# Articles

## Resonance Raman Spectroscopic Studies of the Nitric Oxide Adducts of Cobaltous-Reconstituted Myoglobin and Hemoglobin

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Resonance Raman spectra of the nitric oxide adducts of cobaltous-reconstituted myoglobin and hemoglobin were measured using 406.7-nm excitation. The $\nu$(N–O), $\nu$(Co–NO), and $\delta$(Co–N–O) modes were located at 1613, 576, and 367 $cm^{-1}$, respectively. The band assignments were secured by a normal-mode analysis of their frequencies and isotopic shifts upon substitution of natural-abundance nitric oxide with $^{15}N^{16}O$, $^{14}N^{18}O$, and $^{15}N^{18}O$. The slightly higher $\nu$(Co–NO) and dramatically lower $\delta$(Co–N–O) frequencies compared to those of the Fe(II)–NO linkage were explained on the basis of the consideration of both the bonding interaction between the central metal ions and the bound nitric oxide and the kinematic effect imposed by their significantly different bonding geometries.

### Introduction

Resonance Raman (RR) spectroscopy has been increasingly used to probe the structure, dynamics, and reaction mechanisms of heme proteins.[1] It is well established that the high-frequency RR spectrum, which arises from the skeletal vibrations of the porphyrin ring, is very useful in monitoring the oxidation and spin states of metal ions. The frequencies of these bands were also correlated to the core size of metalloporphyrins.[2] Although the potentially informative, but complex, RR spectra of heme proteins in the low-frequency region are poorly understood at present, those bands associated with metal–axial ligand vibrations can be identified with the help of isotope substitution. Excitation into the Soret or charge-transfer transitions has been effectively used to enhance certain metal–ligand vibrations (metal–ligand stretching, bending, and ligand internal modes). Thus, many of the normal vibrational modes of the exogeneous diatomic ligand adducts (carbon monoxide, nitric oxide, dioxygen, and cyanide) have been detected and assigned.[3] Careful analysis of the frequencies and intensities of these modes permits us to investigate the heme structure and to probe the active-site environment. In this paper, the RR structural characterization of the nitric oxide adducts of cobaltous-substituted myoglobin and hemoglobin is reported. These adducts are of particular interest, inasmuch as they are isoelectronic with the physiologically important oxygenated heme proteins. In addition, cobaltous myoglobin and hemoglobin have been shown to reversibly bind dioxygen,[4] confirming their physiological relevance. This is the first report of the observation and assignment of the three expected normal modes of the Co(II)–NO fragment, confirming a previous detection of the $\nu$(Co–NO) mode.[5] The results are discussed with respect to the structure and bonding of the Co(II)–NO moiety.

### Experimental Section

Cobalt(III) protoporphyrin IX chloride was purchased from Porphyrin Products (Logan, UT) and used without further purification. $Na^{15}NO_2$ (99.5%) was obtained from MSD. The $^{18}O$-labeled nitrites ($NaN^{18}O_2$ and $Na^{15}N^{18}O_2$) were synthesized from the corresponding $^{16}O$ derivatives by acid-catalyzed oxygen atom exchange[6] in bulk solvent $H_2{}^{18}O$ (99.5%, MSD) and characterized by Raman spectroscopy as described previously.[7] Horse heart myoglobin was obtained from Sigma (St Louis, MO) as a lyophilized powder and was purified by passage over a Sephadex G-25 column to remove insoluble impurities. Human hemoglobin was obtained from whole red blood cells according to the published procedure.[8] The apoglobins of both myoglobin and hemoglobin were prepared using Teale's method.[9] The residual heme of the resultant apoglobin preparations was less than 5%. The oxygenated adducts of cobaltous-reconstituted myoglobin and hemoglobin were obtained by the established procedure.[4,10]

The preparations of the nitric oxide adducts of cobaltous myoglobin and hemoglobin were performed by anaerobic decomposition of properly isotope-labeled sodium nitrite with dithionite in the following way. About 0.5 mL of 200 $\mu$M protein was placed in a 5 mm o.d. NMR tube, which was then sealed with a septum. After the tube was vigorously degassed by flushing with argon for several minutes to remove the bound oxygen, an approximately 50-fold excess of a buffered solution of sodium dithionite was added via a gastight syringe. This was followed by addition of an approximately 20-fold excess of sodium nitrite to form the nitric oxide adduct. No difference was noticed between the nitric oxide adduct thus formed and the one formed by exposure of cobaltous myoglobin or hemoglobin solution to nitric oxide gas, as reported previously.[5]

Resonance Raman spectra were acquired on a Spex Model 1403 system equipped with a Spex DM1B data station and a Hamamatsu R-928 photomultiplier. The excitation line was the 4067-Å line from a Coherent Innova Model 100-K3 $Kr^+$ laser. The laser power was about 10 mW at the sample point. The spectrometer was advanced at a scan speed of 0.5

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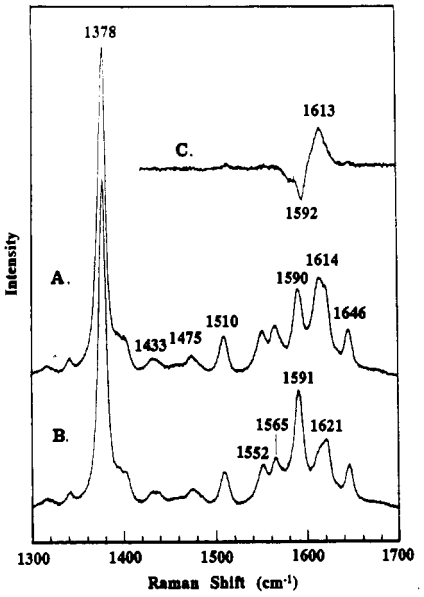


**Figure 1.** High-frequency resonance Raman spectra (406.7-nm excitation) of the nitric oxide adduct of cobaltous myoglobin: trace A, $^{14}N^{16}O$; trace B, $^{15}N^{16}O$; trace C, A–B difference spectrum, obtained by normalizing traces A and B using the 1378-$cm^{-1}$ band. The protein concentration was about 200 $\mu$M in 50 mM potassium phosphate buffer at pH 7.0; laser power at sample was 10 mW.

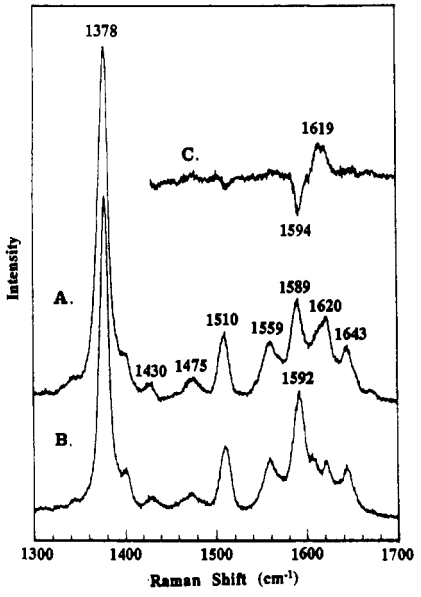


**Figure 2.** High-frequency resonance Raman spectra (406.7-nm excitation) of the nitric oxide adduct of cobaltous hemoglobin: trace A, $^{14}N^{16}O$; trace B, $^{15}N^{16}O$; trace C, A–B difference spectrum, obtained by normalizing traces A and B using the 1378-$cm^{-1}$ band. The concentration of protein was 200 $\mu$M in heme; the protein was dissolved in 50 mM potassium phosphate at pH 7.0; laser power at sample was 10 mW.

$cm^{-1}$/s. The high-frequency spectra were obtained from two scans. The low-frequency spectra were an accumulation of four scans. The sample contained in an NMR tube was positioned in a backscattering geometry and kept spinning to prevent photodissociation and to avoid local thermal degradation of proteins during the spectral acquisition process.

## Results and Discussion

**A. High-Frequency RR Spectra and the Heme Core Structure.** Figures 1 and 2 display the high-frequency resonance Raman spectra (406.7-nm excitation) of the nitric oxide adducts of cobaltous myoglobin and hemoglobin, respectively. The frequencies of all the bands are listed in Table I, along with those of ferrous nitrosyl myoglobin and hemoglobin. The assignments of the bands are made based upon the measurement of depolarization ratios, following the nomenclature of Abe and Kitagawa.[11] The assignments of vinyl vibrations were adopted from those reported by Spiro and co-workers[12] for nickel protoporphyrin IX.

**Table I.** Assignment of High-Frequency Resonance Raman Spectral Bands of Nitric Oxide Adducts of Cobaltous Myoglobin and Hemoglobin

| | (Mb)Co(II)–NO[a] | (Hb)Co(II)–NO[a] | (Mb)Fe(II)–NO[b] | (Hb)Fe(II)–NO[b] |
|---|---|---|---|---|
| $\nu_{10}$ | 1643 | 1643 | 1638 | 1636 |
| $\nu$(C=C) | 1621 | 1620 | 1622 | 1623 |
| $\nu_2$ | 1590 | 1589 | 1584 | 1584 |
| $\nu_{11}$ | 1565 | 1559 | 1560 | 1564 |
| $\nu_{38}$ | 1552 | | 1543 | 1551 |
| $\nu_3$ | 1510 | 1510 | 1501 | 1500 |
| $\nu_{40}$ | 1475 | 1475 | 1470 | 1471 |
| $\delta$(=$CH_2$) | 1433 | 1430 | 1433 | 1431 |
| $\nu_4$ | 1378 | 1378 | 1375 | 1375 |

[a] This work. [b] Reference 14a.

The Soret-excited RR spectra are expected to exhibit primarily polarized modes ($A_{1g}$ symmetry) of the heme group. Thus, the observed spectra show prominent $A_{1g}$ vibrational modes, i.e., $\nu_2$ (1590 $cm^{-1}$), $\nu_3$ (1510 $cm^{-1}$), and $\nu_4$ (1378 $cm^{-1}$). In addition, two $B_{1g}$ modes, $\nu_{10}$ (1643 $cm^{-1}$) and $\nu_{11}$ (1565 $cm^{-1}$), are also enhanced, presumably owing to the Jahn–Teller effect, albeit with weaker intensity. The RR activation of some IR-active $E_u$ modes ($\nu_{38}$ at 1552 $cm^{-1}$ for nitrosyl cobaltous myoglobin), as originally pointed out by Spiro and co-workers,[12] was also confirmed. The $\nu_{37}$ mode was not clearly discernible in the natural-abundance nitric oxide adduct but was detected at 1605 $cm^{-1}$ as a weak feature in the spectrum of the $^{15}NO$ adduct of cobaltous hemoglobin. This difficulty arises from the overlapping of this mode with the stronger $\nu$(N–O) mode. It is found that the double-bond stretch, $\nu$(C=C), of vinyl groups overlaps accidentally with the internal $\nu$(N—O) vibration of the bound nitric oxide. As a result, the frequency of this mode listed in Table I is obtained from the spectra of the $^{15}N$ nitric oxide adducts.

The high-frequency RR spectra of heme proteins are known to exhibit sensitive marker bands for the oxidation and spin states of central metal ions. The frequencies of these bands can also be used to estimate the heme core size, (i.e., the distance from the center of the porphyrin ring to the pyrrole nitrogen ($C_t$–N)), using the established empirical relationship $\nu = k(A - d)$ and the tabulated $k$ and $A$ values.[2d] For the nitric oxide adducts of cobaltous myoglobin and hemoglobin, the spectral pattern as shown in Figures 1 and 2 is characteristic of a low-spin six-coordinate ($\pi$-acid ligand) adduct. The oxidation-state marker, $\nu_4$, occurs at 1378 $cm^{-1}$ and the spin-state marker bands, $\nu_3$ and $\nu_{10}$, appear at 1510 and 1643 $cm^{-1}$, respectively. The $C_t$–N distance of 1.974 Å was obtained from the average of the $C_t$–N values calculated from the observed frequencies of two spin-state markers, $\nu_3$ (1.966 Å) and $\nu_{10}$ (1.982 Å). This value is very close to the one obtained for an appropriate model compound, nitrosyl(tetraphenylporphinato)cobalt(II) (1.978 Å), by an X-ray crystal structural determination.[13]

**B. RR Observation and Assignment of the Vibrational Modes Associated with the Bound Nitric Oxide.** Comparison of traces A and B of Figures 1 and 2 reveals an altered intensity for the nitric oxide adducts of both cobaltous myoglobin and hemoglobin in the 1600-$cm^{-1}$ region upon $^{14}N^{16}O/^{15}N^{16}O$ isotopic substitution. This can be clearly seen in the difference spectrum (trace C), obtained by normalizing traces A and B and subtracting trace

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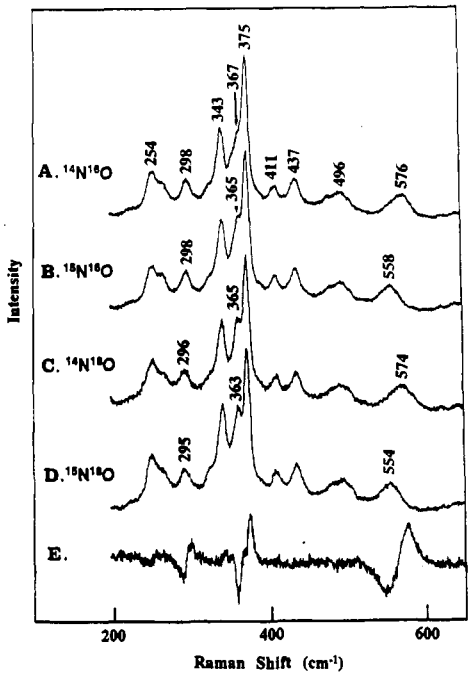


**Figure 3.** Low-frequency resonance Raman spectra (406.7-nm excitation) of the isotopomeric nitric oxide adducts of cobaltous myoglobin. Trace E: difference spectrum (A–D) × 1.5, obtained by normalizing traces A and D using the 437-cm$^{-1}$ band.

B from trace A. In the spectrum of the nitric oxide adduct of cobaltous-reconstituted myoglobin, the positive 1613-cm$^{-1}$ band ($^{14}$NO) shifts to 1592 cm$^{-1}$ ($^{15}$NO). Similarly, the nitrosyl cobaltous hemoglobin exhibits a positive peak at 1619 cm$^{-1}$ ($^{14}$NO) and a negative peak at 1594 cm$^{-1}$ ($^{15}$NO). The observed isotope shifts (21–25 cm$^{-1}$) compare reasonably well with the calculated one (28.9 cm$^{-1}$) based on a two-body vibrator model, thus confirming their assignment to the $\nu$(N–O) mode. The deviation of isotope shifts is, partly, due to experimental uncertainty, since the N–O stretching vibrations are generally weak and overlapped with porphyrin modes; therefore they are determined from the difference spectra.

Figure 3 presents the low-frequency RR spectra of the isotopomeric nitric oxide adducts of cobaltous myoglobin. The spectra are arranged in the order of increasing mass of nitric oxide, and all the bands are labeled. An isotope-sensitive line is clearly identified at 576 cm$^{-1}$ ($^{14}N^{16}O$), which shifts to 558 ($^{15}N^{16}O$), 574 ($^{14}N^{18}O$), and 554 cm$^{-1}$ ($^{15}N^{18}O$). This characteristic zigzig isotope-shift pattern is identical to those observed for the nitric oxide adduct of several ferrous heme proteins.[7,14] Other isotope-sensitive lines are located at 367 and 298 cm$^{-1}$. The 367-cm$^{-1}$ line is overlapped with a neighboring heme mode at 375 cm$^{-1}$ but becomes separated for the $^{15}N^{18}O$ derivative. This mode exhibits a monotonous downshift as the mass of the NO molecule increases from $^{14}N^{16}O$ (367 cm$^{-1}$), through $^{15}N^{16}O$ (365 cm$^{-1}$) and $^{14}N^{18}O$ (365 cm$^{-1}$), to $^{15}N^{18}O$ (363 cm$^{-1}$). Interestingly, an isolated band at 298 cm$^{-1}$ exhibits a small but definite isotopic shift. These three isotope-sensitive lines are manifested as three sets of positive-and-negative peaks in the difference spectrum (trace E).

To secure the vibrational assignments of the observed isotope-sensitive bands, we performed a normal-coordinate analysis of a simple tetraatomic L–Co–N–O model (L stands for *N*-methylimidazole with a dynamical mass of 82 au). The results, along with the experimental data, are presented in Table II. The structural parameters, given in Table II, were adopted from those

**Table II.** Normal-Mode Frequencies and Isotope Shifts of $\nu$(N–O), $\nu$(Co–NO), and $\delta$(Co–N–O) Vibrations of the L–Co–N–O Fragment[b,c] in the Nitric Oxide Adduct of Cobaltous Myoglobin

| mode | obsd ($\Delta^a$) | calcd ($\Delta^a$) | assgnt (PED, %) |
|---|---|---|---|
| $\nu_1$ | 1613 (21) | 1612.3 (30.0, 40.4, 71.3) | 98% $\nu$(NO) |
| $\nu_2$ | 576 (18, 2, 22) | 576.7 (15.6, 3.2, 18.8) | 59% $\nu$(Co–NO), 49% $\delta$(Co–N–O) |
| $\nu_3$ | 367 (2, 2, 4) | 362.0 (1.8, 8.9, 10.5) | 41% $\delta$(Co–N–O), 16% $\nu$(Co–NO), 35% $\nu$(Co–L) |
| $\nu_4$ | not obsd | 229.4 (1.0, 2.5, 3.5) | 83% $\nu$(Co–L) |

[a] The value indicates the isotopic shift of nitric oxide in the order of $^{15}N^{16}O$, $^{14}N^{18}O$, and $^{15}N^{18}O$. [b] Structural parameters for the L–Co–N–O linkage: $d$(Co–L) = 2.10 Å, $d$(N–O) = 1.01 Å, $d$(Co–N) = 1.85 Å, ∠CoNO = 130°, ∠LCoN = 180°. [c] Force constants used: $K$(N–O) = 11.25, $K$(Co–N) = 2.05, $K$(Co–L) = 1.40, $k$(L–Co,Co–N) = 0.20, $k$(Co–N,N–O) = 0.20 mdyn/Å; $H$(Co–N–O) = 0.66, $H$(L–Co–N) = 0.35 mdyn·Å/rad$^2$; $k$(Co–N,Co–N–O) = 0.11 mdyn.

of Co(TPP)NO[13] and Co(TPP)(1-MeIm).[15] The $d$(Co–N) distance was slightly adjusted from 1.833 to 1.850 Å to reflect the structural differences of the cobaltous nitric oxide adduct of myoglobin and Co(TPP)NO, which are six- and five-coordinate, respectively. Scheidt and co-workers[16] have shown that the Fe–NO linkage is longer in a six-coordinate adduct, Fe(TPP)(NO)(1-MeIm), than in a five-coordinate compound, Fe(TPP)(NO).

The initial force field[7] was transferred from those of the ferrous nitric oxide adduct, recently reported for cytochrome P450cam. The Co–L stretching force constant, $K$(Co–L), was set at 1.4 mdyn/Å, a value slightly smaller than that for Fe–L (1.6 mdyn/Å),[17] to take into account a longer Co–L (2.1 Å) vs Fe–L (2.0 Å) bond distance. The only other adjustment from the previous force field is the Co–NO stretching, $K$(Co–N). It was noticed that this force constant has to be reduced from 2.44 for $K$(Fe–N) to 2.05 mdyn/Å to fit the observed frequencies and isotope shifts for the cobaltous nitric oxide adduct of myoglobin. The reduction of the $K$(Co–N) force constant is important, considering the fact that the Co–N bond length (1.85 Å) is longer than that of Fe–N (1.74 Å). This agrees qualitatively with the empirical relationship of bond distance and stretch force constant, such as Badger's rule, which predicts a smaller force constant for a longer bond length. Thus, we are able to model the vibrational characteristics of the cobaltous nitric oxide adduct by meaningfully adjusting some force constants in the force field[7] initially developed for ferrous nitrosyl cytochrome P450cam.

Previously, Yu and co-workers[5] observed and tentatively assigned the Co–NO stretching vibration to a similar isotope-sensitive line observed in this region for the nitric oxide adducts of ferrous monomeric hemoglobin. This empirical assignment is clearly borne out in the present normal-mode analysis. Therefore, we assign the bands observed at 576 and 367 cm$^{-1}$ to $\nu$(Co–NO) and $\delta$(Co–N–O), respectively, although significant mixing of these two modes is evident in their respective potential energy distributions.

The low-frequency RR spectrum of the nitric oxide adduct of cobaltous hemoglobin is shown in Figure 4. The $\nu$(Co–NO) mode is readily recognized from its isotope shift trend from 566 ($^{14}N^{16}O$), through 551 ($^{15}N^{16}O$) and 562 ($^{14}N^{18}O$), to 548 cm$^{-1}$ ($^{15}N^{18}O$). However, the expected $\delta$(Co–N–O) mode cannot be seen clearly in the spectrum of any isotopomers, owing to its overlap with a strong heme vibrational mode at 370 cm$^{-1}$, but is identified at about 360 cm$^{-1}$ in the difference spectrum (trace E). In addition, the band at 295 cm$^{-1}$ also exhibits small downshifts.

We have assigned two of the observed three isotope-sensitive bands in the low-frequency region to the $\nu$(Co–NO) and $\delta$(Co–

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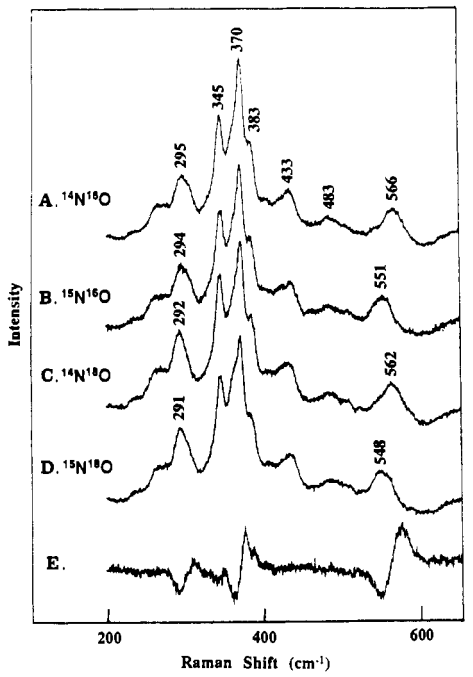


**Figure 4.** Low-frequency resonance Raman spectra (406.7-nm excitation) of the isotopomeric nitric oxide adducts of cobaltous hemoglobin. Trace E: difference spectrum (A–D) × 1.5, obtained by normalizing traces A and D using the 433-cm$^{-1}$ band.

N–O) modes, wishing to point out that the vibrational classification of the bands located at 295 and 298 cm$^{-1}$ (for nitrosyl hemoglobin and cobaltous myoglobin, respectively) is uncertain. We note here that a similar mode has previously been observed for the ferric cyanide and ferrous carbon monoxide adducts and tentatively assigned to the iron–histidine stretching vibrations,[18] on the basis of the fact that it displays isotopic sensitivity toward both the iron and exogenous axial ligands. However, in a recent low-frequency RR spectral study of oxyhemoglobin,[19] this mode was shown to shift in the spectrum of the oxygen-18 adduct and of the oxygenated derivatives of the *meso-d*$_4$ heme reconstituted hemoglobin. The combined result suggests that the observed isotope shift is reasonably attributed to coupling of the metal–axial ligand bending mode to a low-frequency macrocycle distortion (which is likely a heme out-of-plane vibration). Coupling of metal–axial ligand with out-of-plane vibrations was previously observed in a study of bis(imidazolyl) heme.[20] It is very attractive to speculate that this vibrational coupling is important in regulating the ligand-binding reactivity of heme iron.

**C. Comparison of the Bonding and Vibrational Frequencies of the M–NO Fragment for Co(II) and Fe(II) Derivatives.** Table III compares the vibrational frequencies of the M–NO fragment in the nitric oxide adducts of cobaltous and ferrous myoglobin and hemoglobin. It is obvious that $\nu$(N–O) is slightly lower for the cobaltous proteins than for those of the ferrous analogues. The decreased frequency could be the result of increased electron density on the bound nitric oxide, inasmuch as the N–O stretch is relatively independent of the M–N–O geometry. The observed lower $\nu$(N–O) frequency for the cobaltous nitrosyl adduct is also consistent with the reported lower $\nu$(O–O) frequencies for the dioxygen adducts of Co(II) porphyrins compared to those for the Fe(II) analogues.[21]

**Table III.** Comparison of Vibrational Frequencies of the M–NO Fragment in Nitric Oxide Adducts of Cobaltous and Ferrous Myoglobin and Hemoglobin

| | $\nu$(N–O) ($\Delta^a$) | $\nu$(M–NO) ($\Delta^a$) | $\delta$(M–N–O) ($\Delta^a$) |
|---|---|---|---|
| (Mb)Co(II)–NO[b] | 1613 (21) | 576 (18, 2, 22) | 367 (2, 2, 4) |
| (Mb)Fe(II)–NO[c] | 1624 (37) | 554 (8, 2, 12) | 449 (2, 4, 6) |
| (Hb)Co(II)–NO[b] | 1619 (25) | 566 (15, 4, 18) | ~365[e] |
| (Hb)Fe(II)–NO[c] | 1622 (30) | 555 (–, –, 22)[d] | 450 (4, 6, 7) |

[a] The value indicates the isotopic downshift of nitric oxide in the order of $^{15}N^{16}O$, $^{14}N^{18}O$, and $^{15}N^{18}O$. [b] This work. [c] References 7 and 14a. [d] The exact isotope shifts were not determined, owing to an overlapping heme mode. The $^{15}N^{18}O$ shift was obtained from the difference spectrum. [e] This mode was not located accurately. The value shown here indicates the presence of the $\delta$(Co–N–O) mode in this region as detected from the difference spectrum.

Before we discuss the difference of vibrational frequencies between the cobaltous and ferrous nitric oxide adducts, it is instructive to consider the bonding interactions between a transition metal and $\pi$-acid diatomic ligands (X–Y). In this class of compounds, two types of bonding interaction are important, i.e., the forward ligand → metal $\sigma$ and M → L $\pi$-back-bondings. The first results from the overlapping of filled ligand $\sigma$ and unoccupied metal $d_{z^2}$ orbitals, while the second is formed by the electron population flow from the occupied metal $d_\pi$ ($d_{xz}$ and $d_{yz}$) to the unoccupied or partially occupied ligand $\pi^*$ orbitals. It is expected that an enhanced $\pi$-back-bonding will weaken the X–Y bond and strengthen the M–XY linkage. Hoffman and co-workers[22] have shown that the $d_{xz} + \pi^*$ orbital is increasingly destabilized as M–X–Y becomes bent. Thus, the back-bonding is less effective in a more distorted M–X–Y system.

In a series of X-ray crystal structural studies of nitric oxide adducts of iron porphyrins, Scheidt and co-workers[16] have noticed that the Fe–N bond length is about 0.03 Å longer and ∠FeNO is about 10° smaller in six-coordinate Fe(TPP)(NO)(1-MeIm) (1.743 Å, 138.3°) than in five-coordinate Fe(TPP)(NO) (1.717 Å, 149.2°). This is indicative of a decreased $\pi$-back-bonding, upon the coordination of trans 1-MeIm. For Co(TPP)(NO),[13] the Co–N bond length and ∠CoNO were determined to be 1.833 Å and 128.5°, respectively. These results are understandable in light of the previous prediction of a further decreased back-bonding, owing to a very small Co–N–O angle. The shorter N–O bond distance (1.01 Å), compared to 1.12 Å for Fe(TPP)(NO), lends further support.

The observed lower frequencies of the $\nu$(N–O) mode for the nitric oxide adducts of cobaltous proteins compared to those of ferrous derivatives seem to contradict a shorter N–O bond, revealed in the structure of Co(TPP)(NO).[13] However, it should be pointed out that the comparison is not straightforward, because of their different coordination numbers. While no crystal structure is available for a six-coordinate nitric oxide adduct of cobaltous porphyrins, two closely related molecules, $Co(NH_3)_5(NO)^{2+}$ [23] and $Co(en)_2(NO)Cl^+$,[24] were shown to have an N–O bond length of about 1.15 Å. The $\nu$(N–O) frequencies (1610 and 1611 cm$^{-1}$, respectively) are correspondingly lower than that of Co(TPP)-(NO) (1689 cm$^{-1}$). Thus, it can be predicted from the $\nu$(N–O) frequency that the N–O distance in cobaltous nitrosyl myoglobin is about 1.15 Å.

The low-frequency vibrations of the M–NO fragment, i.e., $\nu$(Co–NO) and $\delta$(Co–N–O), are particularly sensitive to both the electronic and the kinematic effect. For the nitric oxide adduct, the observed $\nu$(Co–NO) frequency is significantly higher than $\nu$(Fe–NO), in direct opposition to the expectation based solely upon consideration of electronic bonding strength between the metal and nitric oxide and the available structural data. However,

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the substantial difference in geometry between Fe–N–O and Co–N–O fragments is expected to introduce a dramatic effect, attributable to kinematic factors. In other words, as the M–N–O angle decreases, the $\nu$(Co–NO) frequency is raised and $\delta$(Co–N–O) is decreased rapidly. In fact, our normal-mode simulation *does* indicate that the Co–NO linkage is weaker than the Fe–NO bond, as indicated by their respective force constants. Therefore, we attribute the observed higher $\nu$(Co–NO) and lower $\delta$(Co–N–O) frequencies to mainly a kinematic effect.

Finally, we consider the influence of proteins on the vibrational frequencies of the Co–N–O moiety. It was found that those proteins which possess a distal histidine exhibit this feature at 573–575 $cm^{-1}$, and a band at 554–556 $cm^{-1}$ was observed for those proteins which lack distal histidine.[5] The isoelectronic nature of Co(II)–NO and nearly identical Co–N–O geometry when compared with those of the Fe(II)–$O_2$ fragment may allow the bound nitric oxide to interact effectively with the distal histidine in the heme pocket of myoglobin and hemoglobin, which are evolutionarily designed to stabilize the binding of dioxygen. This interaction, likely through the formation of hydrogen bonding, will enhance $\pi$-back-bonding from Co(II) to the bound NO, thus weakening the bound NO and strengthening the Co–NO bond. In other words, the $\nu$(Co–NO) mode for model compounds and those proteins which lack distal interactions is expected to occur at lower frequencies. It is clear that this consideration is experimentally confirmed.[5] Therefore, as previously recognized by Yu and co-workers,[5] the $\nu$(Co–NO) mode can be used as a good probe of the interaction of distal histidine with the bound NO.

In summary, we have shown that the expected three vibrational normal modes of the nitric oxide adducts of cobaltous-reconstituted heme proteins can be readily observed by using Soret-excited resonance Raman spectroscopy. A normal-mode analysis of these frequencies reveals a Co(II)–NO linkage weaker than the Fe(II)-NO bond, gauged from their respective M–NO stretching force constants. The slightly higher $\nu$(Co–NO) and dramatically lower $\delta$(Co–N–O) frequencies were attributed to both the bonding interaction between the central metal ions and the bound nitric oxide and the kinematic effect imposed by the significantly different bonding geometries of the Co(II)–NO and Fe(II)–NO fragments.

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