Electronic Structure of Reaction Intermediate of Cytochrome P450nor in Its Nitric Oxide Reduction

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Nitric oxide reductase (Nor) isolated from the denitrifying fungus *Fusarium oxysporum* is a cytochrome P450-type heme enzyme (P450nor) and catalyzes a nitric oxide (NO) reduction reaction, in which two NO molecules are converted into nitrous oxide (N_2O) using two electrons directly transferred from NADH: $2NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+$.[1,2] By optically following the reaction of the ferric−NO bound enzyme with NADH, we found that a new species having a Soret band at 444 nm is present as a transient reaction intermediate (**I**) in the NO reduction.[2c,e] The **I** decays to the ferric enzyme whose rate is dependent on the NO concentration, suggesting that the process involves an attack of a second NO molecule on **I** and a release of N_2O. Since the optical spectral feature of **I** is significantly different from those of the ferric−NO ($Fe^{3+}NO$) and the ferrous−NO ($Fe^{2+}NO$) complexes of P450nor, we tentatively assigned **I** as a two-electron reduced species of the $Fe^{3+}NO$ complex, $\{FeNO\}^8$, which is comparable to that of the oxy−iron complex, $\{FeO_2\}^8$. While the two-electron reduced species of the $Fe^{3+}NO$ complex is unprecedented in hemoprotein chemistry, we consider it as a key precursor for the N_2O formation that involves the N−O bond cleavage and the N−N bond formation. To understand the unique chemistry catalyzed by P450nor, the electronic and the ligand coordination structures of **I** should be characterized in more detail. We have therefore undertaken resonance Raman spectroscopic investigation of **I** to clarify the molecular mechanism of the most important process in the NO reduction catalyzed by P450nor.[3]

We have developed a mixed-flow cell[4] for Raman measurements of **I** which has only a few seconds of lifetime. The resonance Raman spectrum of **I** shown as the trace d of Figure 1 (left panel) was obtained at 200 ms after mixing the $Fe^{3+}NO$ complex of P450nor with NADH. The distinct difference between the traces b ($Fe^{3+}NO$ complex) and d indicates that the formation of **I** was completed at 200 ms. The sample solution after five minutes gave the spectrum identical to that of the ferric resting enzyme (Figure 1a, left panel). These observations are consistent with those in the optical spectral measurements reported earlier.[2e]

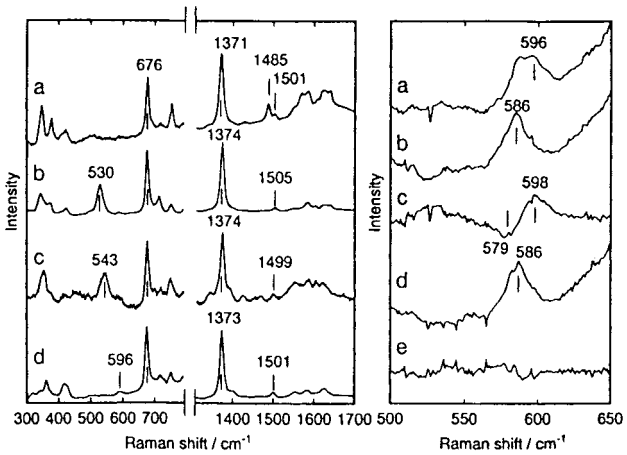

Figure 1. (Left Panel) High (1300∼1700 cm^{-1})- and low (300∼800 cm^{-1})-frequency regions of the resonance Raman spectra for P450nor in ferric resting (trace a), ferric−NO bound (trace b), ferrous−NO bound forms (trace c), and intermediate **I** (trace d). All spectra were obtained at 200 ms after mixing two solutions in 1:1 ratio using the mixed-flow cell. The two solutions were buffer and ferric P450nor for trace a, buffer and ferric−NO bound P450nor for trace b, sodium dithionite (10mM) and ferric−NO bound P450nor for trace c, and NADH (10mM) and ferric−NO bound P450nor for trace d. Intensity scale of the spectra presented in the low-frequency region was expanded three times relative to that of the high-frequency spectra. All solutions contained 100 mM potassium phosphate which was adjusted at pH 7.0. Concentration of proteins before the mixing was 100 μM. The laser excitation wavelength was 441 nm whose intensity at the sample point was 20 mW. Traces a and b measured using the mixed-flow cell were identical to those measured using the rotating cell.[7] (Right Panel) The Fe−NO stretching (ν_{Fe-NO}) region of the resonance Raman spectra for intermediate **I**. Traces a, b, and d were obtained by the same experimental condition as that of trace d of the Figure 1, except for the use of ^{15}NO, and ^{15}NO and D_2O buffers for traces b and d, respectively. Trace c demonstrates the $^{14}NO - {}^{15}NO$ difference spectrum calculated by subtracting trace b from a. Trace e demonstrates the deuterium shift calculated by subtracting trace d from b. The peak observed at 588 cm^{-1} in the spectrum a should arise from the porphyrin vibration because its frequency is insensitive to the $^{14}NO/^{15}NO$ substitution.

Several Raman lines that appear in the high-frequency region are known to indicate the electronic structure of the heme macrocycle. First, the ν_4 lines were observed at 1371∼4 cm^{-1} for the ferric resting, $Fe^{3+}NO$, $Fe^{2+}NO$, and **I** (see Figure 1, left panel). Since the frequency of the ν_4 line reflects the amount of π-electrons present in the heme macrocycle,[6] this observation suggests that the redox states of the macrocycle are similar in all of the complexes examined. The ν_3 lines, whose frequencies reflect the core sizes of heme macrocycles, were observed at 1501 cm^{-1} for **I** and at 1505 cm^{-1} for $Fe^{3+}NO$ and 1499 cm^{-1} for

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(3) The resonance Raman spectra were measured with a JASCO NR-1800 spectrometer which was modified in a single dispersion mode and equipped with a liquid nitrogen cooled CCD detector (Princeton Instrument). Excitation wavelength used for the measurement was 441.6 nm from He-Cd laser (Kimmon, Electronics, CDR80SG).

(4) A new cell was designed that can mix two solutions flowing slowly by referring to the cell designed by Takahashi et al.[5] Flow rate of the mixed solution was 3 mL/min. The incident laser was focused on a point of the channel where the solution is aged 200 ms after the mixing.

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$Fe^{2+}NO$, indicating that the core size of **I** is similar to that of $Fe^{2+}NO$ rather than that of $Fe^{3+}NO$. On the basis of the observations in the high-frequency region, it is obvious that the heme macrocycle does not accept electrons in **I** as a π-anion radical and that the excessive electrons of **I** should reside on the Fe−NO moiety, whose iron is likely in a ferrous state.

The low-frequency region of the resonance Raman spectra is also presented in Figure 1 (left panel). The Fe−NO stretching lines (ν_{Fe-NO}) are observed at 530 and 544 cm^{-1} for $Fe^{3+}NO$ (Figure 1b, left panel) and $Fe^{2+}NO$ (Figure 1c, left panel) complexes, respectively, which were shifted to 525 and 528 cm^{-1} upon substitution of ^{15}NO for ^{14}NO (data not shown).[7] In the spectrum of **I**, these two lines are undetectable; however, a new line appears at 596 cm^{-1} (Figure 1d, left panel or Figure 1a, right panel) which is shifted to 586 cm^{-1} by the ^{15}NO-substitution (Figure 1b, right panel).[8] The $^{14}NO - {}^{15}NO$ difference spectrum (Figure 1c, right panel) shows that only this line is sensitive to the ^{15}NO-substitution. The line cannot be assigned to the Fe═N stretching mode, since the mode usually appears at 700−900 cm^{-1} as reported by model studies.[9] Furthermore, the observed isotope shift (10 cm^{-1})[10] is unlikely to be explained by a simple Fe═N oscillator which should show about 22 cm^{-1} shift as reported for the model compound.[9] Therefore, we interpret that the N−O bond is not cleaved in **I** and assign the line at 596 cm^{-1} as ν_{Fe-NO}.

Our assignment for **I** is consistent with the observation in the model study, in which Choi et al. reported the ν_{Fe-NO} of Fe^{2+}(TPP)(NO) at 525 cm^{-1} and its one-electron reduced product, $Fe^{2+}(TPP)(NO)^-$, at 549 cm^{-1}.[11] Addition of electrons to either the d-orbital of iron and/or the $2p\pi^*$-orbital of NO should facilitate either the metal−ligand electron back-donation or donation, respectively, and eventually strengthens the Fe−NO bond and shifts the ν_{Fe-NO} to a higher frequency.

The Fe−NO unit in **I** should be bent because the isotope shift (10 cm^{-1}) of **I** is larger than that of the ferric−NO complex having a linear Fe−NO unit (5 cm^{-1}).[7] It was also found that the N atom of the NO ligand of **I** does not possess an exchangeable proton,[12] since the $\nu_{Fe-{}^{15}NO}$ line of **I** in D_2O (Figure 1d, right panel) appears at the same frequency as that in H_2O (Figure 1b, right panel). With the upper limit of the deuterium shift estimated using the noise level of the difference spectrum (Figure 1e, right panel), the intensity and line width of $\nu_{Fe-{}^{15}NO}$ is less than 2.2 cm^{-1},[13] and thus the possibilities of a weak hydrogen bonding on the NO or protonation at the O atom cannot be neglected. On the basis of all of the Raman observations in this study, we propose the structure **I** in Figure 2 as the best description of **I**, where a negative charge is delocalized on the NO moiety. Although Averill proposed **2** as the structure of **I**[15] based on a hypothetical hydride (H^-) transfer from NADH to the $Fe^{3+}NO$ to form **I**, we believe it to be unlikely because of the following circumstantial evidence. First, the hydrogen directly attached to the N atom of structure **2** is probably exchangeable with solvent protons. Second, our recent mutagenesis work has suggested that the proton transfer through the specific hydrogen bond network (H_2O- - -Ser286- - -H_2O- - -Asp393- - -solvent) is involved in the intermediate formation.[16] Third, the presence of ordered water molecules in the vicinity of the heme is observed in the crystal structure.[16] Hydride transfer across the water molecules is unlikely to occur.[17]

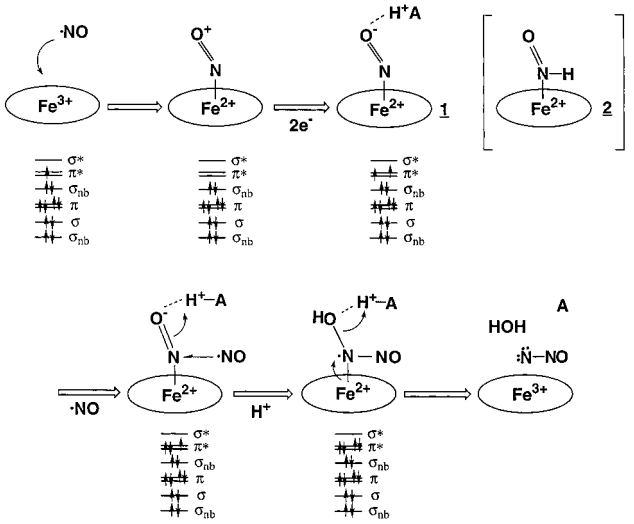

Figure 2. Schematic electronic and coordination structures of the heme in the NO reduction by P450nor. The molecular orbital energy diagrams of the iron-bound NO are shown below the corresponding models.[14] In **1**, a negative charge is delocalized on the NO moiety. In this scheme, A represents a base and could be either H_2O or OH^-.

The structure **1** would be stabilized through hydrogen bonds with the ordered water molecules (A in Figure 2) which interact with their surrounding amino acid residues such as Ser286 and Thr243.[16,18] In addition, the cationic environment of the heme pocket generated by the positively charged cluster of Lys62, Arg64, Lys291, Arg292, and some water molecules, which locates 10∼12 Å above the iron,[16] might contribute to the stabilization of the highly reduced state of **I**. The electronic structure of **I** we proposed, i.e., structure **1**, has never been observed as a physiological reaction intermediate of hemoproteins, but it should be suitable for the hyponitrite mechanism for the N_2O formation proposed by us and our collaborators.[19,2e] In this mechanism, the second NO molecule attacks the N atom of **1** to transiently yield hyponitrite ($HONNO^-$) (the N−N bond formation), which rapidly decomposes into N_2O and H_2O (the N−O bond cleavage) as illustrated in Figure 2. It should be stressed that the thiolate ligation as the iron fifth ligand, which strengthens the Fe−NO bond in **1**, facilitates these reactions.

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(10) Because of the presence of the heme Raman line at 586 cm^{-1}, it is difficult to estimate the isotope shift accurately. The 10 cm^{-1} shift might be smaller than the real shift since the peak-to-trough separation of the NO − ^{15}NO spectrum (Figure 1c, right panel) is 19 cm^{-1}.

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(12) In general chemistry, the nitrogen atom of the iron-bound NO should possess a negative charge in **I**. However, our experimental data do not strongly support this suggestion. One of possible explanations for this discrepancy is that the S^- ligation at the heme 5th axial side raises the electron density on the nitrogen atom, resulting in delocalization of the negative charge on the NO moiety.

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