UV Resonance Raman Detection of a Ligand Vibration on Ferric Nitrosyl Heme Proteins

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The Fe^{3+}-nitric oxide (NO) complexes formed from the reactions of NO with heme proteins are of significant physiological relevance. Microbial denitrification catalyzed by cytochrome P450nor[1,2] is initiated by the formation of an Fe^{3+}-NO complex. The delivery of NO by a blood-sucking insect, *Rhodnius prolixus*, to its victims is via the ferric, not ferrous, NO complex of nitrophorin.[3] The affinity of NO for Fe^{3+} is much lower than that for Fe^{2+}, thereby facilitating the transfer of NO. While vibrational spectroscopy has been applied extensively to explore heme-ligand systems,[4] its application to Fe^{3+} heme-NO complexes has been scarce, primarily because of the weak vibrational signals from the NO moieties. Thus, the development of an efficient and highly sensitive method to detect the ligand vibrations in Fe^{3+} heme NO complexes is necessary. Here, we report an innovative and highly sensitive UV resonance Raman (RR) spectroscopic method for detecting the NO stretching frequency of a variety of Fe^{3+} NO heme proteins, including myoglobin (Mb), horseradish peroxidase (HRP), mammalian heme oxygenase isoform 2 (HO-2),[5] and FixLH.[6]

The 244 nm excited RR spectra of Fe^{3+} myoglobin (metMb) and its NO-bound forms are shown in Figure 1.[7] Strong RR bands at 1559 and 1620 cm^{-1}, which are derived from aromatic amino acids, tyrosine and tryptophan,[8] are observed for metMb and metMb^{14}NO (Figure 1, spectra A and B, respectively). However, metMb^{14}NO has an additional RR band at 1922 cm^{-1} that is absent in the metMb spectrum (Figure 1, spectrum A). This band is shifted to 1884 cm^{-1} upon the replacement of ^{14}NO with ^{15}NO, as shown in Figure 1, spectrum C. Its presence, along with the maximum (1922 cm^{-1}) and minimum (1884 cm^{-1}) derived from the difference spectrum between ^{14}NO and ^{15}NO complexes (Figure 1, spectrum D), gives evidence of an N−O stretching mode [ν(NO)]. Our ν(NO) of 1922 cm^{-1} is in agreement with that observed by IR spectroscopy,[2b] albeit 5 cm^{-1} lower than a previously reported value.[9] The observed ^{15}N-isotopic shift of 38 cm^{-1} is close to that expected for a diatomic NO molecule (35 cm^{-1}).

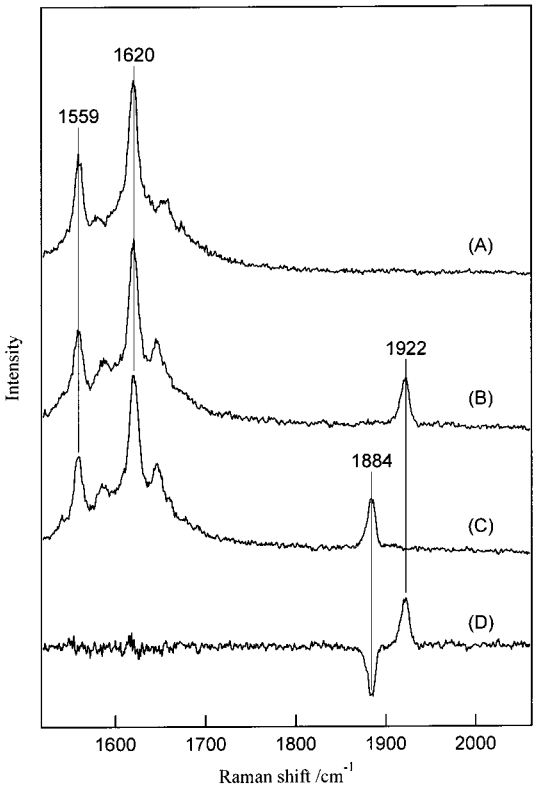

Figure 1. UV resonance Raman spectra for metMb (A), metMb-^{14}NO (B), and metMb-^{15}NO (C). Spectrum D is the difference between spectra B and C. The spectra were obtained at 20 °C using a laser power of 0.3 mW and 5 min accumulation time. The sample was diluted to 0.25 mM with 50 mM phosphate buffer, pH 7.

One advantage of our current UV RR method of measurement, in comparison to that by IR, is that the concentration of protein required to obtain a distinct spectrum is more than 10-fold (0.1−0.4 mM) lower than that used in IR measurements (1−10 mM). Furthermore, the ν(NO) band can be detected without spectral manipulations. Therefore, this method could apply to heme systems that are difficult to concentrate highly.

Soret excitation RR spectroscopy is a popular method for detecting ligand vibrations. In fact, Fe−NO stretching or Fe−N−O bending modes of Fe^{3+}-NO were observed by RR spectroscopy with blue excitation.[10] Furthermore, ν(NO) of Fe^{3+}-NO

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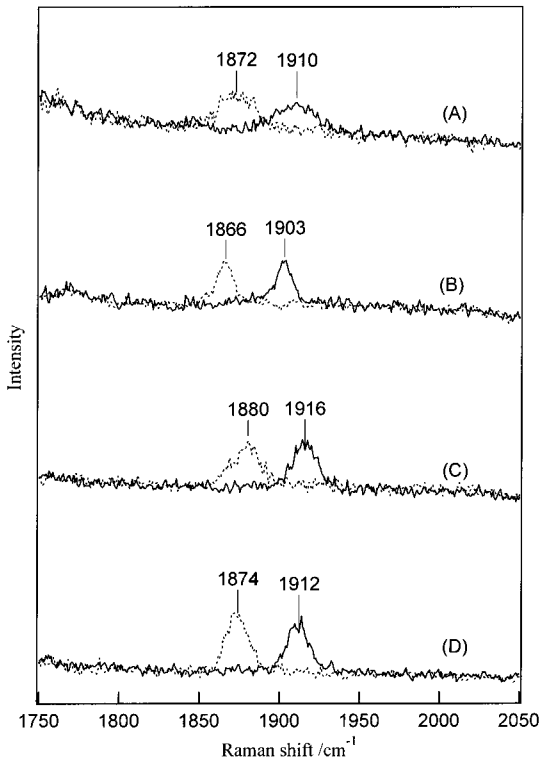
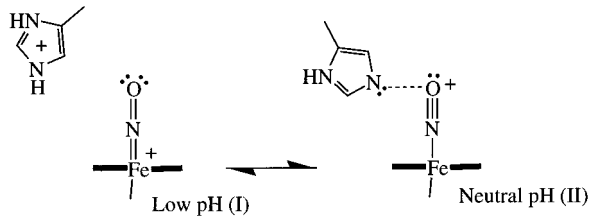


Figure 2. UV resonance Raman spectra for metMb-NO acidic form (A), HRP-NO (B), HO-2-NO (C), and FixLH-NO (D). The solid and dotted lines represent the ^{14}NO and ^{15}NO adducts, respectively. The MetMb-NO acidic form was made by addition of degassed acetic acid into the neutral metMb-NO. Other experimental conditions are the same as those described in Figure 1.

was also obtained in the case of P450nor.[2b] However, ν(NO) Raman bands of the four heme-NO complexes used in this report could not be obtained by Soret excitation RR spectroscopy. This indicates that there is little orbital conjugation between the NO group and the heme. UV RR does not always need such conjugation. Thus, it is likely that these Raman bands are enhanced in resonance with the localized $\pi-\pi^*$ transition.[11]

In addition to the acidic form of metMb, we have applied our novel UV RR technique to measuring the ν(NO) of the Fe^{3+} NO adducts of HRP (Figure 2, spectrum B), HO-2 (Figure 2, spectrum C), and FixLH (Figure 2, spectrum D). The isotopic substitution of ^{14}NO with ^{15}NO renders a set of RR spectra that have bands at 1910, 1903, 1916, and 1912 cm^{-1} for the Mb-NO acidic form, HRP-NO, HO-2-NO, and FixLH−NO, respectively. As these bands have downshifted 36−37 cm^{-1} from those observed for the nonisotopic ^{14}NO fomrs (Figure 2, spectra A−D, solid line, respectively), they are assigned to ν(NO) mode. They are quite similar to those of NP1−NO (1917, 1904 cm^{-1}).[3]

The radical NO has one electron (e^-) in its antibonding (π^*) orbitals. While the removal of this e^- strengthens the N−O bond and yields NO^+, the addition of an e^- weakens the N−O bond, leading to NO^-. The stretching frequency of diatomic NO molecule is very sensitive to its electronic state and widely disperserd as follows; $\nu(NO^+)$, $\nu(NO^\bullet)$, and $\nu(NO^-)$ are 2345, 1876, and 1284 cm^{-1}, respectively.[12] The electronic states of bound NO on the four aforementioned heme proteins are close to that of $NO^\bullet$ and slightly electron deficient, judging from their ν(NO) value (1903−1922 cm^{-1}). In the case of HRP, the trans ligand of NO is a histidine (His) that has an anionic character because of the strong hydrogen bonding of its N_δ proton.[13] Consequently, π donation from the trans ligand to the bound NO is expected, as the case of Fe^{3+} P450cam-NO; its ν(NO) (=1806 cm^{-1}) means strong π donation from the thiolate ligand.[2b] However, the ν(NO) of Fe^{3+} HRP-NO is only 6−19 cm^{-1} lower than that of metMb-NO, HO-2-NO, and FixLH−NO, which all have a neutral His as their trans ligand. Comparatively, the ν(CO) of Fe^{2+} HRP-CO is 40−63 cm^{-1} lower than that of the Fe^{2+}-CO forms of Mb, HO, and FixLH.,[6a,14] The difference in the stretching mode of Fe^{2+} and Fe^{3+} heme complexes is attributed to the effects of the π donation by the trans ligand, effects which are compounded by the presence of a hole in the d_π orbitals of Fe^{3+}.



The ν(NO) of metMb-NO is pH dependent. At pH 4, the distal His of metMb-NO is protonated and swings out into the solvent,[15] thereby removing the interaction between the bound NO and the distal His. If ν(NO) is reflective of the relative contributions of the two resonance structures of $Fe^+=N=O$ and $Fe-N\equiv O^+$ rather than of the trans ligand effects, it would become pH dependent. As suggested by Chance and co-workers, the stability of the Fe-NO structure depends on the protonation of the distal His.[9] If the distal His is the N_δ-H tautomer, the lone pair of electrons on N_ϵ will be located adjacent to the O atom of bound NO, stabilize structure (II), and raise ν(NO). In the case of the Fe^{2+} MbCO complex, the Nϵ-H tautomer of His64 is present, donates a hydrogen bond to the O atom of bound CO, stabilizes the $Fe=C=O$ resonance form, and lowers ν(CO).[4] When the distal histidine is protonated and rotates out into solvent or is replaced with an apolar residue, ν(CO) for Fe^{2+} MbCO increases, whereas for metMbNO these same changes cause a decrease in ν(NO).[9] Thus, the influences imposed by trans ligands and the distal environment on the stretching frequency of the bound ligand are different for Fe^{3+}NO and Fe^{2+}CO.

In conclusion, we demonstrate detection for ν(NO) of Fe^{3+} heme-NO heme proteins having His as an axial ligand by UV RR spectroscopy. The ν(NO) of the four cases indicates that the NO moiety is electron deficient and that the effect of π donation from the proximal His is small. The distal His stabilizes the $Fe-N\equiv O^+$ resonance form of metMb-NO, and the ν(NO) is shifted higher than those of the other NO complexes.

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