Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3640.
Hurst, J. K., & Lane, R. H. (1973) *J. Am. Chem. Soc. 95*, 1703.
Jorter, J. (1976) *J. Chem. Phys. 64*, 4860.
Jwo, J. J., & Haim, A. (1976) *J. Am. Chem. Soc. 98*, 1172.
Larsen, R. P., & Ross, L. E. (1959) *Anal. Chem. 31*, 176.
Latimer, W. M. (1952) *Oxidation Potentials*, 2nd ed., Prentice-Hall, Englewood Cliffs, NJ.
Lenaz, G., Daves, G. D., Jr., & Folkers, K. (1968) *Arch. Biochem. Biophys. 123*, 539.
Leonard, J. J., & Yonetani, T. (1974) *Biochemistry 13*, 1466.
Lim, H. S., Barclay, D. J., & Anson, F. C. (1972) *Inorg. Chem. 11*, 1460.
Lode, E. T., Murray, C. L., & Rabinowitz, J. C. (1974) *Biochem. Biophys. Res. Commun. 60*, 163.
Lowe, D. C., & Bray, R. C. (1978) *Biochem. J. 169*, 471.
Mackey, L. N., Kuwana, T., & Hartzell, C. R. (1973) *FEBS Lett. 36*, 326.
Mizrahi, I. A., Wood, F. E., & Cusanovich, M. A. (1976) *Biochemistry 15*, 343.
Nordmeyer, F., & Taube, H. (1968) *J. Am. Chem. Soc. 90*, 1162.
Norton, K. A., & Hurst, J. K. (1978) *J. Am. Chem. Soc. 100*, 7237.
Norton, K. A., Hulett, L. G., Halko, D. J., & Hurst, J. K. (1979) in *Electron Tunneling in Biological Systems*, University of Pennsylvania, Philadelphia, PA (in press).
Packer, E. L., Sternlicht, H., & Rabinowitz, J. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3278.
Palmer, G., Babcock, G. T., & Vickery, L. E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2206.
Phillips, W. D., McDonald, C. C., Stombaugh, N. A., & Orme-Johnson, W. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 140.
Potasek, M. J. (1978) *Science 201*, 151.
Potasek, M. J., & Hopfield, J. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3817.
Rieder, K., & Taube, H. (1977) *J. Am. Chem. Soc. 99*, 7891.
Rossotti, F. J. C., & Rossotti, H. (1961) *The Determination of Stability Constants and Other Equilibrium Constants in Solution*, McGraw-Hill, New York.
Salemme, F. R. (1977) *Annu. Rev. Biochem. 46*, 299.
Savitzky, A., & Golay, M. (1964) *Anal. Chem. 36*, 1627.
Sieker, L. C., Adman, E., & Jensen, L. H. (1972) *Nature (London) 235*, 40.
Stein, C. A., & Taube, H. (1978) *J. Am. Chem. Soc. 100*, 1635.
Sutin, N. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., Ed.) Vol. 2, Chapter 19, Elsevier, Amsterdam.
Taube, H. (1973) *Surv. Prog. Chem. 6*, 1.
Taube, H. (1977) *Adv. Chem. Ser. No. 162*, 127.
Tweedle, M. F., Wilson, L. J., Garcia-Iniquez, L., Babcock, G. T., & Palmer, G. (1978) *J. Biol. Chem. 253*, 8065.
Vanderkooi, J. M., Landesburg, R., Hayden, G. W., & Owen, C. S. (1977) *Eur. J. Biochem. 81*, 339.
Van Heuvelen, A. (1976) *Biophys. J. 16*, 939.
Wiley, G. A., Hershkowitz, R. L., Rein, B. M., & Chung, B. C. (1964) *J. Am. Chem. Soc. 86*, 964.

Low-Frequency Vibrations in Resonance Raman Spectra of Horse Heart Myoglobin. Iron–Ligand and Iron–Nitrogen Vibrational Modes†

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ABSTRACT: The low-frequency regions (150–700 cm^{-1}) of resonance Raman (RR) spectra of various complexes of oxidized and reduced horse heart myoglobin were examined by use of 441.6-nm excitation. In this frequency range, RR spectra show 10 bands common to all myoglobin derivatives (numbered here for convenience from I to X). Relative intensities of bands IV, V, and X constitute good indicators of the doming state of the heme and, consequently, of the spin state of the iron atom. An additional band is present for several complexes (fluorometmyoglobin, hydroxymetmyoglobin, azidometmyoglobin, and oxymyoglobin). Isotopic substitutions on the exogenous ligands and of the iron atom ($^{56}Fe \rightarrow {}^{54}Fe$) allow us to assign these additional lines to the stretching vibrations of the Fe–sixth ligand bond. Similarly, bands II are assigned to stretching vibrations of the Fe–N(pyrrole) bonds. An assignment of bands VI to stretching vibrations of the Fe–N_ϵ(proximal histidine) bonds is also proposed. Mechanisms for the resonance enhancement of the main low-frequency bands are discussed on the basis of the excitation profiles and of the dispersion curves for depolarization ratios obtained for fluorometmyoglobin and hydroxymetmyoglobin.

In the past few years, resonance Raman (RR)[1] spectroscopy has been extensively used for the study of hemoproteins [Spiro (1975) and references cited therein]. In particular, the intense lines lying between 1100 and 1650 cm^{-1} have been thoroughly studied. This range of frequencies covers the stretching vibrations of C=C and C=N groups of the porphyrin ring and the bending vibrations of C–H groups of the methine bridges (Ogoshi et al., 1972). Since a proper description of the iron porphyrin complex in hemoproteins also implies a knowledge of the bonds between the iron atom and its five or six ligands, we have given our attention to the low-frequency region; previous work in infrared spectra had indeed suggested that

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[1] Abbreviations used: RR, resonance Raman; Mb, myoglobin; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

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iron–ligand vibrations could be expected to lie in the low-frequency region, 120–610 cm^{-1} (Ogoshi et al., 1973).

In RR spectroscopy, with an excitation wavelength lower than 500 nm, the Soret band dominates the scattering process and the resonating vibrational modes should all be totally symmetrical (A-term scattering) (Strekas et al., 1973). On the contrary, the α and β bands prevail when the excitation wavelength is higher than 500 cm^{-1}. Due to B-term scattering, nontotally symmetric vibrational modes are then strongly enhanced, while symmetrical modes are still active (Spiro & Strekas, 1972). Yamamoto et al. (1973) observed strong bands in the 100–800-cm^{-1} range using a 441.6-nm excitation on various hemoproteins but did not propose any assignment. Other authors have described low-frequency bands appearing in RR spectra of hemoglobin, myoglobin, cytochromes, and cytochrome *c* oxidase (Brunner & Sussner, 1973; Brunner, 1974; Salmeen et al., 1973, 1978; Kitagawa et al., 1975; Rimai et al., 1975; Asher et al., 1977; Champion et al., 1978). Using isotopic substitution of the oxygen molecules in oxyhemoglobin, Brunner (1974) assigned an RR band at 567 cm^{-1} to the stretching vibration of the Fe–O bond.

One can further predict that excitation in charge-transfer bands should allow resonance of stretching modes between iron and its axial ligands (Spiro, 1975), insofar as both the transition moments of certain of these bands and the vibrational modes may be perpendicular to the heme plane (*z* polarized). In this respect, using an excitation between 600 and 630 nm, Asher et al. (1977) observed low-frequency lines in RR spectra of different derivatives of methemoglobin and assigned bands to the iron–ligand stretching vibrations (Fe^{III}–OH^-, Fe^{III}–N_3^-, and Fe^{III}–F^-).

Here we report a systematic study of the low-frequency vibrations exhibiting activity in the RR spectra of horse heart myoglobin derivatives due to preresonance on the Soret bands and, probably, to full resonance on charge-transfer bands. Isotopic substitutions allows several band assignments to be made. We compare the following couples of complexes with exogenous ligands: $H_2{}^{16}O$–$H_2{}^{18}O$, $^{16}OH^-$–$^{18}OH^-$, $^{14}N_3^-$–$^{15}N_3^-$, and ($^{14}N_2$)imidazole–($^{15}N_2$)imidazole on one hand and, on the other hand, reconstituted [^{54}Fe]myoglobin to the natural [^{56}Fe]myoglobin. We also discuss correlations of band frequencies with oxidation and spin states. Furthermore, we measure excitation profiles and dispersion curves for depolarization ratios for the main low-frequency bands, which proved useful in assigning electronic absorption bands of some hemoglobin derivatives (Strekas et al., 1973; Asher et al., 1977).

Experimental Section

Protein Preparations. Horse heart myoglobin was prepared according to the method of George & Irvine (1952). After oxidation by ferricyanide, metmyoglobin was finally purified by gel filtration on a Sephadex G-75 column equilibrated with phosphate (0.05 M) and sodium chloride (0.15 M), pH 7.0, buffer. Deoxymyoglobin was prepared by addition of dithionite anaerobically to the sample solution equilibrated with nitrogen gas (Air Liquide S.A., purity 99.998%). To obtain oxymyoglobin, an aliquot of metmyoglobin was reduced by dithionite, then filtered on a Sephadex G-10 column, and finally saturated with pure oxygen. Nitrosylmyoglobin was prepared from metmyoglobin by anaerobic reduction of nitrite by dithionite in a Thunberg tube. To prepare the various metmyoglobin derivatives, ligands were added up to minimal saturating concentrations. The protein concentration was adjusted to 1 mM with Bistris (0.05 M) and sodium chloride (0.15 M), pH 7.0, buffer. For aquometmyoglobin and hydroxymetmyoglobin, the pH was adjusted with Bistris (0.05 M) and sodium chloride (0.15 M), pH 6.0, buffer and with carbonate (0.05 M) and sodium chloride (0.15 M), pH 10.4, buffer, respectively. Before we recorded RR spectra, the samples were filtered on Millipore filters (0.22-μm pore diameter).

Stable isotopes, with an isotopic enrichment of 95%, were purchased as $Na^{15}N_3$, ($^{15}N_2$)imidazole, $H_2{}^{18}O$, and $^{54}Fe_2O_3$ from the Bureau des isotopes stables of the Centre d'Etudes Nucléaires de Saclay. In order to exchange $H_2{}^{18}O$, myoglobin was concentrated through a dialysis bag in dry Sephadex G-10 until one obtained a paste. This paste was then diluted with either $H_2{}^{18}O$ or $H_2{}^{16}O$ (control experiment) to obtain a final concentration of 1 mM. The pH value was then adjusted to 6.0 or 10.4 by addition of solid monosodium phosphate or solid sodium carbonate. To obtain [^{54}Fe]myoglobin, the globin was prepared by the method of Rossi-Fanelli et al. (1958). The iron (^{54}Fe and ^{56}Fe for control) incorporation in protoporphyrin was performed according to Yonetani & Asakura (1968). The incorporations of the hemes and the purifications of reconstituted myoglobins were achieved by the method of Tamura et al. (1973). Optical spectra and polyacrylamide gel electrophoresis showed that, in these respects, each pair of reconstituted myoglobins was identical with the nontreated samples.

Resonance Raman Spectra. Various lines (454.5–528.7 nm) from an argon laser and the 441.6-nm emission of He–Cd laser (Spectra Physics) were used. Light scattered at about 90° from the excitation beam was analyzed through a double grating Raman spectrometer (Coderg PHO) with an effective resolution of 12 cm^{-1}. A grazing incidence method was employed in order to minimize reabsorption effects. A laser power of 50 mW was used. This resulted in only 1 mW impinging on the surface of the sample. In order to minimize the nonresonant Raman scattering from the glass, cuvettes were built on which the observation surface was made of a microscope coverslip 0.15-mm thick. A time constant of 5 s was used in the detection system. In the studies of the 200–650 cm^{-1} region, improvement in the signal to noise ratio of RR spectra was achieved by summation by use of a multichannel analyser (Didac 4000, Intertechnique). For the isotopic experiments where 10 scans were accumulated, a significant frequency shift was considered to be significant when equal to or higher than 1 cm^{-1}.

Depolarization ratios (ρ_1) of the main low-frequency bands were measured for fluoride, hydroxide, and cyanide complexes of metmyoglobin by use of the 441.6-nm excitation and for hydroxymetmyoglobin with excitation wavelengths from 441.6 to 514.5 nm. A given spectral region was scanned several times, alternatively with parallel and perpendicular analysis, and the $I_\parallel$ and $I_\perp$ signals were each averaged by summation in two different subgroups of the multichannel analyser. No polarization scrambler was used, and the experimental values of ρ_1 were corrected for the differential spectral sensitivities of the apparatus in vertical and horizontal polarized light.

Excitation profiles were obtained for the parallel polarized component ($I_\parallel$) of RR bands of fluorometmyoglobin and hydroxymyoglobin. Ammonium sulfate (0.25 M) was added to the solutions, and the strong band at 983 cm^{-1} was used as an internal standard. Excitation profiles were further corrected for the differential spectral sensitivity of the spectrometer. No noticeable reabsorption effects occurred under the present conditions, as checked on samples of different concentrations.

For oxy- and nitrosylmyoglobin, the extent of photodissociation under laser illumination was monitored by the ob-

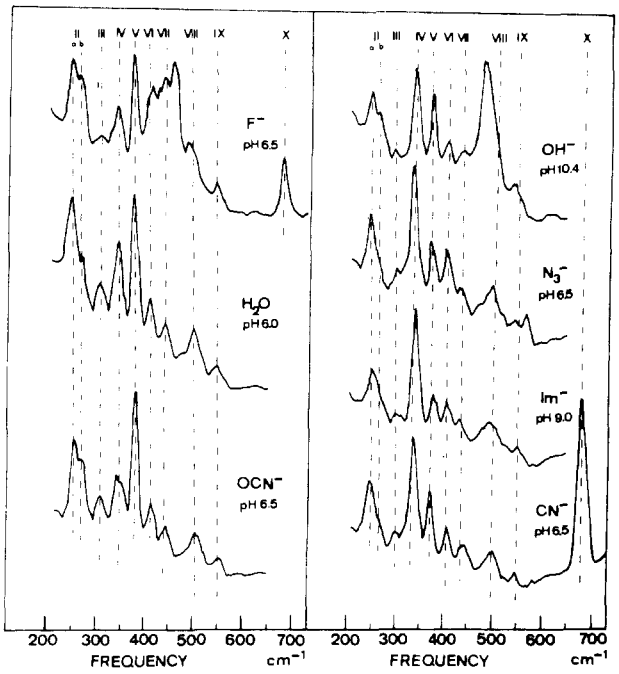

FIGURE 1: Resonance Raman spectra of horse heart metmyoglobin with various ligands. Conditions: excitation wavelength is 441.6 nm; scanning is 25 cm^{-1}/min; slit width is 8 cm^{-1}; laser power is 50 mW; summation of six scans; heme concentration is 1 mM; nature of the ligand and pH conditions are indicated on the right side of the spectra.

servation of the intense high-frequency RR bands at 1377 cm^{-1} for oxy- and nitrosylmyoglobin and at 1357 cm^{-1} for deoxymyoglobin (Spiro & Strekas, '974; Rimai et al., 1975). With a laser power of 50 mW which gave a power level of 1 mW at the samples, a weak shoulder could be seen at 1357 cm^{-1}, showing the presence of a small proportion of deoxymyoglobin produced by photolysis. However, the frequencies of the RR bands for oxy and nitrosyl derivatives were not affected by a 10-fold variation of incident laser power, i.e., when we decreased the output from 50 to 5 mW. The RR spectra reported in this work for the oxy and nitrosyl derivatives can thus be considered to be free from artifacts that could arise from partial photodissociation.

Results

RR Spectra of Oxidized Myoglobin Derivatives

Description of the RR Spectra. RR spectra of nine different metmyoglobin derivatives were recorded by use of 441.6-nm excitation (Figure 1 and Table I). Although some derivatives are unequivocally characterized by the positions of specific bands, RR spectra of these derivatives all contain common bands which are enumerated in Table I together with the actual frequencies for each myoglobin derivative. These bands are numbered for convenience from I to X. Beside these common bands, some derivatives show an additional band (at 462 cm^{-1} for fluorometmyoglobin, at 490 cm^{-1} for hydroxymetmyoglobin, and at 570 cm^{-1} for azidometmyoglobin). In the region of band IV, the cyanate derivative yields a doublet at 341 and 354 cm^{-1} and the thiocyanate derivative yields a line at 341 cm^{-1} with a shoulder at 349 cm^{-1} (Figure 1 and 2).

The main lines of the low-frequency region of RR spectra of fluorometmyoglobin, hydroxymetmyoglobin, and cyanometmyoglobin are all polarized (Table II).

Differences in the positions and in the relative intensities of lines II, IV, V, and X characterize the RR spectra of high-spin and of low-spin derivatives (Figure 1). Purely

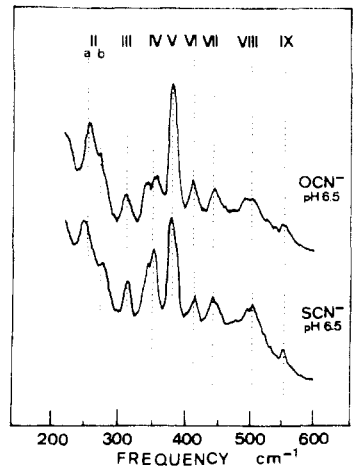

FIGURE 2: Resonance Raman spectra of cyanatometmyoglobin and thiocyanatometmyoglobin. Excitation wavelength is 454.5 nm; same conditions as in Figure 1.

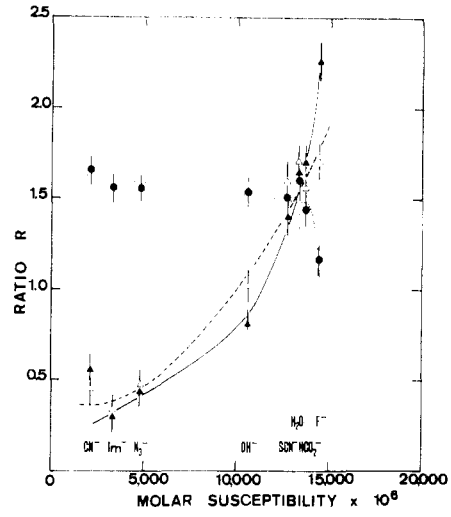

FIGURE 3: Variations of R_1 and R_2 ratios with molar susceptibility of horse heart metmyoglobin derivatives. R_1 = ratio of intensity of band V to that of band IV at 441.6-nm (▲) and 454.5-nm (△) excitations; determinations of R_1 from accumulated spectra. R_2 = ratio of intensities of the X to IV band at 441.6-nm excitation (●); determination from single scans. The molar susceptibility values are taken from Beetlestone & George (1964).

high-spin derivatives yield a doublet in region II. The relative intensity of the line at 271 cm^{-1} (II_b) increases with an increasing high-spin to low-spin ratio. The low-spin derivatives present only one band near 254 cm^{-1}. Band IV is more intense than band V when the derivative is essentially low spin, while the reverse occurs for the high-spin form. These relations are visualized in Figure 3 where the ratio (R_1) of the intensity of band V to that of band IV is plotted as a function of the molar susceptibilities of the different derivatives. For 441.6- and 454.5-nm excitations, R_1 shows a decrease with increasing low-spin character. The cyanide derivative constitutes an exception, however. The ratio (R_2) of the intensity of band X to that of band IV also showed an interesting correlation with the magnetic susceptibility. R_2 was high for the cyanide and imidazole derivatives and decreased for the fluoro derivative.

Isotopic Substitutions. Isotopic substitutions of the exogenous ligands ($^{16}OH^- \rightarrow {}^{18}OH^-$ and $^{14}N_3^- \rightarrow {}^{15}N_3^-$) resulted in significant shifts of some lines in the RR spectra of the respective derivatives. The sensitive lines were the following: 490 cm^{-1} for the hydroxy derivative and 570 cm^{-1} for the azide

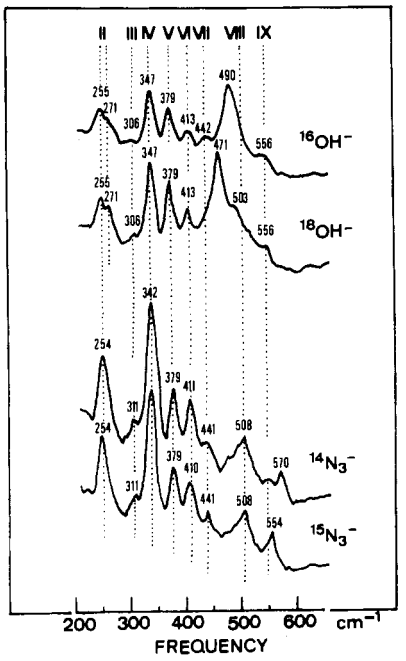

FIGURE 4: Resonance Raman spectra of [^{16}O]hydroxymetmyoglobin, [^{18}O]hydroxymetmyoglobin (upper spectra), [^{14}N]azidometmyoglobin, and [^{15}N]azidometmyoglobin (lower spectra). Same conditions as in Figure 1.

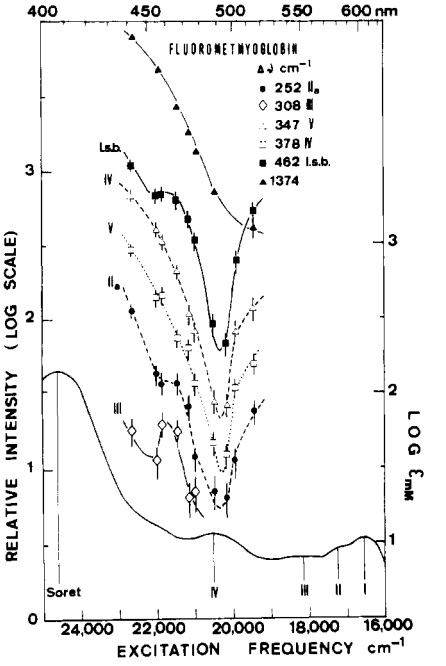

FIGURE 5: Absorption spectrum and excitation profiles of the main low-frequency RR bands of fluorometmyoglobin. The profiles are displaced for clarity on an arbitrary log intensity scale (left log scale).

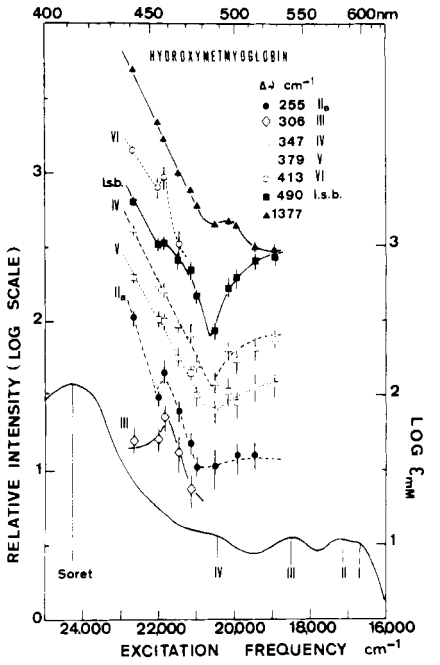

FIGURE 6: Absorption spectrum and excitation profiles of the main low-frequency RR bands of hydroxymetmyoglobin. Same representation as in Figure 5.

derivative (Figure 4 and Table III). For this latter, the line at 411 cm^{-1} (band VI) also showed a slight but significant displacement.

The main sensitive lines are those described above as "specific" for the derivative. The finding that isotopic substitutions of the respective ligands influence precisely these bands thus confirms their earlier qualification as "ligand specific".

We could not observe any band shift upon isotopic substitution of the exogenous ligands in the RR spectra of the aquo and imidazole derivatives, under identical excitation conditions. We recall that these derivatives show only the common lines I to X but no "specific band".

Substitution of ^{56}Fe by ^{54}Fe in fluoro-, aquo-, hydroxy-, azido-, and cyanometmyoglobin produced significant shifts in bands II$_a$, VI, and VIII and also, whenever existing, in the ligand-specific bands (Table III).

These results thus demonstrate that at least four low-frequency bands of RR spectra of horse heart myoglobin complexes excited at 441.6 nm arise from modes of the Fe-(N)$_4$N$_\epsilon$L grouping, where (N)$_4$ represents the four pyrrolic nitrogen atoms of the porphyrin and N$_\epsilon$ and L are respectively the nitrogen atom of the proximal histidine and the exogenous ligand.

Excitation Profiles of RR Lines of Fluorometmyoglobin and Hydroxymetmyoglobin. We measured the excitation profiles of bands II$_a$ and III–VI and ligand-specific of fluorometmyoglobin and hydroxymetmyoglobin, from 441.6 to 514.5 nm (Figures 5 and 6). All of these profiles exhibited minima near 490 nm and more or less marked shoulders close to 460 nm, with further increases in intensities at shorter wavelengths. Identical values in isotopic shifts for the ligand-specific bands of fluorometmyoglobin and hydroxymetmyoglobin at 441.6 and 514.5 nm showed that these bands originate in the same fundamental modes throughout the excitation range 441.6–514.5 nm.

The profile of the 1375-cm^{-1} band was also obtained for comparison: the band increased monotonically with shorter wavelengths, as previously observed (Strekas et al., 1973), but we observed an additional shoulder at 496 nm for hydroxymetmyoglobin.

Dispersion Curves for Depolarization Ratios. We measured the depolarization ratios (ρ_1) from 441.6 to 514.5 nm of bands IV and V, of the ligand-specific band, and of the band at 1377 cm^{-1} of hydroxymetmyoglobin (Figure 7). The depolarization ratios are close to 0.17 at 441.6 nm, and probably all of them reach maxima near 490 nm. All the bands appear to remain polarized throughout this excitation range. However, band V may become depolarized or even antipolarized near 490 nm.

RR Spectra of Reduced Myoglobin Derivatives

In the 200–650-cm^{-1} range, the spectra of deoxy-, oxy-, and nitrosylmyoglobin contain the same common features as

Table I: Low-Frequency Vibrations of Various Myoglobin Derivatives

band (cm^{-1})	oxidized derivatives									reduced derivatives			assignment
	Mb$^+$-F^-	Mb$^+$$H_2O$	Mb$^+$$HCOO^-$	Mb$^+$$SCN^-$	Mb$^+$$OCN^-$	Mb$^+$$OH^-$	Mb$^+$$N_3^-$	Mb$^+$$Im^-$	Mb$^+$$CN^-$	Mb	MbO_2	MbNO	
I_a			160 (vwsh)	164 (vwsh)	162 (vwsh)	148 (vwsh)				109			
I_b		194 (vwsh)	192 (vwsh)		187 (vwsh)	188 (vw)	187 (vw)	193 (vwsh)	180	140 (sh)	158 (vwsh)	157 (vwsh)	
I_c		214 (vwsh)	214 (vwsh)	214 (vwsh)	214 (vwsh)		217 (vwsh)	216 (vwsh)	217 (vwsh)				
II_a	252	249	252	249	255	255	254	255	254	222	218	218	ν[Fe–N(pyrrole)]
II_b	270	272 (sh)	270 (sh)	271 (sh)	270 (sh)	271 (sh)				243 (sh)			
III	308	308	309	306	307	306	311	309	306	305	305	306	
IV	347	346	347	341	341	347	342	347	347	345	344	344	heme in-plane deformation
V	378	378	378	349 (sh)	354 (sh)								
VI	416	411	412	377	378	379	379	384	378	373	373	374	heme in-plane deformation
VII	446	442	442	414	413	413	411	413	413	408	409	409	ν[Fe–N_ϵ(histidine)]
VIII	502	503	502	442	441	442	441	441	444	442	438	438	
IX	556	552	552	505	505	503	508	501	505	501	502	500	heme deformation + [Fe–N(pyrrole)]?
X	679	678	678	555	555	556	554	553	554	551	552	552	
lsb	462			679	680	678	677	679	677	675	680	680	heme deformation
						490	570				577		ν(Fe–sixth ligand)

[a] Excitation wavelength is 441.6 nm; experimental conditions are as in Figure 1; determination is from single scans for bands I; for bands from II to X, determination is from six accumulated scans; sh represents shoulder; vw represents very weak; lsb represents ligand-specific band.

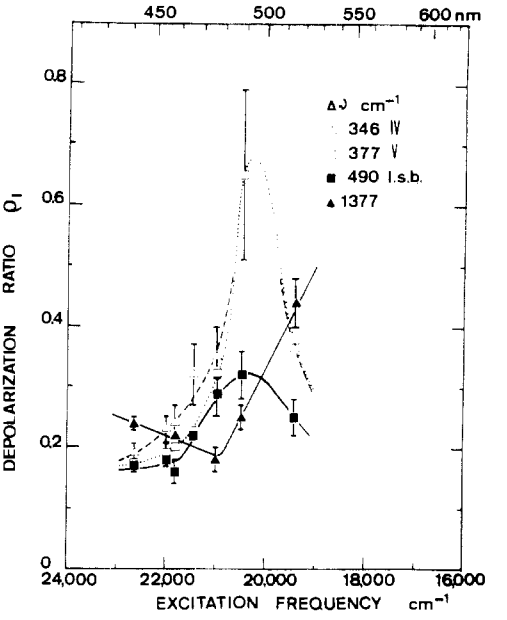

FIGURE 7: Variation with excitation frequency of depolarization ratios of the main low-frequency lines of the RR spectrum of hydroxymetmyoglobin. Determination from accumulated spectra.

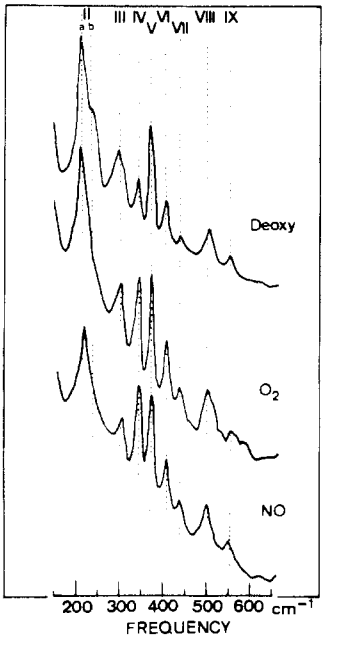

FIGURE 8: Resonance Raman spectra of reduced myoglobin derivatives. Excitation wavelength is 441.6 nm; same conditions as in Figure 1.

exhibited by the metmyoglobin derivatives (Figure 8 and Table I). However, the bands of region II have markedly different frequencies while bands III, V, and VI have slightly different positions. An additional weak line is present in the spectrum of oxymyoglobin at 577 cm^{-1}. Isotopic substitution of the iron of deoxymyoglobin induced a single line, at 501 cm^{-1}, to shift down by 1.5 cm^{-1}.

At 441.6 nm, R_1 has the value 2.85 for deoxymyoglobin, 1.20 for oxymyoglobin, and 1.00 for nitrosylmyoglobin. It thus appears qualitatively that the same correlation exists between R_1 and the spin state of the reduced derivatives.

Discussion

Ligand-Specific Bands

Isotopic substitutions in hydroxymetmyoglobin and azidometmyoglobin show that the specific lines at 490 and 570 cm^{-1}, respectively, involve displacements of the external ligand

Table II: Depolarization Ratios (ρ_l) of the Main Low-Frequency Bands in the Resonance Raman Spectra of Fluorometmyoglobin, Hydroxymetmyoglobin, and Cyanometmyoglobin[a]

	band										
	II										
derivative	a	b	III	IV	V	VI	VII	VIII	IX	X	lsb
Mb^+F^-	0.12	0.16	0.39	0.19	0.18			0.24	0.39	0.19	0.16
Mb^+OH^-	0.17	0.14		0.19	0.17	0.35	0.23		0.32	0.17	0.17
Mb^+CN^-	0.20			0.23	0.23	0.26	0.32	0.21	0.28	0.26	

[a] Excitation wavelength is 441.6 nm; determination is from accumulated spectra; lsb represents ligand-specific band.

Table III: Lines Sensitive to Isotopic Substitution of Ligands or of the Iron Atom in the Resonance Raman Spectra of Some Myoglobin Derivatives[a]

derivative MbL	line (cm^{-1})	type of line	isotopic substitution	exptl shift, $\Delta\nu_{exptl}$ (cm^{-1})	theoretical shift for ν(Fe-L), $\Delta\nu_{calcd}$ (cm^{-1})
Mb^+F^-	251	II_a	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1	
	462	lsb	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+2	+2 ν(Fe-F^-)
Mb^+H_2O	249	II_a	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1	
	411	VI	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1.5	
Mb^+OH^-	413	VI	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1	
	490	lsb	$^{16}OH^- \rightarrow {}^{18}OH^-$	−19	−22 ν(Fe-OH^-)
			$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+2	+2 ν(Fe-OH^-)
$Mb^+N_3^-$	254	II_a	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1	
	411	VI	$^{14}N_3^- \rightarrow {}^{15}N_3^-$	−1.5	
			$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1.5	
	508	VIII	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+2	
	570	lsb	$^{14}N_3^- \rightarrow {}^{15}N_3^-$	−16	−16 ν(Fe-N_3^-)
			$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+2	+2 ν(Fe-N_3^-)
Mb^+CN^-	413	VI	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1	
	505	VIII	$^{56}Fe^{III} \rightarrow {}^{54}Fe^{III}$	+1.5	
Mb	501	VIII	$^{56}Fe^{II} \rightarrow {}^{54}Fe^{II}$	+1.5	

[a] Excitation wavelength is 441.6 nm; same experimental conditions as in Figure 1; for these experiments, 10 scans are accumulated for each isotopic species; lsb represents ligand-specific band; L represents exogenous sixth ligand.

and of the iron atom (Figure 4 and Table III). The displacements calculated by assuming a stretching vibration of a diatomic oscillator ($\Delta\nu_{calcd}$) are close to the experimental values ($\Delta\nu_{exptl}$) (Table III). From these results, we may assign bands at 490 and 570 cm^{-1} to stretching vibrations $\nu(Fe^{III}-O)$ and $\nu(Fe^{III}-N)$ of hydroxymetmyoglobin and azidometmyoglobin, respectively. A line at 462 cm^{-1} is characteristic of fluorometmyoglobin. This line is iron sensitive (Table III) and may thus be assigned to the stretching vibration $\nu(Fe^{III}-F)$.

These assignments are in agreement with those made by Asher et al. (1977) using an excitation range from 600 to 640 nm, except for azidometmyoglobin. These authors assigned a band at 497 cm^{-1} to a $\nu(Fe^{III}-O)$ mode in hydroxymethemoglobin, a band at 413 cm^{-1} to $\nu(Fe^{III}-N)$ in azidomethemoglobin, and a doublet at 443 and 471 cm^{-1} to $\nu(Fe^{III}-F)$ in fluoromethemoglobin. Our results show that the vibration at 413 cm^{-1} is unspecific of the exogenous ligand (band VI in Figure 1 and Table I).

Bands in far-infrared and in RR spectra of different fluoride–iron porphyrin complexes occurring between 579 and 606 cm^{-1} were previously assigned to stretching vibrations $\nu(Fe^{III}-F)$ (Ogoshi et al., 1973; Kincaid & Nakamoto, 1973; Spiro & Burke, 1976). On the other hand, the frequencies of this vibration are 471 cm^{-1} (Asher et al., 1977) and 462 cm^{-1} (this work) for hemoglobin and for myoglobin, respectively. This discrepancy between both sets of measures may be explained simply. The fluoride–iron porphyrin complexes are five coordinated and the iron atom is out of the plane of the porphyrin toward the fifth ligand (Figure 9e). In fluorohemoproteins, the iron atom is also high spin but is six coordinated and is displaced toward the nitrogen atom (N_ϵ) of the proximal histidine (Figure 9b). These differences may well account for the discrepancy of Raman frequencies of Fe–F

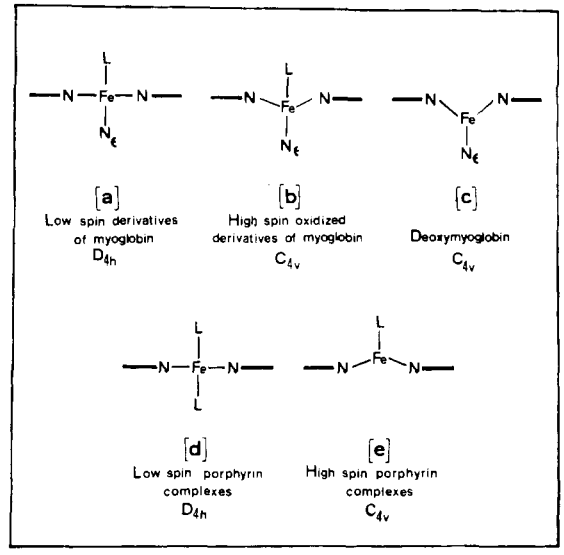

FIGURE 9: Conformations and local symmetries around the iron atom in myoglobin (a, b, and c) and for porphyrin complexes (d and e). Schematic representation viewed parallel to the plane of the macrocycle; L represents exogenous sixth ligand; N_ϵ represents histidine nitrogen.

bonds in free porphyrins and hemoproteins (Asher et al., 1977). Consequently, the high-spin oxidized complexes of free porphyrins constitute inadequate models for the simulation of sites in hemoproteins.

Except aquometmyoglobin, ligand-specific lines are present in the RR spectra of derivatives with significant high-spin ratios, i.e., fluorometmyoglobin (about 100%), hydroxymetmyoglobin (70%), and azidometmyoglobin (25%). The weakness of the ligand-specific band of azidometmyoglobin

(570 cm^{-1}) may be correlated with its low ratio. Moreover, no ligand-specific line is present for the essentially low-spin derivatives imidazolo- and cyanometmyoglobin. The fact that the low-spin forms are centrosymmetrical (Figure 9a) and the high-spin forms have no symmetry center (Figure 9b) may account for this difference through the concomitant differences in their electronic absorption spectra.

A specific line at 577 cm^{-1} characterizes the reduced derivative oxymyoglobin. This frequency is close to the 567-cm^{-1} value assigned to $\nu(Fe^{II}-O)$ by Brunner (1974) in RR spectra of oxyhemoglobin. Chottard & Mansuy (1977) assigned a band at 549 cm^{-1} to ν(Fe–NO) in RR spectra of nitrosylhemoglobin excited at 541.5 nm. This line had significant activity with 454.5-nm excitation. However, we did not find any specific band with 441.6- and 454.5-nm excitation on nitrosylmyoglobin. Moreover, a line is present at about 552 cm^{-1} (band IX) in the spectra of all the reduced derivatives.

Lines Sensitive to Mass, Oxidation, or Spin State of the Iron Atom

Bands II. Bands II are spin sensitive and are upshifted upon heme reduction. Moreover, components II_a of the high-spin oxidized derivatives fluoro- and aquometmyoglobin and of azidometmyoglobin are iron sensitive.

According to Ogoshi et al. (1973), the Fe–N stretching coordinate predominates in two vibrations observed between 250 and 280 cm^{-1} in the far-infrared spectra of high-spin ferric octaethylporphyrin complexes Fe(OEP)L (L = F^-, Cl^-, Br^-, I^-, and N_3^-). Two Fe–N stretching modes indeed are expected to be infrared active (A_1 and E) under C_{4v} symmetry (Figure 9e). For the D_{4h} low-spin complexes of ferric octaethylporphyrin Fe(OEP)L_2 (Figure 9d), only one Fe–N stretching mode was assignable (Ogoshi et al., 1973).

RR spectra of the low-spin complexes protohemin and protohemin–imidazole obtained at 441.6 nm each showed a line in this region at 255 and 276 cm^{-1}, respectively (Verma & Bernstein, 1974a). With the same excitation wavelength, RR spectra of high-spin oxidized derivatives of myoglobin gave two bands, II_a and II_b, between 250 and 280 cm^{-1}. Only one band was present in this region for the essentially low-spin compounds. Although two Fe–N stretching modes (A_1 and E) are also expected to be Raman active under C_{4v} symmetry, the activity of a E mode in Soret preresonance conditions remains difficult to explain.

The frequencies of bands II_a of all high-spin oxidized derivatives fall between 249 and 252 cm^{-1}. The frequencies of bands II of low-spin oxidized compounds are significantly higher, falling between 254 and 255 cm^{-1}. On the contrary, for the reduced compounds, the frequencies of bands II_a are lower for the low-spin derivatives (218 cm^{-1}) than that for the high-spin derivative (222 cm^{-1} for deoxymyoglobin). These slight frequency shifts might be correlated with the differences in length of Fe–N(pyrrole) bonds existing between high-spin and low-spin forms (Hoard, 1975; Perutz, 1970). The lengths and, consequently, the stretching vibrations of Fe–N(pyrrole) bonds are governed by several factors such as the oxidation state and the coordination number of the metal. According to Clark (1965) and as shown in Table IV, an increase in the oxidation number results in an increase of the frequency of bands II_a, while an increase in the coordination number tends to decrease this frequency. The coordination number appears to be preponderant compared to the oxidation and spin state.

All these observations lead us to think that bands II_a are Fe–N(pyrrole) stretching modes. Bands II_b might be nonpure modes, which should explain their apparent insensitivity to iron substitution. Indeed, Warshel (1977) predicted deformation modes of the heme in this region.

Table IV: Effects of Spin State, Coordination Number, and Oxidation Number of Myoglobin Derivatives upon the Raman Frequencies of Bands II

spin state of myoglobin derivative	coordination no.	oxidation no.	frequency (cm^{-1})	
			II_a	II_b
high-spin ferric	6	3	249–252	270–272
low-spin ferric	6	3	254–255	
high-spin ferrous	5	2	222	243
low-spin ferrous	6	2	218	

Bands IV and V. Bands IV and V are slightly sensitive to the oxidation state of the iron atom but not to its mass. The ratio R_1 of their relative intensities constitutes an indicator of the spin state of the derivatives (Figure 3).

Lines are present at the same frequencies (about 350 and 380 cm^{-1}) in far-infrared spectra of metalloprotoporphyrins. The intensity of the 350-cm^{-1} infrared line, relative to that of the 380-cm^{-1} line, is metal sensitive (Boucher & Katz, 1967). In infrared spectra of metal-free protoporphyrin, likely homologous bands are found at 360 and 382 cm^{-1}, respectively (Boucher & Katz, 1967). Homologous bands are also present in RR spectra of protohemin complexes and various porphyrins (Verma & Bernstein, 1974a,b; Spaulding et al., 1975; Spiro & Burke, 1976; Kitagawa et al., 1976).

These facts suggest an origin of these bands in in-plane deformation modes of the macrocycle. Indeed, Warshel (1977) predicted that heme expansion should result in a very strong RR band at 360 cm^{-1}, a value intermediate between the frequencies of bands IV and V.

Band VI. Band VI occurs at 412 and 409 cm^{-1} for oxidized and for reduced derivatives, respectively, and is sensitive to the oxidation state of the iron atom. This line is also sensitive to the mass of iron for the oxidized derivatives. No homologous bands are found at these frequencies, neither in the infrared spectra of metalloporphyrins (Boucher & Katz, 1967) nor in the RR spectra of iron mesoporphyrin complexes (Spiro & Burke, 1976). However, weak lines are present at 415 and 417 cm^{-1} in the RR spectra obtained at 441.6 nm from protohemin and protohemin–imidazole complexes, respectively (Verma & Bernstein, 1974a). RR spectra of cytochromes *c* and c_3 and cytochrome *c* oxidase also contain a line at the same frequency as band VI (Kitagawa et al., 1975; Salmeen et al., 1978). It is interesting that the iron of myoglobin, of *c*-type cytochromes, and of cytochrome *c* oxidase binds a nitrogen atom of a histidyl residue as a fifth ligand.

These observations lead us to tentatively assign bands VI to the stretching vibrations ν[Fe–N_ϵ(proximal histidine], although such a mode may not necessarily constitute a group frequency with respect to the stretching Fe–N(pyrrole) modes.

Band VIII. Band VIII is a broad band which is sensitive to iron mass for azidometmyoglobin, cyanometmyoglobin, and deoxymyoglobin only (Table III). A dependence on the oxidation state of the iron atom is also observed. An homologous band was found in infrared spectra of metalloporphyrins (Boucher & Katz, 1967). No line occurs at the frequency of band VIII in the RR spectra of protohemin and protohemin–imidazole complexes, but weak bands were recorded at 488 and 536 cm^{-1} with excitation at 441.6 nm (Verma & Bernstein, 1974a; Spaulding et al., 1975). Weak bands are also present at 481 and 526 cm^{-1} in RR spectra of nickel octaethylporphyrin (Spaulding et al., 1975). Bands were found at 515 and 518 cm^{-1} in the RR spectra of reduced cytochrome *f* and of reduced cytochrome *c*, respectively (Kitagawa et al., 1975).

Finally, bands VIII are broad in RR spectra of myoglobin and might correspond to at least two modes which are resolved in the RR spectra of porphyrins. We propose that these modes contain both porphyrinic and Fe–N(pyrrole) vibrations.

Band X. Band X of RR spectra of myoglobin derivatives is not sensitive to iron mass. From Figure 3, the relative intensities of bands X seem to be constant except for fluorometmyoglobin where band X is weak (Figures 1 and 3).

Homologous bands were found in all the RR spectra of hemoproteins (Salmeen et al., 1973; Kitagawa et al., 1975; Rimai et al., 1975; Asher et al., 1977; Chottard & Mansuy, 1977; Salmeen et al., 1978), of metal-free porphyrins (Verma & Bernstein, 1974b) and of metalloporphyrins (Verma & Bernstein, 1974a; Spaulding et al., 1975; Spiro & Burke, 1976; Kitagawa et al., 1976). The intensity of bands X appears to constitute a good indicator of the doming of the heme in various metallooctaethylporphyrins (Kitagawa et al., 1976). Indeed, band X is strong for planar complexes. On the contrary, high-spin complexes which are domed show weaker lines near 674 cm^{-1} (Kitagawa et al., 1976). The weakening observed for band X of fluorometmyoglobin which is essentially high spin shows the same analogy. Band X was assigned to a breathing vibrations of the macrocycle (Kitagawa et al., 1976).

Origin of the Resonance Enhancement of the Main Low-Frequency Bands

Up to 14 bands are resonance-enhanced in the 100–700-cm^{-1} range for the derivatives of oxidized myoglobin. At least 11 of these bands are certainly polarized at 441.6 nm and thus arise from totally symmetric modes of the heme. This number appears high compared to the total number of 19 A_1 modes expected for the heme if we assume C_{4v} symmetry and assume identical substituents on C_b atoms. This suggests that the effective symmetry of the heme should be considered lower than C_{4v} when lower intensity bands are considered. However, four polarized bands, II_a, VI, VIII, and ligand-specific, are attributable to totally symmetric modes of the $Fe(N)_4N_\epsilon L$ grouping, in agreement with the number of four A_1 vibrations expected in C_{4v} symmetry.

In RR spectra of both fluorometmyoglobin and hydroxymetmyoglobin excited near the Soret band, the intensities of bands II–VI as well as of the ligand-specific band increase nearly parallel to the intensity of the skeletal band at 1377 cm^{-1}, which clearly derives mostly from a A-type resonance with the Soret band (Strekas et al., 1973) (Figures 5 and 6). The low-frequency bands thus also primarily arise in this region from A-type resonances with the Soret band. Their depolarization ratios are much lower than 0.33, suggesting that the Soret band remains nearly degenerate.

Interestingly enough, the z-polarized ν(Fe–L) modes, giving rise to the ligand-specific bands, behave with respect to the x,y polarized Soret bands exactly as do the x,y polarized, $\nu(C{=}N) + \nu(C{=}C)$ modes (Lapidot & Irving, 1976; Lutz et al., 1976; Kitagawa et al., 1977) which give rise to the 1377-cm^{-1} bands. Thus, it appears that the symmetry type of the modes, here A_1, is a more important parameter in the resonance process than are the relative orientations of the nuclear displacements and of the electronic transition moments, as first suggested by Kitagawa et al. (1976) for iron octaethylporphyrin.

The excitation profiles of the 1377-cm^{-1} bands are essentially monotonous from 515 to 441 nm. The profile of hydroxymetmyoglobin exhibits a shoulder at 496 nm. This latter shoulder may arise from a weak additional resonance involving the corresponding 0–1 vibronic level of the β band (Shelnutt et al., 1976). The position of the 0–0 level calculated on this basis is 532 nm, a reasonable value. The profiles of the low-frequency bands depart from those of the 1377-cm^{-1} bands for wavelengths longer than 470 nm, presenting deep troughs near 490 nm, at the level of electronic band IV. This discrepancy most probably cannot be ascribed merely to the fact that the relevant 0–1 transitions of the β band are expected near 518 nm instead of near 496 nm. Indeed, the depolarization ratios of the low-frequency bands of hydroxymetmyoglobin also behave differently than that of the 1377-cm^{-1} band, reaching maxima at the level of the intensity minima. Moreover, all the excitation profiles of these bands show shoulders at 460 nm, indicative of another resonance process likely involving an electronic transition present as a weak shoulder near 458 nm in the absorption spectra of hydroxymetmyoglobin and of fluorometmyoglobin, and perhaps more clearly observed in their magnetic circular dichroism spectra (Vickery et al., 1976).

The ligand-specific RR bands are specifically observed for derivatives with high-spin or mixed-spin character, which should have charge-transfer transitions in their visible absorption spectra. Such transitions should occur in the blue region, but their positions were controversial, being fixed either at 490 nm (Brill & Williams, 1961) or at 539 nm (Eaton & Hochstrasser, 1968). Although not necessarily z polarized (Eaton & Hochstrasser, 1968), these transitions should more particularly promote resonance of modes involving the Fe–L grouping (Asher et al., 1977).

The present Raman data suggest that a charge-transfer transition should be present in both fluorometmyoglobin and hydroxymetmyoglobin either at 460 nm, where an additional resonance process occurs, or at 490 nm, as suggested by Brill & Williams (1961). In both hypotheses, a destructive interference between A-type Soret resonance and A-type charge-transfer resonance should be responsible for the 490-nm trough in the excitation profile (Matsuzaki & Maeda, 1974; Stein et al., 1976).

Electron Distribution in Oxymyoglobin

Controversies have developed about the oxidation state of the iron atom in oxyhemoproteins since Weiss' suggestion (1964) of a superoxide complex (Fe^{III}–O_2^-) involving low-spin Fe^{III}. Arguments in favor of this suggestion have been forwarded from the examination of the high-frequency region of the RR spectra (Yamamoto et al., 1973; Spiro, 1975). However, the low-frequency region of these spectra, as recorded in this study, does not support this hypothesis. Indeed, the frequencies of bands II and IV–VI of this region of the RR spectra of oxymyoglobin classify this compound as having a valence of II (Fe^{II}–O_2).

On the other hand, the activity of the stretching vibrations of the Fe–O bond has been demonstrated previously by use of 488-nm excitation for oxyhemoglobin (Brunner, 1974) and 441.6-nm excitation in this work for oxymyoglobin. These activities should be particularly sensitive to charge-transfer transitions (Asher et al., 1977). Evidences for unresolved transitions between 440 and 500 nm in the optical spectra of oxymyoglobin have come from magnetic circular dichroism spectra (Vickery et al., 1976) and from the polarized absorption spectra of a single crystal of oxyhemoglobin (Makinen & Eaton, 1973). We note that, in the conditions utilized in this study, no Fe–exogenous ligand vibration is detectable in essentially low-spin ferric forms. Surprisingly enough, the Fe–O bond vibration is active in our spectrum; moreover, the ratio of the intensities of bands V to IV (R_1), which classed the derivatives in order of high- to low-spin ratios (R_1 = 2.85

for Mb and R_1 = 1.00 for MbNO), gave for oxymyoglobin a number (R_1 = 1.20) intermediate between those for deoxy- and nitrosylmyoglobin. These facts would tend to classify oxymyoglobin as being not purely low spin. It is of interest to note that a recent X-ray crystallographic work (Phillips, 1978) has shown that the iron atom in sperm whale oxymyoglobin lies considerably out of the heme plane (by 0.33 Å). Although performed on oxyhemoglobin, the magnetic susceptibility measurements of Cerdonio et al. (1977) may be relevant to this point.

The present study thus demonstrates that a least four low-frequency bands of the RR spectra of myoglobin derivatives excited in the blue region involve modes from the $Fe(N)_4N_\epsilon L$ grouping and hence are directly sensitive to the coordination number, oxidation state, and spin state of the iron atom. Conclusions drawn about these parameters from consideration of low-frequency bands thus appear more precise and more reliable than those previously drawn on high-frequency skeletal RR bands which can only be indirectly sensitive to the state of the iron atom. The RR spectra in the low-frequency region is thus likely to be a sensitive tool for the study of minute variations of heme geometry and electronic structure believed to modulate the reaction of hemoproteins with their ligands.

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References

Asher, S. A., Vickery, L. E., Schuster, T. M., & Sauer, K. (1977) *Biochemistry 16*, 5849–5856.

Beetlestone, J., & George, P. (1964) *Biochemistry 3*, 707–714.

Boucher, L. J., & Katz, J. J. (1967) *J. Am. Chem. Soc. 89*, 1340–1345.

Brill, A. S., & Williams, R. J. P. (1961) *Biochem. J. 78*, 246–253.

Brunner, H. (1974) *Naturwissenschaften 61*, 129–130.

Brunner, H., & Sussner, H. (1973) *Biochim. Biophys. Acta 310*, 20–31.

Cerdonio, M., Congiu-Castellano, A., Mogno, F., Pispisa, B., Romani, G. L., & Vitale, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 398–400.

Champion, P. M., Gunsalus, I. C., & Wagner, G. C. (1978) *J. Am. Chem. Soc. 100*, 3743–3751.

Chottard, G., & Mansuy, D. (1977) *Biochem. Biophys. Res. Commun. 77*, 1333–1338.

Clark, R. J. H. (1965) *Spectrochim. Acta 21*, 955–963.

Eaton, W. A., & Hochstrasser, R. M. (1968) *J. Chem. Phys. 49*, 985–995.

George, P., & Irvine, D. H. (1952) *Biochem. J. 52*, 511–517.

Hoard, J. L. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., Ed.) pp 317–380, Elsevier, Amsterdam.

Kincaid, J., & Nakamoto, K. (1976) *Spectrosc. Lett. 9*, 19.

Kitagawa, T., Kyogoku, Y., Iizuka, T., Ikeda-Saito, M., & Yamanaka, T. (1975) *J. Biochem. 78*, 719–728.

Kitagawa, T., Abe, M., Kyogoku, Y., Ogoshi, H., Watanabe, E., & Yoshida, Z. (1976) *J. Phys. Chem. 80*, 1181–1186.

Kitagawa, T., Abe, M., Kyogoku, Y., Ogoshi, H., Sugimoto, H., & Yoshida, Z. (1977) *Chem. Phys. Lett. 48*, 55–58.

Lapidot, A., & Irving, C. S. (1976) *Proc. Int. Conf. Stable Isot., 2nd 1975*, 427–444.

Lutz, M., Klēo, J., Gilet, R., Henry, M., Plus, R., & Leicknam, J. P. (1976) *Proc. Int. Conf. Stable Isot., 2nd 1975*, 462–469.

Makinen, W., & Eaton, W. A. (1973) *Ann. N.Y. Acad. Sci. 206*, 210–222.

Matsuzaki, S., & Maeda, S. (1974) *Chem. Phys. Lett. 28*, 27–30.

Ogoshi, H., Saito, Y., & Nakamoto, K. (1972) *J. Chem. Phys. 57*, 4194–4202.

Ogoshi, H., Watanabe, E., Yoshida, Z., Kincaid, J., & Nakamoto, K. (1973) *J. Am. Chem. Soc. 95*, 2845–2849.

Perutz, M. F. (1970) *Nature (London) 228*, 726–734.

Phillips, S. E. V. (1978) *Nature (London) 273*, 247–248.

Rimai, L., Salmeen, I., & Petering, D. H. (1975) *Biochemistry 14*, 378–381.

Rossi-Fanelli, A., Antonini, E., & Caputo, A. (1958) *Biochim. Biophys. Acta 30*, 608–615.

Salmeen, I., Rimai, L., Gill, D., Yamamoto, T., Palmer, G., & Hartzell, C. R. (1973) *Biochem. Biophys. Res. Commun. 52*, 1100–1107.

Salmeen, I., Rimai, L., & Babcock, G. (1978) *Biochemistry 17*, 800–806.

Shelnutt, J. A., O'Shea, D. C., Yu, N., Cheung, L. D., & Felton, R. H. (1976) *J. Chem. Phys. 64*, 1156–1165.

Spaulding, L. D., Chang, C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517–2525.

Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169–189.

Spiro, T. G., & Strekas, T. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2622–2626.

Spiro, T. G., & Strekas, T. C. (1974) *J. Am. Chem. Soc. 96*, 338–345.

Spiro, T. G., & Burke, J. M. (1976) *J. Am. Chem. Soc. 98*, 5482–5489.

Stein, P., Miskowski, V., Woodruff, W. H., Griffin, J. P., Werner, K. G., Gaber, B. P., & Spiro, T. G. (1976) *J. Chem. Phys. 64*, 2159–2167.

Strekas, T. C., Packer, A. J., & Spiro, T. G. (1973) *J. Raman Spectrosc. 1*, 197–206.

Tamura, M., Asakura, T., & Yonetani, T. (1973) *Biochim. Biophys. Acta 295*, 467–479.

Verma, A. L., & Bernstein, H. J. (1974a) *J. Raman Spectrosc. 2*, 163–173.

Verma, A. L., & Bernstein, H. J. (1974b) *Biochem. Biophys. Res. Commun. 57*, 255–262.

Vickery, L., Nozawa, T., & Sauer, K. (1976) *J. Am. Chem. Soc. 98*, 343–350.

Warshel, A. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 273–300.

Weiss, J. J. (1964) *Nature (London) 202*, 83–84.

Yamamoto, T., Palmer, G., Gill, D., Salmeen, I. T., & Rimai, L. (1973) *J. Biol. Chem. 248*, 5211–5213.

Yonetani, T., & Asakura, T. (1968) *J. Biol. Chem. 243*, 4715–4721.