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

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

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 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 of brucine (dioxoisobacteriochlorin) chromophore.⁹ The roles of the two heme centers in catalysis remain unresolved, although the heme d₁ is presumed to be the site at which nitrite is reduced. Substantial evidence has been adduced for the existence of an electrophilic heme–nitosyl 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, however, too fast to be detected even by stopped-flow studies.¹² We report herein the results of FTIR studies of the heme cd 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;

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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 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 N₂.¹⁵⁻¹⁸ In contrast, NO has been seldom studied in this regard, because while NO by 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 νNO 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 νNO at 1865 cm⁻¹ for ¹⁵NO and the NO complex of metHb was reported to give a νNO at 1925 cm⁻¹ for ¹⁴NO,²⁰ but no spectra were shown.

Consequently, we began by examining the FTIR spectra of concentrated samples of metHb.²¹ Optical spectra of metHb treated with 1 atm NO (not shown) show that a new species with λmax 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 (λmax = 540 and 570 nm⁻²). 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 Δλ/Δν ≈ 0 cm⁻¹ and νNO = 1615 and 1587 cm⁻¹ for ¹⁴NO and ¹⁵NO, respectively, in good agreement with earlier work.²² At higher energies, however, a new feature is seen that decays with time. This feature is very sharp (Δλ/Δν ≈ 8 cm⁻¹) and appears at 1925 and 1889 cm⁻¹ for ¹⁴NO and ¹⁵NO, respectively. This feature is most reasonably assigned to the metHb–NO species, which can be formulated as containing either a heme Fe³⁺–NO⁻ unit or a heme Fe²⁺–NO⁺.
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, 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 peaks due to $^{15}$NO+ originate from a minor NO-bound species. The FTIR spectra of solutions 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.$^{23}$ 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 $cd_1$ NiR.$^{24}$

In contrast, the heme 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 $cd_1$ 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$^-$ in the mechanism proposed for the enzyme.$^{26}$

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

<table>
<thead>
<tr>
<th>species</th>
<th>$^{15}$N$^{16}$O frequency (cm$^{-1}$)</th>
<th>$^{15}$N shift (obs) (cm$^{-1}$)</th>
<th>$^{15}$N shift (calc) (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ox $cd_1$ NiR--NO</td>
<td>1910</td>
<td>36</td>
<td>34.3</td>
</tr>
<tr>
<td>metHb--NO</td>
<td>1925</td>
<td>36</td>
<td>34.5</td>
</tr>
<tr>
<td>ox HRP--NO</td>
<td>1865$^{b}$</td>
<td>36</td>
<td>29.6</td>
</tr>
<tr>
<td>deoxyHb--NO</td>
<td>1616$^{c}$</td>
<td>28</td>
<td>29.6</td>
</tr>
<tr>
<td>metHb--NO</td>
<td>1925$^{c}$</td>
<td>36</td>
<td>29.6</td>
</tr>
</tbody>
</table>

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

The FTIR spectra of D$_2$O 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. The other details are same as in Figure 1.

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