NO, presented as ^{14}NO minus ^{15}NO difference spectra. Other details are same as in Figure 1.

Scheme 1. Proposed Mechanism for Formation of NO from Nitrite at the Heme d_1 Site of Heme cd_1 Nitrite Reductase[26]

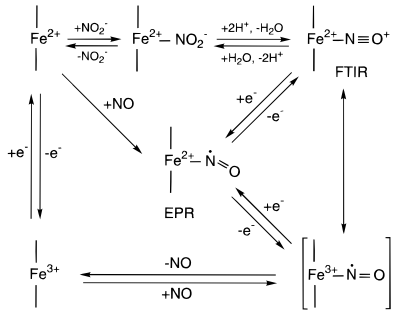

Table 1. Reported Values for N−O Stretching Frequencies in Heme Protein−NO Adducts

species	$^{14}N^{16}O$ frequency (cm^{-1})	^{15}N shift (obs) (cm^{-1})	^{15}N shift (calc) (cm^{-1})
ox cd_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 $^{15}N^{16}O$ complex. [c] Data from ref 19b.

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