

well to the experimental spectrum. However, the correspondence is not sufficient enough to allow an exact value for S_{FF} to be determined from the lipid dispersions. The small deviation in the region 15–35 ppm seems to indicate that the experimental spectrum is either less isotropic, i.e., more ordered, than eq 5 assumes or the initial dead time in the acquisition of the FID could have distorted the line shape. More work is needed to resolve these two possibilities. Spectra obtained for both deuterated and nondeuterated samples dispersed in water were found to have practically identical line shapes. Liposomes containing only 25% water were found to have line shapes somewhat broader than those from liposomes containing 70% water (results not shown). The high sensitivity of the ^{19}F probe to the water content of the liposomes is being investigated further. We are currently also examining the 12,12-difluoro- and 4,4-difluorophospholipid analogues by ^{19}F NMR spectroscopy. Additionally, computer programs taking into account other motions that could affect the dipolar interaction and the chemical shift anisotropy are being investigated.

Conclusions. The high-field ^{19}F NMR spectra of oriented and nonoriented phospholipids specifically labeled in the 2-acyl chain have shown fluorine to be a sensitive probe for motion occurring in the hydrocarbon portion of a model lipid membrane. It will be of interest to examine the effect of incorporating cholesterol and proteins into model bilayers containing the difluoromethylene group at various positions along the 2-acyl chain.

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Resonance Raman Spectroscopic Studies of Axial Ligation in Oxyhemoglobin, Oxymyoglobin, and Nitrosylmyoglobin†

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ABSTRACT: Raman intensity measurements for the $\text{Fe}-\text{O}_2$ stretching band of HbO_2 (Hb = hemoglobin) have been used to construct an excitation profile, which shows that resonance enhancement occurs mainly via the B and Q transition; no contribution is detectable from an out-of-plane charge-transfer transition. Direct coupling of $\nu_{\text{Fe}-\text{O}_2}$ to the porphyrin $\pi-\pi^*$ transitions is explained on the basis of competition between the π^* orbitals of porphyrin and O_2 for $\text{Fe } d_\pi$ electrons. The RR spectrum of MbNO (Mb = myoglobin) at pH 8.4 is due solely to six-coordinate heme-NO, but lowering the pH to 5.8 converts the RR spectrum to one characteristic of five-coor-

dinate heme-NO, consistent with Fe-ImH (ImH = imidazole) dissociation via protonation. The Fe-NO stretching frequencies are at 553 and 596 cm^{-1} for the high- and low-pH forms, as expected, but the low-pH form shows an additional ^{15}NO -sensitive band, at 573 cm^{-1} , which is assigned to Fe-N-O bending in the five-coordinate complex. The RR spectrum of MbO_2 shows a shoulder at $\sim 270 \text{ cm}^{-1}$, which shifts down by $\sim 3 \text{ cm}^{-1}$ upon $^{18}\text{O}_2$ substitution, and is suggested to contain the Fe-ImH stretching mode. The weakness of $\nu_{\text{Fe-ImH}}$, relative to $\nu_{\text{Fe}-\text{O}_2}$, is attributable to the lack of ImH involvement in the heme π bonding.

Resonance Raman (RR) spectroscopy is currently being applied as a probe of structure and dynamics in heme proteins (Spiro, 1981, 1983; Asher, 1982; Rousseau et al., 1979). There

has been special interest in the signatures of the axial ligation, particularly for O_2 and NO complexes. The $\text{Fe}-\text{O}_2$ stretching mode, $\nu_{\text{Fe}-\text{O}_2}$, was identified via its $^{18}/^{16}\text{O}_2$ isotope shift in the oxyhemoglobin (HbO_2) RR spectrum, in an early study by Brunner (1974), and it has naturally been of great interest as a probe of the $\text{Fe}-\text{O}_2$ bond strength (Nagai et al., 1980a; Tsubaki et al., 1980; Walters et al., 1980). In this study we evaluate the scattering mechanism for $\nu_{\text{Fe}-\text{O}_2}$ via its excitation

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profile and show that its intensity is due to direct coupling with the porphyrin $\pi-\pi^*$ electronic transitions. The Fe-NO stretching frequency, $\nu_{\text{Fe-NO}}$, has likewise been identified via its $^{15}/^{14}\text{NO}$ isotope shift in the HbNO RR spectrum (Chottard & Mansuy, 1977), and its upshift on converting the quaternary structure from R to T provided direct evidence for Fe-proximal imidazole bond breaking in the T state (Stong et al., 1980). In the present work we find that MbNO (Mb = myoglobin) undergoes proximal imidazole bond breaking via protonation at low pH and provides particularly clear RR signatures for five- and six-coordinate heme-NO.

The bond to the proximal imidazole (ImH) is also of great interest, as the only covalent link between the heme group and the globin in Hb and Mb. The Fe-ImH stretching frequency has been identified in deoxyHb and deoxyMb (Kincaid et al., 1979; Kitagawa et al., 1979; Hori & Kitagawa, 1980) and shown to be sensitive to the Hb quaternary structure, particularly for the α chains (Nagai et al., 1980a; Nagai & Kitagawa, 1980). However, $\nu_{\text{Fe-ImH}}$ has not previously been identified in any ligated form of Hb or Mb. We now present evidence, from the O_2 isotope shift, that $\nu_{\text{Fe-ImH}}$ gives rise to a very weak band at $\sim 270\text{ cm}^{-1}$ in the MbO₂ RR spectrum. An attempt to locate the mode in MbNO has been inconclusive.

Experimental Procedures

Raman spectra were obtained with a Spex 1401 double monochromator equipped with a cooled photomultiplier and photon counting electronics. The data were collected digitally with a MINC (DEC) computer and were smoothed via Gaussian filtration (Linden & Ferrige, 1980) [$e^{(A/t)-(B/t^2)}$, where the parameters A and B are adjusted empirically to minimize noise without distorting the band shapes] of the Fourier transform (Cooley & Tukey, 1965) of the spectra. Excitation was provided by Spectra Physics 171 Kr⁺ and 170 Ar⁺ lasers, with 25–50 mW incident at the sample. A backscattering geometry was used, with samples contained in spinning NMR tubes or in a larger diameter spinning cell held horizontally, with its optical flat perpendicular to the collection axis. The samples were cooled with a flow of N₂ which was passed through a heat exchanger immersed in dry ice.

Mb solutions were prepared by dissolving metMb (Sigma, sperm whale) in pH 5.8 (citrate-phosphate) or pH 8.4 (Tris) buffer and filtered or centrifuged to remove suspended particles. The CO adduct was prepared by deaerating the solution with CO and adding a small excess of sodium dithionite and was desalted with a Sephadex G-25 column equilibrated with CO. The NO and O₂ adducts were prepared by injecting samples of COMB into cells filled with ^{14}NO or $^{16}\text{O}_2$ (Matheson) or ^{15}NO or $^{18}\text{O}_2$ (Prochem). HbO₂ ($\sim 5\text{ mM}$ in heme) was prepared from whole blood, as described by Stong et al. (1980).

Results and Discussion

Resonance Enhancement of $\nu_{\text{Fe-O}_2}$ in HbO₂. Figure 1 is a plot of the Raman intensity of the HbO₂ Fe-O₂ stretching mode at 571 cm^{-1} measured against Na₂SO₄ and NaNO₃ internal standards, for Kr⁺ and Ar⁺ laser wavelengths from the Soret (B) to the α,β (Q_{0,1}) absorptions. It is clear that the excitation profile tracks the absorption spectrum quite faithfully and that $\nu_{\text{Fe-O}_2}$ is enhanced strongly in the B (413 nm) band, and less strongly in the Q (530, 590 nm) bands.

$\nu_{\text{Fe-O}_2}$ was first identified by Brunner (1974) via its $^{18}/^{16}\text{O}_2$ shift in HbO₂ and has been of much interest as a monitor of the Fe-O₂ bond strength. It shows little apparent change between R and T oxy states of the mutant Hb's, Kansas and

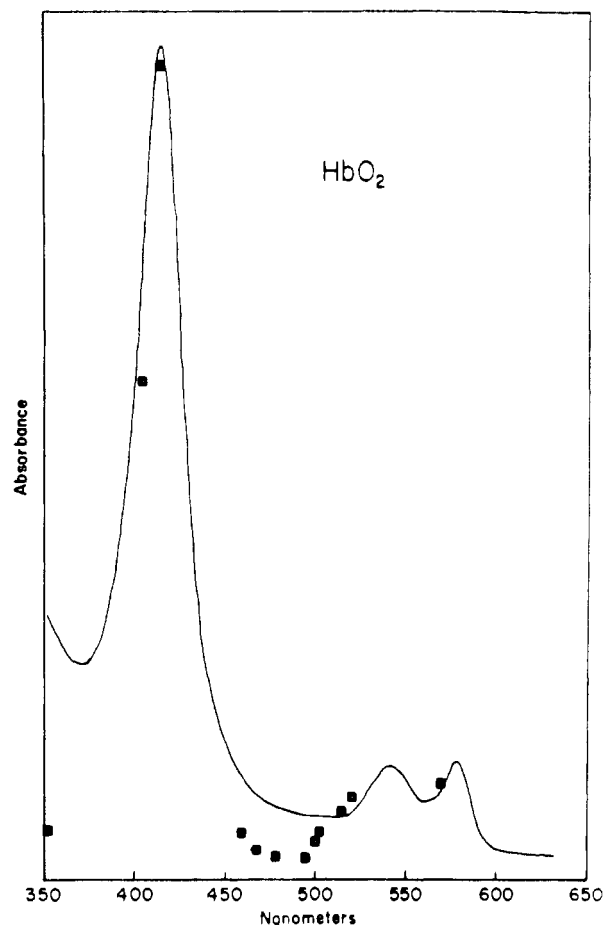


FIGURE 1: Excitation profile of the Fe-O₂ stretching mode in HbO₂. Na₂SO₄ served as an internal standard for all excitation wavelengths except 4067 and 4131 Å. NaNO₃ served as an internal standard in samples excited at the latter two wavelengths (because of interference with $\nu_1(\text{SO}_4^{2-})$ of a B-enhanced porphyrin mode). The relative NO₃⁻ and SO₄²⁻ intensities were measured in a separate experiment and used to normalize the data points in the B band. Absorption corrections were negligible in the backscattering geometry employed (Shriver & Dunn, 1974).

Milwaukee (Nagai et al., 1980a), and also is insensitive to the substitution of formyl for peripheral vinyl heme groups in MbO₂ (Tsubaki et al., 1980). Small downshifts have been observed for "picket fence" heme-O₂ adducts with hindered imidazole axial bases, suggesting a minor contribution from the altered Fe-O₂ bond strength to the lowered O₂ affinity (Walters et al., 1980). These measurements have been carried out with excitation in the 4579–5145-Å region, and it has sometimes been assumed (Spiro, 1981) that enhancement was due to an out-of-plane electronic transition, single crystal absorption spectroscopy having identified a weak broad z-polarized absorption at $\sim 475\text{ nm}$ in MbO₂ (Makinen & Eaton, 1973). This transition has been assigned to either O₂ → Fe (Eaton et al., 1978) or a porphyrin(a_{2u}) → Fe(d_{z2}) charge transfer transition (Churg & Makinen, 1978a,b). Resonance enhancement of $\nu_{\text{Fe-O}_2}$ is expected in either case, since the Fe-O₂ bond would be weakened in the excited state. However, the excitation profile in Figure 1 shows no extra enhancement near 475 nm, and the z-polarized transition at this wavelength evidently does not contribute significantly to the $\nu_{\text{Fe-O}_2}$ resonance enhancement. This is probably due to its low transition moment (Makinen & Eaton, 1973). The $\nu_{\text{Fe-O}_2}$ band is enhanced via the B and Q transitions, roughly in proportion to their transition moments, implying that although the Fe-O₂ bond is perpendicular to the porphyrin plane, its stretching mode is nevertheless directly coupled to the in-plane

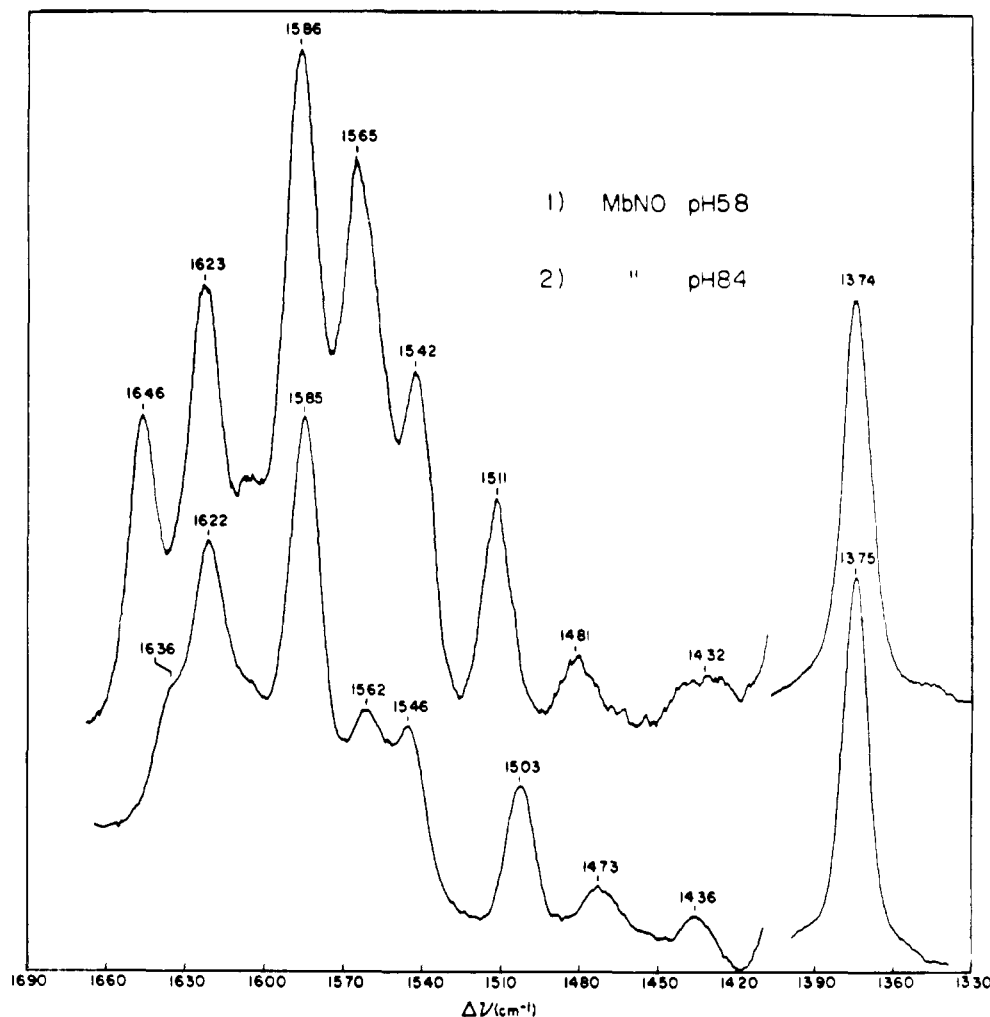


FIGURE 2: High-frequency resonance Raman spectra of five- and six-coordinate MbNO, with 4131-Å excitation. Conditions: (1) above 1390 cm^{-1} , 2000 counts/s full scale, 5-s time constant; below 1390 cm^{-1} , 20 000 counts/s full scale, 1-s time constant; (2) above 1390 cm^{-1} , 10000 counts/s full scale, 5-s time constant; below 1390 cm^{-1} , 50000 counts/s full scale, 1-s time constant. Power output 100 mW; spectral slit width 10 cm^{-1} ; scan speed 0.5 cm^{-1}/s .

$\pi-\pi^*$ transitions. There is, of course, no symmetry restriction against such coupling, since the mode has A_1 symmetry, and its scattering tensor has elements α_{xx} and α_{yy} , which are associated with in-plane electronic transitions.

The coupling mechanism can be understood with reference to the π -acceptor properties of O_2 and of porphyrin. In low-spin Fe^{II} porphyrins certain of the skeletal vibrational frequencies are strongly influenced by the extensive back-donation of $\text{Fe } d_\pi$ electrons into the low-lying empty π^* orbitals (e_g) of porphyrin (Spiro & Strekas, 1974). The pattern of vibrational frequency shifts has recently been examined in detail (Choi et al., 1982b) and shown to be related to the nodal structure of the e_g orbitals. Axial ligands which themselves have low-lying π^* -acceptor orbitals compete for the d_π electrons and reverse the skeletal frequency shifts (Spiro & Burke, 1976).

In the case of O_2 (Spiro & Strekas, 1974) and NO (Strong et al., 1980), the competition is sufficiently effective that the skeletal frequencies are essentially the same as those observed for low-spin Fe^{III} porphyrins. The extent of back-donation to porphyrin, and therefore the energy of the e_g orbitals, should be sensitive to the $\text{Fe}-\text{O}_2$ bond length. The resulting modulation of the $\pi-\pi^*$ transitions via $\text{Fe}-\text{O}_2$ stretching provides a plausible mechanism for resonance enhancement of $\nu_{\text{Fe}-\text{O}_2}$. Because of the π competition a definite origin shift along the $\text{Fe}-\text{O}_2$ coordinate can be expected in the $\pi-\pi^*$ excited states,

resulting in A term RR scattering (Tang & Albrecht, 1970; Spiro & Stein, 1977).

This mechanism should also produce enhancements for other π -acceptor ligands. Indeed the $\text{Fe}-\text{NO}$ stretching mode of HbNO shows similar RR excitation behavior, the intensity rising in both the B and Q bands (Chottard & Mansuy, 1977). Since the original submission of this paper, Tsubaki et al. (1982) and Armstrong et al. (1982) have identified the $\text{Fe}-\text{CO}$ stretch in HbCO, using B band excitation. Tsubaki et al. (1982) referred to unpublished excitation profiles for $\nu_{\text{Fe}-\text{O}_2}$, $\nu_{\text{Fe}-\text{CO}}$, and $\nu_{\text{Fe}-\text{NO}}$, all following the visible absorption spectra, and advanced an enhancement mechanism similar to the one discussed here.

Five- and Six-Coordinate MbNO. The RR spectrum of HbNO has been of considerable interest because of changes in skeletal mode frequencies observed upon adding inositol hexaphosphate (Szabo & Barron, 1975; Scholler & Hoffman, 1979; Stong et al., 1980; Nagai et al., 1980b). These have been interpreted (Perutz et al., 1976) in terms of Fe -proximal imidazole bond breaking in half the Hb chains upon conversion from R to T quaternary structure. The inference that bond breaking occurs in the α chains (Perutz et al., 1976) has been confirmed via RR spectroscopy, with Hb selectively reconstituted with meso-deuterated heme (Nagai et al., 1980b).

We were able to obtain clean six- and five-coordinated heme-NO RR spectra in myoglobin, by changing the pH.

Table I: High-Frequency Raman Bands (cm^{-1}) of MbNO^a

assignment	pH 8.4	pH 5.8	Δ	Δ_{calc}^b
$\nu_{10}(\text{B}_{1g})^c$	1636	1646	10	9
$\nu_{\text{C}=\text{C}}^d$	1622	1623	1	
$\nu_{37}(\text{E}_u)$	1603	1605	2	6
$\nu_2(\text{A}_{1g})$	1585	1586	1	8
$\nu_{11}(\text{B}_{1g})$	1562	1565	3	6
$\nu_{38}(\text{E}_u)$	1546	1542	-4	10
$\nu_3(\text{A}_{1g})$	1503	1511	8	8
$\nu_{28}(\text{B}_{2g})$	1473	1481	8	7
$\delta=\text{CH}_2^e$	1436	1432	-4	
$\nu_4(\text{A}_{1g})$	1375	1374	-1	

^a Excited at 4131 Å. ^b Five- vs. six-coordinated frequency shifts calculated for a 0.018-Å contraction of $\text{C}_t\text{-N}$, using the core size coefficients of Choi et al. (1982b)—see text. ^c Mode numbering and symmetries as for NiOEP (Abe et al., 1978); assignments based on Choi et al. (1982a). ^d $\nu_{\text{C}=\text{C}}$ -vinyl $\text{C}=\text{C}$ stretching mode. ^e $\delta=\text{CH}_2$ -vinyl CH_2 scissors mode.

Figure 2 compares RR spectra for MbNO, using B band excitation at 4131 Å, at pH 8.4 and pH 5.8. At pH 5.8 the highest frequency RR band is at 1646 cm^{-1} , characteristic of five-coordinate heme-NO (Stong et al., 1980). No trace of this band is left at pH 8.4; it has shifted to 1636 cm^{-1} , characteristic of six-coordinate heme-NO. We conclude that the proximal imidazole of MbNO dissociates via protonation with a pK_a between 5.8 and 8.4.

With B excitation all the high-frequency porphyrin skeletal modes are observed except for the anomalously polarized ν_{19} (Abe et al., 1978). Two vinyl modes (Choi et al., 1982a), the $\text{C}=\text{C}$ stretch and one of the $=\text{CH}_2$ scissors modes, at 1622 and 1436 cm^{-1} , also show up clearly. Table I lists the observed frequencies and their assignments.

All of the skeletal modes above 1450 cm^{-1} have been shown to correlate inversely with the porphyrin center-to-nitrogen ($\text{C}_t\text{-N}$) distance for a series of heme complexes (Choi et al., 1982b), the coefficients varying with the degree of involvement of methine bridge stretching in the mode. The observed upshift of several of the MbNO modes upon dissociating the proximal imidazole can be partly understood on this basis. When *N*-methylimidazole (*N*-MeIm) is dissociated from (*N*-MeIm)(NO)FeTPP (TPP = tetraphenylporphine), $\text{C}_t\text{-N}$ is known to contract, from 2.008 (Scheidt & Piciulo, 1976) to 1.990 Å (Scheidt & Frisse, 1975), as the Fe-pyrrole bonds shorten slightly and the Fe moves out of the porphyrin plane (by 0.21 Å), relieving the nonbonded interactions between the pyrrole N atoms and the coordinated NO (Scheidt & Frisse, 1975). When this $\text{C}_t\text{-N}$ contraction, 0.018 Å, is used with the frequency correlations (Choi et al., 1982b) to calculate the expected upshifts, the agreement is remarkably good for ν_{10} , and also for ν_3 and ν_{28} . However ν_{37} , ν_2 , ν_{11} , and ν_{38} fail to shift up by as much as expected (ν_{38} actually shifts down). All of these modes are sensitive (Choi et al., 1982b) to π back-bonding and must be affected by the altered electronic structure associated with the shorter Fe-NO distance and the out-of-plane displacement of the Fe atom (Scheidt & Frisse, 1975).

RR spectroscopy provided definite evidence for proximal imidazole bond breaking in half the chains of T state HbNO, since the Fe-NO stretching mode at 553 cm^{-1} was supplemented by an additional ^{15}N -sensitive band at 592 cm^{-1} upon addition of IHP (inositol hexaphosphate) (Stong et al., 1980). The frequency difference between the two bands was consistent with the differences in Fe-NO distances, as estimated via Badger's rule (Stong et al., 1980). The MbNO RR spectra bear out this interpretation, as shown in Figure 3. At pH 8.4, there is a band at 553 cm^{-1} , the $\nu_{\text{Fe-NO}}$ frequency expected for

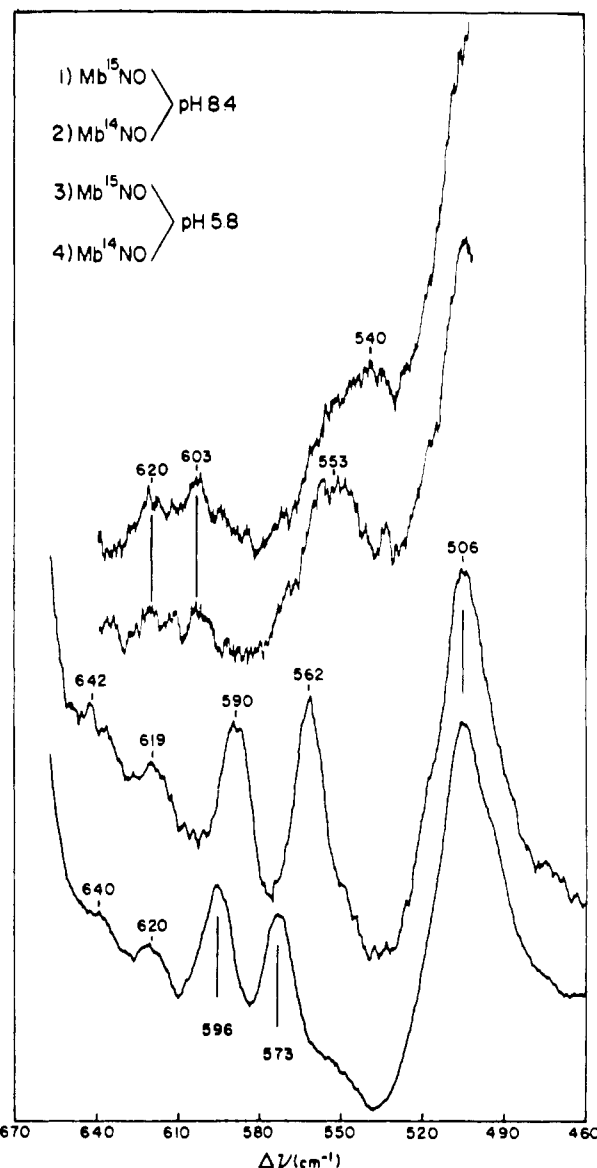


FIGURE 3: Intermediate frequency resonance Raman spectra of five- and six-coordinate MbNO, with 4131-Å excitation. Conditions: at pH 8.4, scan speed $0.5\text{ cm}^{-1}/\text{s}$, 5-s time constant; at pH 5.8 scan speed $0.1\text{ cm}^{-1}/\text{s}$, 20-s time constant. Power output 100 mW; spectral slit width 10 cm^{-1} ; 1000 counts/s full scale.

six-coordinate heme-NO, which shifts to 540 cm^{-1} upon ^{15}NO substitution. At pH 5.8 this band is gone and is replaced by one at 596 cm^{-1} , shifting to 590 cm^{-1} upon ^{15}NO substitution, as expected for five-coordinate heme-NO. The lowered isotope shift for the five-coordinate heme-NO, also observed for HbNO (Stong et al., 1980), can be attributed to three factors: (1) Loss of the trans imidazole ligand increases the extent of Fe motion in the Fe-NO mode. Since Fe is more massive than NO, this is not a large effect; a diatomic oscillator calculation shows the lowering of the ^{15}N shift to be $<3\text{ cm}^{-1}$ from this source. (2) An increase in the Fe-N-O angle, θ , would increase the effective mass of the NO ligand and suppress the ^{15}N shift. To a first approximation, the effective mass is expected to be ~ 30 (O moves in concert with N) at $\theta = 180^\circ$ and ~ 14 (O does not move) at $\theta = 180^\circ$, varying roughly as $\cos \theta$. For these two extremes, $\Delta^{15}\text{N}$ for the 596-cm^{-1} band is calculated for an Fe-NO diatomic oscillator to be 7 and 16 cm^{-1} . Dissociation of *N*-MeIm from (*N*-MeIm)(NO)FeTPP does increase θ , from 142° (Scheidt & Piciulo, 1976) to 149° (Scheidt & Frisse, 1975), but the associated increase in \cos

θ is insufficient to account for an appreciable change in $\Delta^{15}\text{N}$. In the proteins, however, θ may be lower, and $\Delta\cos\theta$ would then be higher for a given $\Delta\theta$. It is notable that for the six-coordinate heme-NO, and also for HbO_2 , the large ^{15}N (or ^{18}O) frequency shift can only be reproduced, in simple triatomic oscillator calculations (vide infra), if the NO mass is set close to 14 (16 for O_2). (3) There may be greater mixing of $\nu_{\text{Fe-NO}}$ with porphyrin out-of-plane mode in the out-of-plane (Scheidt & Piciulo, 1976) five-coordinate structure.

At pH 5.8 an additional band is seen, at 573 cm^{-1} , which also shifts, to 562 cm^{-1} , on ^{15}NO substitution. We tentatively assign it to the $\delta_{\text{Fe-N-O}}$ bending mode; on the basis of the G matrix element (Wilson et al., 1955) for the Fe-N-O bending coordinate, without any allowance for interaction, the expected ^{15}NO frequency shift of this mode is 14 cm^{-1} . The frequency of this mode in transition metal nitrosyls is quite variable (Nakamoto, 1978); it can be as high, or higher, than that of the Fe-NO stretch. The relatively high RR intensity of $\delta_{\text{Fe-N-O}}$ in the pH 5.8 MbNO spectrum may be due to substantial mixing with $\nu_{\text{Fe-NO}}$, because of their proximity. The band is not seen for T-state HbNO (Stong et al., 1980); it may occur at a slightly lower frequency, where it would be obscured by the 553-cm^{-1} (six-coordinate) band.

Fe-ImH Stretching Mode in MbO₂ and MbNO. While $\nu_{\text{Fe-O}_2}$ was identified quite early, in the RR spectrum of HbO_2 (Brunner, 1974), the Fe-proximal imidazole stretch, $\nu_{\text{Fe-ImH}}$, has so far escaped detection in this spectrum. Its frequency, however, is a matter of considerable interest as a measure of the strength of the bond connecting the Fe-O₂ unit with the protein. Perutz (1976, 1980) has argued that the molecular "tension" associated with the lowered O₂ affinity of T-state Hb must be exerted, at least in part, via the Fe-ImH bond. In deoxyHb, the Fe-ImH band has been located in the RR spectrum (Kincaid et al., 1979; Kitagawa et al., 1979), and a definite effect of quaternary state has been found (Nagai et al., 1980a). A substantial frequency decrease between R and T states is seen specifically for the α chains (Nagai & Kitagawa, 1980). This downshift has been attributed to molecular strain per se (Nagai & Kitagawa, 1980), or to a decrease in the H bonding of the imidazole N₃ proton to the protein backbone (Stein et al., 1980). It is the oxyHb T state which is of greatest interest, however, since tension should be maximally expressed in the ligated form of the T quaternary structure. As noted above the Fe-O₂ stretching frequency does not appear to change significantly on converting mutant oxyHb's to the T state (Nagai et al., 1980a,b). The question remains whether the Fe-ImH frequency is affected by the HbO_2 R-T transition.

We have searched for $\nu_{\text{Fe-ImH}}$ in the MbO₂ RR spectrum and tentatively identify it with a 272-cm^{-1} shoulder that shifts down $\sim 3\text{ cm}^{-1}$ upon $^{18}\text{O}_2$ substitution, as shown in Figure 4. The downshift is expected because $\nu_{\text{Fe-ImH}}$ is coupled with $\nu_{\text{Fe-O}_2}$. Using an Fe-ImH force constant of 1.63 mdyne/\AA from the symmetric and asymmetric Fe-(ImH)₂ stretching frequencies, 200 (M. Mitchell, S. Choi, and T. G. Spiro, unpublished results) and 377 cm^{-1} (Ogoshi et al., 1973), of (ImH)₂Fe^{III}OEP⁺ and $\nu_{\text{Fe-O}_2} = 572\text{ cm}^{-1}$, one can calculate (Herzberg, 1945) that $K_{\text{Fe-O}_2} = 2.30\text{ mdyne/\AA}$ and $\nu_{\text{Fe-ImH}} = 263\text{ cm}^{-1}$ for a triatomic $-\text{CH}_2\text{ImH-Fe-O}_2$ oscillator with mass 82 (histidine) and 16 (O₂) ligands. The experimental value of the $\nu_{\text{Fe-O}_2}$ $^{18}\text{O}_2$ shift is better reproduced with a mass 16 O_2 (22 cm^{-1}) than a mass 32 O_2 (17 cm^{-1}). The predicted $^{18}\text{O}_2$ shift of ν_{FeImH} is 3 cm^{-1} .

The remaining features of the spectra in Figure 4 have been assigned for other protoheme complexes, with the aid of $^{54/56}\text{Fe}$,

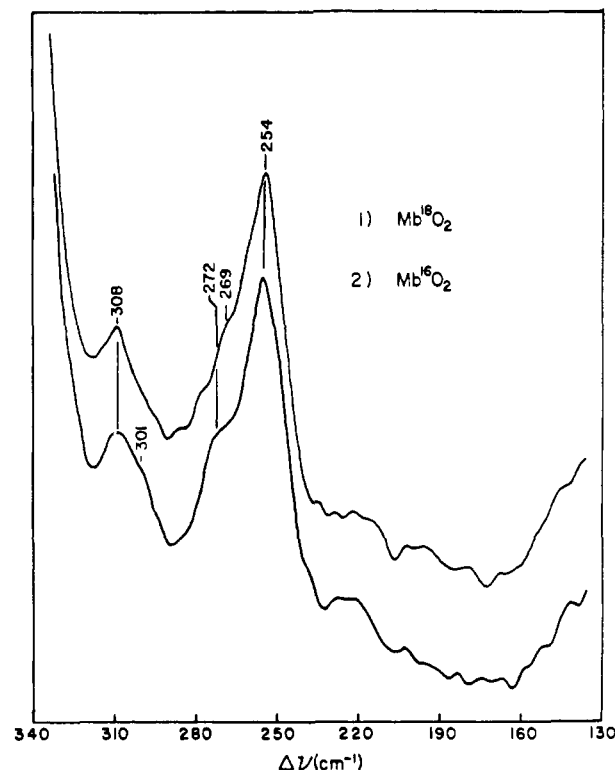


FIGURE 4: Resonance Raman spectra of MbO₂ with 4067-Å excitation; power output 55 mW; spectral slit width 10 cm^{-1} ; 500 counts/s full scale. Digital data collection parameters: 1 cm^{-1} increment, 10 s/increment .

and meso- and vinyl-deuteration shifts (Choi & Spiro, 1983). The 254-cm^{-1} band is believed to be the out-of-plane pyrrole tilting mode, while the broad $\sim 305\text{-cm}^{-1}$ band contains two modes, a vinyl bend, at 308 cm^{-1} , and a methine out-of-plane deformation, at 301 cm^{-1} . The latter loses intensity in the Mb¹⁸O₂ spectrum and may be coupled to $\nu_{\text{Fe-ImH}}$ and $\nu_{\text{Fe-NO}}$. An additional band is expected at $\sim 270\text{ cm}^{-1}$ (Choi & Spiro, 1983), assigned to the lowest frequency A_{1g} skeletal mode, ν_9 (Abe et al., 1978). This mode probably underlies the ^{18}O -sensitive shoulder at 272 cm^{-1} . The weakness of this shoulder (note that the 346-cm^{-1} band, which is prominent in the low-frequency region but is still much weaker than the strong high-frequency modes, is off scale in the spectra), together with the spectral congestion, naturally lessens one's confidence in the $\nu_{\text{Fe-ImH}}$ assignment, but the 272-cm^{-1} feature appears to be the only plausible candidate. If $\nu_{\text{Fe-ImH}}$ is not at 272 cm^{-1} , then its intensity is even lower than that of the 272-cm^{-1} shoulder. This conclusion augurs poorly for the utility of this mode as a RR probe of protein structure. We tried the $^{18/16}\text{O}_2$ experiment with HbO₂. The HbO₂ RR spectrum has a stronger porphyrin band at $\sim 290\text{ cm}^{-1}$; there were hints of $^{18/16}\text{O}_2$ shifts in contours of this band, but the effects were at the limit of signal-to-noise. Further improvements in sensitivity will be needed if $\nu_{\text{Fe-ImH}}$ is to be monitored in different quaternary states.

Clearly $\nu_{\text{Fe-ImH}}$ gives much weaker RR scattering than does $\nu_{\text{Fe-O}_2}$. The difference in enhancement between the two axial modes can be understood on the basis of the back-bonding arguments presented above. In contrast to O₂, ImH is not an effective π acceptor and does not compete effectively with the porphyrin e_g orbitals for Fe d_π electrons (Spiro & Burke, 1976). Consequently excitation to the $\pi-\pi^*$ states should have a smaller effect on the Fe-ImH bond than on the Fe-O₂ bond.

We have also examined the low-frequency region of MbNO in hopes that $\nu_{\text{Fe-ImH}}$ could be identified by its absence at pH

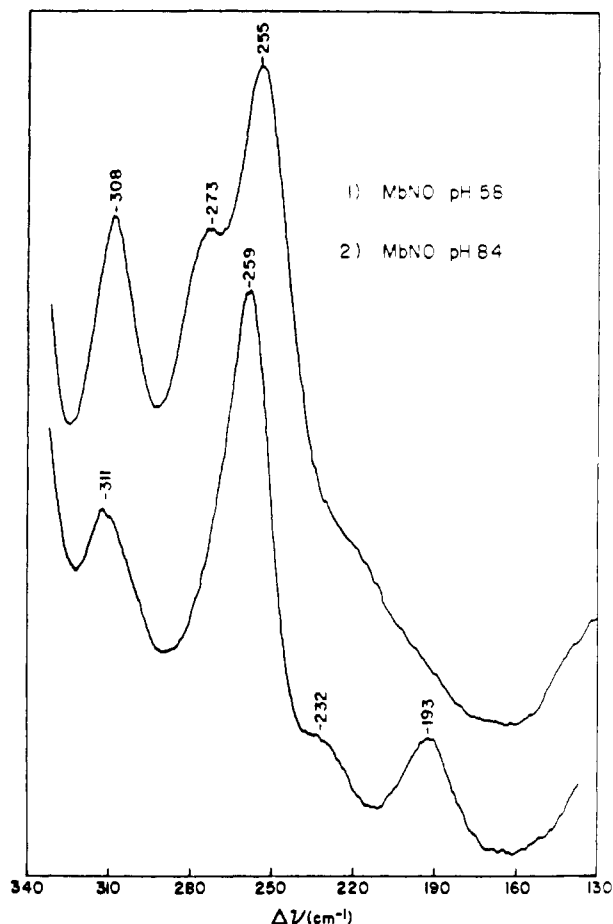


FIGURE 5: Low-frequency resonance Raman spectral of five- and six-coordinate MbNO with 4131-Å excitation. Conditions: scan speed 0.1 cm^{-1}/s ; 20-s time constant; power output 100 mW; spectral slit width 10 cm^{-1} ; 2000 counts/s full scale.

5.8, where the Fe-ImH bond is broken. The frequency of $\nu_{\text{Fe-ImH}}$ is expected to be lower in MbNO than in MbO₂ because of the trans influence of NO. The Fe-ImH bond in (N-MeIm)(NO)FeTPP, at 2.180 Å (Scheidt & Piciulo, 1976), is 0.11 Å longer than the Fe-ImH bond in the O₂ adduct of the "picket fence" heme, (N-MeIm)(O₂)FeT_{ppv}PP, (Jameson et al., 1978). Using Badger's rule (Herschbach & Laurie, 1961) we estimate that this lengthening should reduce $K_{\text{Fe-ImH}}$ from 1.63 to 1.26 $\text{mdyn}/\text{\AA}$. A $-\text{CH}_2\text{ImH-Fe-NO}$ triatom calculation with $\nu_{\text{Fe-NO}} = 550 \text{ cm}^{-1}$ and masses 82 and 14 for histidine and NO gives $\Delta\nu_{\text{Fe-}^{15}\text{NO}} = 13 \text{ cm}^{-1}$, in agreement with the experiment, and $K_{\text{Fe-NO}} = 1.94 \text{ mdyn}/\text{\AA}$. $\nu_{\text{Fe-ImH}}$ is predicted to be at 235 cm^{-1} and to shift 2 cm^{-1} on ^{15}NO substitution.

Figure 5 shows the low-frequency RR spectra of MbNO, at pH 8.4 and 5.8, with 4131-Å excitation at high sensitivity. At pH 8.4 (six-coordinate) there is a band at 232 cm^{-1} which might contain $\nu_{\text{Fe-ImH}}$. However, the pH 5.8 spectrum (five-coordinate) also contains a shoulder at this position, and other heme complexes sometimes show a $\sim 230\text{-cm}^{-1}$ band (Choi & Spiro, 1983) which may be due to a low-frequency B_{1g} or B_{2g} skeletal mode (Abe et al., 1978). The expected 2- cm^{-1} $^{15}/^{14}\text{NO}$ frequency shift is too small to be detected for such a weak, overlapping band. The pH 8.4 spectrum shows an additional band at 193 cm^{-1} , which is absent at pH 5.8, but this frequency seems too low for $\nu_{\text{Fe-ImH}}$, and the band may be due to still another skeletal mode, which is not enhanced for the five-coordinate species. Another intensity change is seen for the 273- cm^{-1} band (ν_9) at pH 5.8, which is absent at

pH 8.4. There are also distinct frequency shifts of the 255- and 308- cm^{-1} six-coordinate bands to 259 and 311 cm^{-1} for the five-coordinate species.

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