stored and averaged by using program DSORTH;[32] 847 reflections were measured once and 1948 had more than one contributor to average ($R_1 = \sum Av|I - Av(I)|/\sum Av|I| = 0.016$; $R_2 = \sum|wI - wAv(I)|/\sum wI| = 0.021$); 2677 independent reflections with $I > 3\sigma(I)$ were used to refine the structure.

The independent data set was then transferred to a PDP11/60 computer and the indices of unobserved reflections were generated. The structure was solved by using the Enraf-Nonius SDP/V16 package[33] and all nonhydrogen atoms were located in an E map.[34] Hydrogen atoms were introduced but not refined by their computed coordinates with a C-H distance of 0.95 Å and an isotropic thermal parameter of 7 Å^2.

Refinement converged to $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$ and $R_2 = ((\sum w|F_o| - |F_c|))^2/\sum wF_o^2)^{1/2} = 0.027$ and 0.041, respectively, refining all nonhydrogen atoms with anisotropic thermal parameters. The estimated standard deviation of a unit weight observation was 0.96 with a fudge factor of 0.08.

The final difference Fourier map showed no significant electron density above background.

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Supplementary Material Available: Contacts less than 3.2 Å (Table III), positional and thermal parameters of all nonhydrogen atoms (Tables IV and V), and hkl, F_o, and F_c times 10 for all observed structure factors (Table VI) (16 pages). Ordering information is given on any current masthead page.

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Resonance Raman Spectra of Nitrosyl Heme Proteins and of Porphyrin Analogues[1]

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Abstract: Addition of inositol hexaphosphate (IHP) to nitrosylhemoglobin (NOHb) produces a new resonance Raman (RR) band at 592 cm^{-1}, identifiable with Fe-NO stretching via its ^{15}NO shift, in addition to the 553-cm^{-1} Fe-NO band observed without IHP. This observation supports previous evidence that the R → T switch induces the Fe-imidazole bonds of half the NOHb heme groups to break. The high-frequency RR spectra are similar to those reported by Szabo and Barron,[2] although slight additional shifts on IHP binding are observed. A series of analogue heme complexes have been studied and the RR spectra effects of NO, CO, imidazole, and imidazolate are analyzed in terms of π back-bonding and trans labilization. The IHP-induced shifts in NOHb are consistent with Fe-imidazole bond breaking, but additional protein influences are apparent. An alternate hypothesis, that the R → T shift in NOHb involves protonation of bound imidazolate, is not supported by the RR spectra.

Introduction

In recent studies of hemoglobin (Hb) allostery, nitrosyl (NO) Hb has played a central role because of the large changes in heme electronic properties induced by the binding of organic phosphates which alter the protein quaternary structure.[3,4] While other forms of Hb, such as high-spin derivatives of metHbA[5] or ligated forms of Hb Kansas[6] or carp Hb,[7] are also switched from the R to the T quatenary structure on addition of phosphate effectors, NOHb is the only one to show marked alterations in the spectral signatures of the heme group.[2-5,8-10] These have been interpreted as resulting from a weakening or breaking of the bond between the iron atom and the proximal imidazole ligand of two of the four Hb chains, presumably the α chains, in the T quaternary structure.[4,10] Because its odd electron is partially transferred to the iron d_{z^2} orbital, the NO ligand is strongly trans labilizing; the trans axial bond is quite long in six-coordinate nitrosyl adducts of iron tetraphenylporphine,[11a] and the five-coordinate adduct, without a trans axial ligand, is readily formed and crystallized.[11b] This weakness of the trans axial bond apparently renders it susceptible to further weakening, or breaking, under the constraints of the T quaternary structure of NOHb, although only in half the chains. The most direct evidence in support of this view is the observation of two NO stretching frequencies in the infrared spectrum of T-state NOHb,[10] attributable to five- and six-coordinate NO heme, and also the superposition of three- and nine-line electron spin resonance spectra, characteristic of five-coordinate NO heme and of six-coordinate NO heme with nitrogenous sixth ligands.[12] Hille et al.[13] have recently analyzed NOHb ESR spectra as a function

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of saturation, pH, and inositol hexaphosphate (IHP) and have concluded that there is an equilibrium between five- and six-coordinate NO heme in the T-state α chains, which is influenced by the binding of protons and IHP.

Chevion et al.[14] have challenged the bond-breaking interpretation since they observed a different three-line ESR spectrum in T-state NOHb, whereas they were able to reproduce the previously reported three-line spectrum under denaturing conditions e.g., 60 °C, or aerobic conditions which produce a sharp reduction of pH. Chevion et al. suggested that the transition from their three-line spectrum to the nine-line spectrum might be associated with loss of the N-1 proton of the proximal imidazole. In this view the sixth ligand in R-state NOHb would be imidazolate ion and this would revert to neutral imidazole in the α chains, upon conversion to the T state.

The resonance Raman (RR) spectrum of NOHb was reported by Szabo and Barron,[2] who observed that addition of inositol hexaphosphate (IHP) altered the spectrum in only one respect: a 1644-cm^{-1} shoulder on the band at 1633 cm^{-1} developed into a band of equal intensity. This is the only instance where the R–T switch, as induced by IHP, has shown any appreciable effect on the RR spectrum.[15a] No change at all is seen for deoxyHb from carp[9] or modified with *N*-maleimidomethyl ether;[15b] for aquometHB, a slight change is observed in the relative intensities of bands associated with high- and low-spin forms, but there are no frequency shifts.[8] Subsequently, Perutz et al.[4] interpreted the 1644–1633-cm^{-1} pair of NOHb as arising from five- and six-coordinated NO heme in the T quaternary structure. Several porphyrin RR bands are known to be somewhat sensitive to the effects of changes in axial ligation,[16,17] and it seemed to us odd that just one band should be altered on loss of imidazole coordination in NOHb. We have rexamined the NOHb RR spectrum and confirm the observations of Szabo and Barron,[2] although two other bands show barely detectable frequency shifts on IHP addition. In addition we have located the Fe–NO stretching mode, recently reported by Chottard and Mansuy,[18] and have found that part of its intensity shifts up by 39 cm^{-1} upon IHP addition. Comparison of the high-frequency spectra of five- and six-coordinate NO, and also CO, heme analogues show that the interpretation of the IHP-induced changes as arising from the breaking of imidazole–iron bonds is plausible, although there are additional protein influences on the frequencies. On the other hand, comparison with imidazolate heme analogues establishes that the IHP-induced Raman shifts are not due to changes in imidazole protonation. RR spectra are also reported for nitrosoferricytochrome *c* and for a NO ferriheme. The attempted preparation of NOmetMb is shown to result in reduction to NO ferroheme, probably with the imidazole–iron bond broken. Since the original submission of this manuscript Scholler et al.[19] have reported an EPR and RR study of NOHb; they also find that addition of imidazole to nitrosoiron(II) protoporphyrin IX dimethyl ester produces a RR shift which is the reverse of the IHP-induced shift for NOHb.

Experimental Section

Iron(III) (mesoporphyrin IX dimethyl ester) fluoride ($Fe^{III}MP(F)$) was prepared as reported previously.[15] Hemin chloride and octaethylporphine (OEP) were from Strem. Iron octaethylporphine chloride was prepared as previously described.[15a] Potassium *tert*-butoxide was from Aldrich. Methylene chloride was obtained from Burdick and Jackson Laboratories. Pyridine was distilled and stored over potassium hydroxide. Imidazole was recrystallized from benzene, and 1-methylimidazole was distilled under reduced pressure. Nitric oxide (Matheson) was passed through a 2-ft column of potassium hydroxide prior to use. Carbon monoxide (Matheson) was used without further purification. Myoglobin and cytochrome *c* were obtained from Sigma Chemical Co. All other chemicals were reagent grade.

Preparation of Hemoglobin. Approximately 40 mL of fresh human blood was allowed to stand for 12 h at 4 °C, after which time the plasma was removed by pipet. The red cells were then diluted to twice their volume with 0.9% saline solution and centrifuged at 4000*g* for 10 min. The top layer was decanted and the washing with saline and centrifugation were repeated until the liquid on top was clear (two or three times). After the cells were lysed for 30 min by dilution with distilled water, they were centrifuged at 32000*g* for 1 h and the Hb solution on top was removed by pipet and dialyzed against pH 7 phosphate buffer for 12 h. The visible spectrum showed the final concentration of O_2Hb to be 2–3 mM. Approximately 7 mL of O_2Hb solution containing five or six crystals of $Na_2S_2O_4$ (to remove excess O_2) were run through a Sephadex column (previously washed with pH 7.3 phosphate buffer) to remove organic phosphates and $Na_2S_2O_4$ (as $Na_2S_2O_4$ reacts with NO).

Preparation of NO Heme Proteins. Oxygen had to be rigorously excluded from the reaction vessel as the NO_2 formed from O_2 + NO readily denatures heme proteins. About 3 mL of a 2 mM heme protein solution (buffered at pH 7.8 by phosphate) was placed in a three-neck round-bottom flask and stirred very slowly. For Hb, a carbon monoxide line was attached to the flask with a water bubbler to prevent evaporation of the protein solution. After 20 min, the Hb was completely converted to COHb.

After the protein solution was carefully degassed, argon was allowed to flow through the reaction flask for 0.5 h, after which time NO was flowed through the flask containing the stirred heme solution for 10 min. The excess NO was then removed by flushing with argon. The sample was transferred to an appropriate spectral cell in a stream of argon. For some experiments an atmosphere of NO was maintained over the samples throughout the spectral measurements. UV–visible absorption spectra were checked with literature data.[20]

Isotope experiments were done by decomposing $Na^{14}NO_2$ or $Na^{15}NO_2$ (Merck isotopes) with a single crystal of sodium dithionite. The resulting ^{14}NOHb was identical in every respect with the ^{14}NOHb prepared as described above.

Porphyrin Complexes. (1) $[(ImH)_2Fe^{III}OEP]^+$. Ca. 25 mg of imidazole was added to 2 mL of 1 mM iron(III) octaethylporphyrin chloride in methylene chloride.

(2) $[(Im)_2Fe^{III}OEP]^-$. Ca. 0.05 mL of *tert*-butyl alcohol saturated with potassium *tert*-butoxide was added to (1). Imidazolate heme complexes have been characterized by Valentine and co-workers.[21]

(3) $(NO)(ImH)Fe^{II}OEP$. Argon was flushed through (1) dissolved in 5% MeOH in methylene chloride in a Raman spinning cell, and NO was then bubbled in for 10 min. Under these conditions Fe^{III} is reduced to the Fe^{II} NO complex.

(4) $[(NO)(Im)Fe^{II}OEP]^-$. Argon was flushed through (2) in a spinning cell, and NO was then bubbled in for 10 min. The absorption spectrum was similar to that of the neutral complex (λ_{max} 565 (α), 533 (β), 480 nm (shoulder)), except that the β band maximum shifted to 542 nm.

(5) $(NO)Fe^{II}MP$. A trace of methanol was added to 1 mM iron(III) mesoporphyrin fluoride in methylene chloride in a spinning cell. The solution was flushed with argon, and NO was bubbled in for 10 min.

(6) $(NO)(1\text{-MeIm})Fe^{II}MP$ and $(NO)(py)Fe^{II}MP$. The procedure was the same as that for (5) but a slight excess of 1-methylimidazole or pyridine was added prior to argon flushing.

(7) $(CO)Fe^{II}MP$. A 1 mM methylene chloride solution of iron(III) mesoporphyrin fluoride in a spinning cell was covered with water and flushed with argon. A small quantity of sodium dithionite was added; the cell was quickly sealed and shaken vigorously until the CH_2Cl_2 layer changed from red-brown to orange, indicating the formation of the hemochrome. CO was then bubbled in for 5 min.

(8) $(CO)(1\text{-MeIm})Fe^{II}MP$ and $(CO)(py)Fe^{II}MP$. The procedure was the same as that for (7), but a slight excess of 1-methylimidazole or pyridine was added prior to argon flushing.

In all cases complex formation was monitored spectrophotometrically. The NO adducts were checked with ESR, and showed three- and nine-

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Table I. Resonance Raman Frequencies (cm^{-1}) for Heme NO and CO Complexes

$(NO)Fe^{II}PP$	$(NO)(ImH)Fe^{II}PP$	$(NO)Fe^{II}OEP$	$(NO)(ImH)Fe^{II}OEP$	$[(ImH)_2Fe^{III}OEP]^+$	$[(Im)_2Fe^{III}OEP]^-$
		1317 (ap)[a]	1317	1323	1323
1374	1375	1377 (p)	1378	1377	1377
		1406 (p)	1407		
		1455			
		1477			
1508	1508	1509 (p)	1508	1504	1504
1563	1562	1572 (dp)	1569	1566	1566
1585	1583	1590 (ap)	1585	1586	1585
1643	1637	1646 (dp)	1641	1638	1638
$(CO)Fe^{II}MP$	$(CO)(py)Fe^{II}MP$	$(NO)Fe^{II}MP$	$(NO)(py)Fe^{II}MP$	$[(NO)(Im)Fe^{II}OEP]^-$	$(NO)Fe^{III}MP$
1129 (ap)	1130 (p)	1131 (ap)			
1165	1161 (dp)				
1228	1226 (dp)	1228			
1305	1313 (ap)	1309	1315	1319	1303
1375	1374 (p)	1377	1375	1378	1361
1403	1400 (ap)	1410	1411	1407	1409
1411	1407 (dp)	1414	1408		
1503	1500 (p)	1510	1502	1508	1500
1576	1570 (dp)	1576	1570	1569	1579
1590	1588 (ap)	1588	1586	1591	1581
1601	1600 (p)	1598	1596		
1638	1633 (dp)	1645	1640	1640	1642

[a] ap = anomalously polarized; dp = depolarized; p = polarized.

line spectra for five- and six-coordinate complexes as previously reported.[12]

Raman, ESR and Visible Spectra. Resonance Raman spectra were obtained as described previously.[15] ESR spectra were taken at room temperature and at 77 K with a Varian E12 spectrometer with X-band bridge. Visible spectra were recorded on a Cary 118 spectrophotometer.

Results and Discussion

Analogues. Table I lists the prophyrin frequencies observed for the NO and CO complexes of iron(II) protoporphyrin IX (PP), mesoporphyrin IX (MP), and octaethylporphyrin (OEP). PP is the porphyrin contained in Hb. MP and OEP have been used as protein-free analogues,[16a] because they lack the peripheral vinyl groups of protoporphyrin, which contribute RR bands and clutter the spectrum.[16b] While the porphyrin vibrational pattern is not significantly altered,[16a] there are nevertheless observable frequency differences among the different porphyrins which are relevant to a detailed comparison with heme protein spectra. The largest effect is seen for the depolarized band (III, vide infra) at ~1570 cm^{-1} in the MP and OEP complexes, which decreases by ~10 cm^{-1} in PP, apparently reflecting a coupling with the vinyl groups.

Several porphyrin vibrational frequencies have been shown to be sensitive to heme electronic structure and stereochemistry.[16,17,22] The three bands that show some sensitivity to IHP addition in NOHb are monitors of iron oxidation state or of electron-donating or -withdrawing properties of the axial ligands.[16,17] In Table II, the frequencies of these bands, previously labeled I, III, and V,[16a] are listed for $Fe^{II}MP$ complexes with ligands of variable π-acceptor properties. There is a continuous increase in all three frequencies with increasing π acidity. This behavior is interpreted[16,17] in terms of competition between the axial ligands and the porphyrin ring for the iron d_π electrons. The greater the electron withdrawal by the axial ligands, the less the back-donation into the porphyrin π^* orbitals. Consequently, the porphyrin bonds are strengthened and the ring vibrational frequencies increase.

Nitric oxide is an even stronger π-acceptor ligand than carbon monoxide, and the NO mesoporphyrin complexes show the highest frequencies for these bands. The frequencies of $(NO)(py)Fe^{II}MP$ are as high as those of $[(ImH)_2Fe^{III}MP]^+$, in which an electron is removed from Fe^{II}. The frequencies of $(NO)Fe^{II}MP$ are even higher. Addition of a trans axial ligand would be expected to increase the electron density at the iron atom, and the back-bonding to the porphyrin ring, thereby accounting for decreased porphyrin frequencies relative to the five-coordinate complex. A similar effect is evident for CO. The frequencies of five-coordinate $(CO)Fe^{II}MP$ are lowered by almost the same amount as those of $(NO)Fe^{II}MP$ upon binding a trans axial pyridine.

Table II. Marker Band Frequencies (cm^{-1}) for NO and CO Mesoporphyrin Complexes

derivative[a]	I (p)	III (dp)	IV (ap)	V (dp)
$(pip)_2Fe^{II}MP$[b]	1358	1537	1583	1620
$(py)_2Fe^{II}MP$[b]	1365	1555	1582	1622
$[P(OEt)_3]_2Fe^{II}MP$[b]	1368	1559	1590	1629
$(CO)(py)Fe^{II}MP$[c]	1374	1570	1588	1633
$(CO)Fe^{II}MP$[c]	1375	1574	1590	1638
$(NO)(py)Fe^{II}MP$[c]	1377	1570	1586	1640
$(NO)Fe^{II}MP$[c]	1377	1576	1588	1645
$[(NO)Fe^{III}MP]^+F^-$[c]	1361	1572	1581	1645

[a] Abbreviations: ap = anomalously polarized; p = polarized; dp = depolarized; MP = mesoporphyrin IX dimethyl ester; pip = piperidine; py = pyridine; Et = ethyl; Im = imidazole. [b] From ref 15. [c] Present study.

Also listed in Table II are the frequencies of band IV,[16a] the anomalously polarized band near 1580 cm^{-1}. This band has been diagnosed as a spin-state marker,[16b] since conversion from low- to high-spin hemes is accompanied by a marked lowering in its frequency. This has been interpreted in terms of associated structural changes, principally expansion of the porphyrin core,[22] and for nonplanar hemes an added contribution from doming of the prophyrin ring.[22c] The band shows essentially no dependence on oxidation state per se.[16] But the expanded data of Table II do show that good π-acceptor ligands increase the frequency to the vicinity of 1590 cm^{-1}. This would be consistent with a more contracted porphyrin core, due to the increase in positive charge on the iron atom.

The frequencies of the bisimidazole iron(II) porphyrins classify imidiazole as an ineffective π acceptor relative to porphyrin.[16a] We had expected that imidazolate ion might be an effective π donor. The sensitivity of heme RR frequencies to π-donor ligands has been demonstrated in the case of cytochrome P450, in which cysteine thiolate is believed to be an axial ligand.[23] However, the porphyrin frequencies are the same within 1 cm^{-1} for the bisimidazole and bisimidazolate complexes of $Fe^{III}OEP$ (Table

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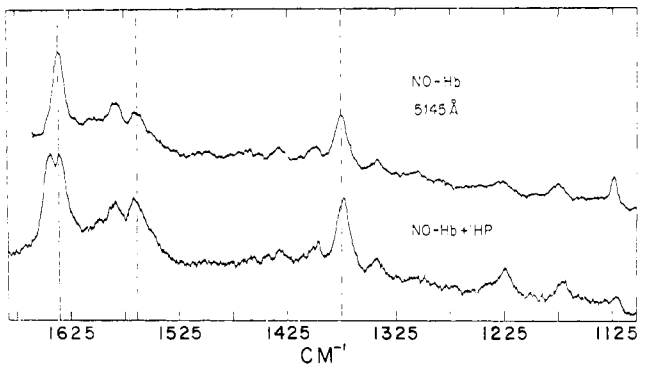

Figure 1. High-frequency Raman spectra of NOHb (1 mM) before and after addition of IHP (10 mM) with 514.5-nm Ar^+ laser excitation (100 mW). Spectral slit width = 10 cm^{-1}, time constant = 2 s; scanning speed = 50 cm^{-1}/min.

I), indicating little interaction of imidazole, in either neutral or anionic form, with the porphyrin π^* levels. The deprotonation of imidazole in the NO complex produces a 5-cm^{-1} increase in band IV, but otherwise little effect. This increase of band IV indicates a contraction of the porphyrin core (by ~0.01 Å[22c]). This might reflect decreased steric interaction between the pyrrole N atoms and the bound NO, since imidazolate is expected to be a stronger σ donor and would exert a stronger trans labilization.[11]

Heme Proteins. RR spectra of NOHb with and without IHP are shown in Figure 1, and the frequencies are listed in Table III, as are those of NOMb (Mb = myoglobin). The solutions were prepared under strictly anaerobic conditions, and denaturation can be excluded. The NOHb spectra are in agreement with those of Szabo and Barron.[2] The only marked change on adding IHP is the reduction in intensity of the 1633-cm^{-1} band and the growth of the 1644-cm^{-1} shoulder into a band of equal height. Careful measurement indicates, however, a slight but perceptible shift of the 1375-cm^{-1} band to 1373 cm^{-1}, and of the 1564-cm^{-1} band to 1566 cm^{-1}. Since the spectral changes are associated with alteration in half of the Hb heme groups, the slightly shifted bands must represent unresolved doublets. The actual frequencies for the altered hemes are estimated to be 1371 and 1568 cm^{-1}.

From the analogue complexes, it can be expected that, if the R → T shift involved imidazolate protonation in half the chains, as suggested by Chevion et al.,[14] the effect would be to shift half the band IV (1582 cm^{-1}) intensity *down* by 5 cm^{-1}. This is not observed. If the R → T shift induced a breaking of the Fe–imidazole bonds in half the chains, then the effect should be to shift half the intensity of bands III and V *up* by 2–5 and 5–6 cm^{-1}, respectively. The observed pattern is close to expectation. Bands III and V do indeed shift up, by 4 and 11 cm^{-1}. The increase in band V is about twice as large as expected; also the 4-cm^{-1} lowering of band I is not observed in the analogue complexes, for which this frequency is constant, or increases 1–2 cm^{-1}, upon removal of the sixth ligand.

These slight differences with respect to the analogues suggest additional protein influences on the heme structure. One such influence might be a donor interaction between the distal imidazole of Hb and the N atom of the iron-bound NO ligand, as suggested by Maxwell and Caughey[10] to explain the fact that the N–O stretching frequency of R-state NOHb is 15 cm^{-1} lower than that in (NO)(1-MeIm)Fe^{II}PP, unless the latter is examined in pure 1-MeIm as solvent. (We were unable to examine RR spectra in pure 1-MeIm because of its strong Raman scattering.) This interaction would decrease the π acidity of the NO ligand and might produce a lowering of band V; its frequency in R-state Hb is 4 cm^{-1} lower than in the analogue (NO)(ImH)Fe^{II}PP. For NOMb, on the other hand, this lowering is not seen (Table III). The donor interaction is presumably abolished when the bond to the proximal imidazole is broken in the T-state α chains, as ν_{NO}[10] and band V are at the frequencies observed for (NO)Fe^{II}PP.

When NO was passed through a solution of metMb, a color change was observed, but the ESR spectrum of the frozen solution demonstrated that the derivative contained Fe^{II} rather than Fe^{III}.

Table III. Resonance Raman Frequencies (cm^{-1}) for Heme Protein NO Complexes

NOHb	NOHb + IHP	NOMb	Fe^{III}Mb + NO	(NO)Fe^{III}-cyt *c*
553 (vw)[a]	553 (vw)			
	592 (vw)			
678			677	691 (p)
757				755 (dp)
800				800 (dp)
				811 (p)
978				
999				
1090				1049 (p)
1125 (p)	1125		1128	1123 (p)
1180 (dp)	1180		1177	1172 (dp)
1225 (dp)	1225		1227	1230 (dp)
1306 (ap)	1306	1306	1308	1322 (ap)
1341	1341		1345	
1375 (p)	1373	1376	1376	1377 (p)
1399 (ap)	1399	1399	1401	
1433 (dp)	1433			1408 (dp)
1464 (w)				
1500 (p)	1500	1503	1508	1505 (p)
1540 (w)				
1564 (dp)	1566	1564	1565	1568 (dp)
1582 (ap)	1582	1587	1584	1587 (ap)
1582 (p)	1582	1583	1585	1588 (p)
1633 (dp)	1633	1637	1645	1638 (p)
	1644			

[a] vw = very weak; w = weak; p = polarized; ap = anomalously polarized; dp = depolarized.

The ESR spectrum was of the three-line variety, typical of five-coordinated NOFe^{II} hemes, rather than the nine-line variety, seen for NOMb, and typical of six-coordinate NOFe^{II} hemes with nitrogeneous axial ligands.[12,13] We infer that addition of NO to metMb leads to reduction of the Fe^{III} heme, and that in the resulting NOFe^{II} derivative the iron–imidazole bond is broken (possibly via reaction of the imidazole with a product of the reduction reaction). Consistent with this, the marker RR band frequencies are similar to those of five-coordinate (NO)Fe^{II}PP, as shown in Table III.

Fe^{III} Derivatives. It is possible to make a (NO)Fe^{III} heme derivative with cytochrome *c*.[20] Addition of NO to ferricytochrome *c* produces a spectral change, and the frozen solution gives no detectable ESR spectrum (consistent with pairing of the NO and Fe^{III} odd electrons). The RR frequencies of (NO)Fe^{III} cyt *c* are given in Table I. They are nearly the same as those for NOMb, which contains Fe^{II}. This suggests that the electron distribution in the porphyrin ring is essentially unaffected by the iron oxidation state in six-coordinate NO complexes. The implication is that the difference in charge is almost entirely accommodated by the NO ligand. The electronic flexibility of coordinated NO,[24] with its available NO^- and NO^+ oxidation levels, is well known. Thus, the dominant resonance forms in the Fe^{II} and Fe^{III} heme derivatives could be formulated as $Fe^{2+}NO$ and $Fe^{2+}NO^+$, or as $Fe^{3+}NO^-$ and $Fe^{3+}NO$. Since the porphyrin RR frequencies are as high as those of low-spin Fe^{III} derivatives, the Fe^{3+} formulation might be preferred. It needs reemphasizing,[16] however, that the electron redistribution may be largely in the π system, and that, by the same criterion, the $Fe^{II}CO$ hemes should be formulated as mainly $Fe^{3+}CO^-$.

Wayland and Olson[25] have shown that it is possible to coordinate NO to $(Cl^-)Fe^{III}$TPP (TPP = tetraphenylporphine), although the presence of protic agents brings about reduction to Fe^{II} complexes. Bubbling NO into a degassed CH_2Cl_2 solution of $(F^-)Fe^{III}$MP caused a disappearance of the ESR spectrum and a shift in the absorption spectrum, with an α, β doublet of almost equal intensity at 565 and 535 nm and the Soret band at 417 nm. These changes are reversible on degassing the solution, and are clearly indicators

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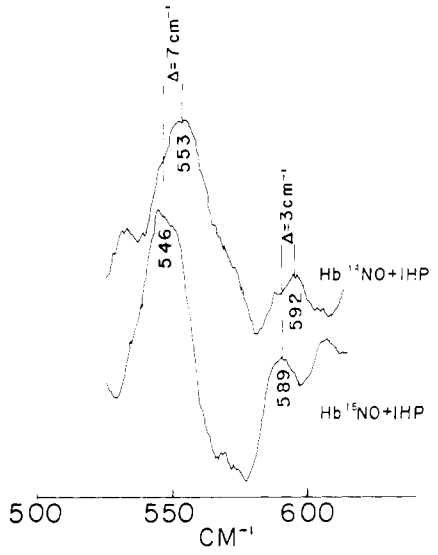

Figure 2. Low-frequency Raman spectra of NOHb (2 mM) before and after addition of IHP (20 mM), pH 7.0, with 454.5 mM Ar^+ laser excitation (60 mW). Spectral slit width = 5 cm^{-1}. Each spectrum is the accumulation of nine complete scans consisting of 1-cm^{-1} steps, 4 s per step.

of the formation of a reversible NO complex. The RR frequencies of this complex are given in Tables I and II. The band III and V frequencies are as high as those of $(NO)Fe^{II}MP$, suggesting that we are dealing with the analogous five-coordinated Fe^{III} complex.

Unexpectedly, however, the band I frequency is shifted dramatically (16 cm^{-1}) to lower frequency; its position, 1361 cm^{-1}, is similar to that of low-spin Fe^{II} hemes without π-acceptor axial ligands (see the entry for $(pip)_2Fe^{II}MP$ in Table II). Band IV is also 7 cm^{-1} lower than for $(NO)Fe^{II}MP$. An effect that might account for these shifts would be an out-of-plane displacement of the iron atom that is greater in $[(NO)Fe^{III}MP]^+$ than in the isoelectronic $(CO)Fe^{II}MP$ (which does not show similar band I and IV lowerings) or in $(NO)Fe^{II}MP$. In the analogous $(NO)Fe^{II}TPP$ (TPP = tetraphenylporphine) the iron atom is 0.21 Å out of the plane toward the NO ligand.[11b] A greater displacement in $[(NO)Fe^{III}MP]^+$ might result from increased π interaction resulting from removal of the odd electron, or from interaction of the cationic complex with the counteranion (possibly on the NO side of the heme). The effect is not specific for F^-, as addition of NO to $(Cl^-)Fe^{III}MP$ produced the same RR spectrum.

Band IV is known to be sensitive to the structural concomitants of out-of-plane displacement.[22c] Band I, however, is primarily sensitive to π delocalization in the porphyrin ring.[16,17] Its sharp decrease in $[(NO)Fe^{III}MP]^+$ might result from *forward* donation from the high-lying filled a_{2u} porphyrin π orbital[26] to the empty iron orbital, which would be a $3d_{z^2}$–$4p_z$ hybrid, trans to the NO ligand. Such an interaction would become more favorable with increasing out-of-plane displacement, since the overlap of the porphyrin orbital with the more extended iron orbital would increase. Unfortunately there are no structural data currently available with which to test the hypothesis.

Attempts to prepare a six-coordinate $Fe^{III}NO$ heme, to model the $(NO)Fe^{III}$cyt *c* spectrum, failed. Addition of nitrogenous ligands to solutions containing $[(NO)Fe^{III}MP]F$ either reduced the complex or displaced the NO.

Fe–NO Stretch. Recently Chottard and Mansuy[18] have reported the detection of the Fe–NO stretching mode as a weak band in the RR spectrum of NOHb at 549 cm^{-1}, shifting to 539 cm^{-1} on ^{15}NO substitution. We have confirmed this finding, although the frequencies we observe are slightly higher, 553 and 546 cm^{-1} for ^{14}NOHb and ^{15}NOHb. When IHP is added, an additional band is found, at 592 and 589 cm^{-1} for ^{14}NOHb and ^{15}NOHb, respectively, as shown in Figure 2. The isotope shift implicates this band as another Fe–NO mode. We assign it to the altered NO heme complexes in half the chains, the remnant 553 band arising from the unaltered NO hemes. The two bands need not have the same intensity, since the resonance enhancement factors may differ if the electronic structure is altered.

Unfortunately we have been unable to obtain Fe–NO stretching frequencies for the analogue complexes. The bands are extremely weak, even in the protein spectra, and seem to be even weaker for the protein-free analogues in CH_2Cl_2. The observed shift is, however, in the right direction if the Fe–imidazole bonds are broken in half the hemes, in view of the weakening of the Fe–NO bond expected upon binding of a sixth ligand. When *N*-methylimidazole is added to $(NO)Fe^{II}TPP$, the Fe–NO distance increases from 1.717[11b] to 1.743 Å.[11a] The extent of the frequency shift is consistent with this effect. Using a diatomic oscillator approximation for the presumed five-coordinate Fe–NO stretch at 592 cm^{-1}, and a linear triatomic approximation for the six-coordinate Fe–NO stretch at 533 cm^{-1} (with point mass NO and imidazole ligands, and an assumed value of the Fe–imidazole force constant of 2.0 mdyn/Å), we calculate[27] 4.03 and 3.17 mdyn/Å, respectively, for the Fe–NO force constants. From Badger's rule[28] ($r = d_{ij} + (a_{ij} - d_{ij})k^{-1/3}$ where the parameters a_{ij} and d_{ij} are 2.35 and 0.85 for bonds connecting first- and third-row atoms) the difference in force constant is associated with a bond lengthening of 0.032 Å, close to the 0.026 Å expansion observed for the *N*-methylimidazole adduct of $(NO)Fe^{II}TPP$. Considering the approximate nature of the vibrational calculation and of Badger's rule, the agreement is quite satisfactory.

Conclusions

1. The observation of a second Fe–NO RR band of NOHb, induced by IHP, 39 cm^{-1} above the first band supports the IR and EPR evidence that the Fe–imidazole bonds are broken for half the NO hemes in the T quaternary structure.

2. The RR frequency shifts induced by NO coordination to Fe^{II} heme are those expected for a strong π-acceptor ligand, which inhibits π back-bonding from Fe^{II} to the porphyrin π^* orbitals as effectively as does oxidation to Fe^{III}. Porhyrin back-bonding increases slightly, and the associated RR frequency shifts are reduced, upon coordination of a sixth ligand, trans to NO. This effect is also observed for CO heme.

3. Imidazole and imidazolate give essentially the same porphyrin frequencies when bound to heme; neither ligand appears to interact significantly with porphyrin π^* orbitals. The band IV frequency of NO heme increases by 5 cm^{-1} when a trans axial imidazole is deprotonated, indicative of a slight contraction of the porphyrin core.

4. In six-coordinate NO hemes the RR frequencies are essentially the same for $Fe^{III}((NO)Fe^{III}$cyt *c*) as for $Fe^{II}((NO)(py)Fe^{II}MP)$, indicating that the one-electron difference is taken up mostly by the coordinated NO. Five-coordinate $(NO)Fe^{III}MP$, however, shows marked frequency shifts, in a pattern not previously observed for heme complexes, which are suggested to result from π forward donation from the prophyrin to the iron $4p_z$–$3d_{z^2}$ hybrid orbital in a substantially out-of-plane structure.

5. The IHP-induced shift of NOHb band V, from 1633 to 1644 cm^{-1}, is in the right direction for Fe–imidazole bond breaking, but is twice as large as expected from the analogue complexes, suggesting additional protein influences, as does the slight decrease in band I.

6. If instead the R → T switch involved protonation of imidazolate bound to NO heme, the only expected consequence for the RR spectrum would be a 5-cm^{-1} decrease in band IV. This is not observed.

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