Direct observation by FTIR spectroscopy of the ferrous heme-NO+ intermediate in reduction of nitrite by a dissimilatory heme cd1 nitrite reductase.

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Nitrite by a Dissimilatory Heme $d_{1}$ Nitrite Reductase

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Dinitrification 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 dinitrification is responsible for evolution of $N_2(g)$ from the biosphere and geosphere to replenish the atmosphere. In addition, dinitrification 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 dinitrification, $N_2O$, is a “greenhouse gas” that has also been linked to ozone destruction in the stratosphere.4 In most, if not all, organisms, dinitrification occurs in four steps via the sequence:

$$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2 \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$$

The step involving the reduction of nitrite has been the focus of substantial attention and considerable controversy,2,5 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 dinitrification.

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 of heme-containing enzymes, the $d_{1}$ NiR’s.5 All examples characterized to date are dimers, with each monomer containing both a $c$ and a $d_{1}$ (dioxoisobacteriochlorin) chromophore.6 The roles of the two heme centers in catalysis remain unresolved, although the $d_{1}$ 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$^{3+}$→NO$^-$ or Fe$^{3+}$→NO$^-$), based primarily on isotopic exchange and trapping experiments.9,10 Its formation from nitrite is, however, too fast to be detected even by stopped-flow studies.11 We report herein the results of FTIR studies of the $d_{1}$ 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.

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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 NO.5,15–18 In contrast, NO has been seldom studied in this regard, because 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 $\nu_{NO}$ values of 1587 and 1615 cm$^{-1}$ were reported for $^{14}$NO and $^{15}$NO, respectively.19 In these studies, NO complex of ferric horseradish peroxidase (HRP) was reported to give a $\nu_{NO}$ at 1865 cm$^{-1}$ for $^{15}$NO$^{19a}$ and the NO complex of metHb was reported to give a $\nu_{NO}$ 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 $\lambda_{max}$ 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 ($\lambda_{max} = 540$ and 570 nm).21 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 $\Delta\nu_{NO} < 10$ cm$^{-1}$ and $\nu_{NO} = 1615$ 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 very sharp ($\Delta\nu_{NO} = 8$ 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$^{2+}$–NO unit or a heme Fe$^{2+}$–NO$^+$ species formed by reaction of NO product with the oxidized enzyme;
NO\textsuperscript+ 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,\textsuperscript{14} suggesting the existence of significant NO\textsuperscript+ character. (In addition, a small amount of a second oxidized species is observed at 1905 and 1870 cm\textsuperscript{-1} for \textsuperscript{14}NO and \textsuperscript{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 \textit{cd} NiR in the \textit{αβ} region show peaks at 524 and 558 nm, attributed to the heme \textit{c}, and a peak at 640 nm, attributed to the \textit{h}emeheme 1,\textsuperscript{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 \approx 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 \textit{cd} NiR.\textsuperscript{24} In contrast, the heme \textit{d} 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 \textit{cd} NiR in the presence of NO are shown in Figure 2 as \textsuperscript{14}NO minus \textsuperscript{15}NO difference spectra. Other details are the same as in Figure 1.  

**Scheme 1.** Proposed Mechanism for Formation of NO from Nitrite at the Heme \textit{d} 1 Site of Heme \textit{cd} 1 Nitrite Reductase\textsuperscript{26}

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

\begin{center}
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{species} & \textbf{\textsuperscript{14}N/\textsuperscript{16}O frequency (\textit{cm}\textsuperscript{-1})} & \textbf{\textsuperscript{15}N shift (obs) (\textit{cm}\textsuperscript{-1})} & \textbf{\textsuperscript{15}N shift (calc) (\textit{cm}\textsuperscript{-1})} \\
\hline
\textit{ox cd} \textit{d} NiR•NO & 1910 & 36 & 34.3 \\
methHb•NO & 1925 & 36 & 34.5 \\
ox HRP•NO & 1865\textsuperscript{b} & 28 & 29 \\
deoxyHb•NO & 1616.5\textsuperscript{b} & & \\
methHb•NO & 1925\textsuperscript{b} & & \\
\hline
\end{tabular}
\end{center}

\textsuperscript{a}HRP = horseradish peroxidase. Data from ref 19a. \textsuperscript{b}Frequency for \textsuperscript{15}NO complex. \textsuperscript{c}Data from ref 19b.

The overlap with the intense amide I band around 1600 cm\textsuperscript{-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 \textit{c} and \textit{d} of the analogous heme \textit{cd} NiR from \textit{Pseudomonas aeruginosa} form complexes with NO,\textsuperscript{24} and our own results suggest that this is also true for the \textit{P. stutzeri} enzyme. Whether the unstable oxidized \textit{d}1•NO species that is formed is best formulated as an Fe\textsuperscript{2+}•NO or Fe\textsuperscript{3+}•NO complex is, to some extent, a matter of semantics. The value of \nu\textsubscript{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\textsuperscript{3+} has occurred, resulting in a species with considerable NO\textsuperscript+ character.\textsuperscript{27} The value of \nu\textsubscript{NO} observed for the oxidized \textit{cd} d1 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\textsuperscript{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.

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