Resonance Raman Spectra of Heme Proteins. Effects of Oxidation and Spin State[1]

Thomas G. Spiro* and Thomas C. Strekas

Contribution from the Chemistry Department, Princeton University, Princeton, New Jersey 08540. *Received June 23, 1973*

Abstract: Resonance Raman frequencies are reported for the following heme proteins: ferri- and ferrocytochrome c, oxy- and deoxyhemoglobin, and fluoro- and cyanomethemoglobin. Laser excitation in the Soret and in the α–β band regions brings out different sets of Raman bands *via* different resonance scattering mechanisms. This discrimination, plus the observation of modes with antisymmetric scattering tensors, which are forbidden in nonresonance Raman scattering, permits the acquisition of an unusually complete set of vibrational frequencies and leads to reliable correlations among the various heme Raman spectra. Approximate assignments are suggested for the high-frequency (1000–1650 cm^{-1}) porphyrin ring modes. Reduction of Fe(III) to Fe(II) leads to a small general lowering of these frequencies, while much larger decreases accompany conversion from low- to high-spin state. The former trend can be understood in terms of changes in π back donation, while the latter trend is attributed to structural changes accompanying the spin state change. Six bands are identified which offer promise as oxidation and/or spin state markers. They classify both oxy- and carbonmonoxyhemoglobin as low-spin Fe(III) hemes. Apparently π back donation to either O_2 or CO leaves the iron atom with about the same charge as in low-spin Fe(III) hemes.

Resonance Raman spectroscopy offers promise as a structure probe for biological molecues. Laser excitation within an electronic absorption band can produce large Raman enhancements for vibrational modes of the chromophore. Resonance enhanced spectra have been reported recently for retinal,[2a] carotenes,[2b] a photoreceptor-like pigment,[3] chlorophyll,[4] rubredoxin,[5] adrenodoxin,[6] vitamin B_{12},[7] hemoglobin,[8–10] and cytochrome c.[10–12] Particular interest attaches to the heme proteins in view of the high quality Raman spectra obtainable for dilute solutions (10^{-3}–10^{-6} *M*) and their potential for monitoring heme structural changes which may be associated with oxygen binding or electron transfer. Preliminary Raman spectra reported from different laboratories[8–12] differ in detail. These differences can be traced to the different laser wavelengths used for excitation. Considerable progress has been made in understanding the scattering mechanisms which determine the form of the resonance Raman spectra of heme chromophores and which selectively enhance different vibrational modes at different wavelengths.[13–15] We present here results of a systematic study of these spectra, encompassing heme proteins containing iron(II) and iron(III) in both high- and low-spin states.

Experimental Section

Protein Samples. Hemoglobin was prepared from human whole blood as the oxy or CO derivative by a modification of the Drabkin[16] procedure. Heme concentration was determined by the pyridine hemochrome method[17] for stock solutions. Ferric hemoglobin was prepared by oxidation with 10–20% molar excess of potassium ferricyanide and dialyzed against distilled water for 24–36 hr with several changes of water. Concentration of heme was determined by the pyridine hemochrome method and as the aquomet derivative[18] at pH 5.8 in phosphate buffer. Methemoglobin fluoride and cyanide were prepared by addition of NaF or NaCN until no further change in the visible spectrum was observed. Solutions used in Raman experiments which required an internal intensity reference, 0.4–0.8 *M* $(NH_4)_2SO_3$, were checked for changes in either Raman or absorption spectra. No significant differences were observed. Deoxyhemoglobin was prepared from oxyhemoglobin by addition of solid sodium dithionite or by equilibrating with Ar gas. Complete conversion was determined spectrophotometrically.[19] Cytochrome c, Sigma type III (horse heart), was used as purchased. The reduced and oxidized states were obtained by treatment with dithionite and ferricyanide, respectively, and checked in the visible absorption region[20] for complete conversion.

Spectra. Raman spectra were run in 1-mm diameter glass capillary cells or in a rotating cell. Typical concentrations ranged from 0.5 to 0.1 m*M* in heme. The spectrometer is based on a Spex 1401 double monochromator with a cooled ITT FW 130 phototube. A Coherent Radiation Model 52 argon ion laser was used for excitation. Spectra obtained at 5145, 4965, and 4579 Å exciting lines are reported here. All spectra were recorded with transverse excitation, polarized perpendicular to the scattering direction. A polarization scrambler was fixed in the scattering path at all times. A polaroid disk placed between the sample and scrambler was used to obtain polarization measurements.

(1) This work was supported by Public Health Service Grants HL-12526 and GM-13498.

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Table I. Resonance Raman Frequencies of Heme Proteins[a]

Ferrocytochrome c	Ferricytochrome c	Oxyhemoglobin	Cyanomethemoglobin	Fluoromethemoglobin	Deoxyhemoglobin
1620 w, dp	1630 vs, dp	1640 vs, dp	1642 vs, dp	1608 vs, dp	1607 s, dp
		1620 m, p	1625 m, p	1623 m, p	1622 m, p
		1606 w, p	1605 w, p	1586 m, p	1590 w, p
1584 s, ap	1582 s, ap	1586 s, ap	1588 s, ap	1555 s, ap	1552 s, ap
1594 m, p	1582 s, p	1583 m, p	1583 m, p	1565 s, p	1565 m, p
1548 m, dp	1562 s, dp	1564 m, dp	1564 m, dp	1565 s, dp	1546 m, dp
		1552 m, dp	1552 w, dp		1557 m, dp
1483 m, p	1502 m, p	1506 m, p	1508 m, p	1482 w, p	1473 m, p
		1431 w, dp	1432 w, dp	1428 m, dp	1427 w, dp
1399 m, ap	1412 m, ap	1400 w, dp	1400 w, dp	1395 m, dp	1397 w, dp
1362 vs, p	1374 vs, p	1377 s, p	1374 vs, p	1373 vs, p	1358 vs, p
		1342 m, ap	1345 w, ap	1340 m, ap	1338 m, ap
1312 vs, ap	1316 w, ap	1305 w, ap	1308 w, ap	1309 w, ap	1305 w, ap
1230 m, dp	1250 w, dp	1225 w, dp		1220 vw	1215 w, dp
1174 m, dp	1170 w, dp	1173 m, dp	1172 w, dp	1175 m	1176 w, dp
1129 w, ap	1127 w, ap	1133 w, ap	1137 vw, ap	1135 vw, ap	1137 w, ap
		1096 vw			
		1088 vw			
752 m, dp		755 w, dp	763 w, dp	760 w, dp	760 w, dp
692 m, p		678 w, p	674 w, p		675 w, p

[a] Symbols: s = strong, m = medium, w = weak, v = very, p = polarized, dp = depolarized, ap = anomalously polarized. Relative intensities are for 4579-Å (Soret region) excitation for polarized bands and for 5145-Å (α–β region) excitation for depolarized and anomalously polarized bands.

Results

The Raman data obtained in this study are summarized in Table I, which lists observed vibrational frequencies for ferri- and ferrocytochrome c, oxy- and deoxyhemoglobin, and methemoglobin cyanide and fluoride. This group of proteins offers representatives of heme groups in both Fe(II) and Fe(III) oxidation states and in both low- and high-spin states.[19] The data below 650 cm^{-1} are incomplete. Many weaker bands occur in this region and are currently under study. Other hemoglobin derivatives examined include carbonmonoxyhemoglobin, which gives the same spectra as oxyhemoglobin, methemoglobin azide, which gives the same spectra as the cyanide, and aquo- and hydroxymethemoglobin, which, being spin state mixtures,[19] give spectra which display both high-spin (fluoride) and low-spin (cyanide) bands (*vide infra*). Myoglobin derivatives have also been examined and found to give the same spectra as their hemoglobin analogs except for anticipated spin state differences.[19] By "same spectra" we mean here that major bands, the ones listed in Table I, occur at the same frequencies, with similar relative intensities. The resonance Raman spectra contain suggestions of many weak features, some of which may bear further study. In particular, weak bands in the low-frequency region show some differences among otherwise similar proteins and are currently under investigation. Also, we have begun a study of protein-free metalloporphyrins. Preliminary results show no inconsistencies with the data and interpretations offered here.

Table I also gives qualitative intensities for the Raman bands and their states of polarization: (p) polarized, $\rho_l < 3/4$; (dp) depolarized, $\rho_l = 3/4$; and (ap) anomalously polarized, $\rho_l > 3/4$. Here ρ_l, the depolarization ratio for linearly polarized light scattered at 90°, is the ratio of scattered intensity perpendicular and parallel to the incident polarization. Anomalous polarization is diagnostic for *antisymmetric* scattering,[13,21,22] which is allowed only in the resonance region (*vide infra*). In assembling the data for Table I, it was necessary to gather spectra at different exciting wavelengths for each protein, inasmuch as different vibrational modes come into resonance at different wavelengths, as discussed below. The effect is demonstrated in Figure 1, which compares spectra obtained for oxy- and deoxyhemoglobin at 5145- and 4579-Å excitation. Examination of different scattering regions is also useful in resolving overlapping vibrational modes of different symmetries. Figure 2 plots the data of Table I in a correlation diagram, which is useful in comparing corresponding vibrational modes for the different proteins.

Discussion

Scattering Mechanisms. When Raman spectra are excited in the region of allowed electronic absorption bands, the vibrational modes which are expected to show enhancement are the same ones which lend intensity to the electronic spectrum; *i.e.*, they are vibronically active modes.[21–26] These may be of two varieties: (A) modes which connect the ground state to the excited state involved in resonance through the Frank–Condon overlap, or which change the energy of the resonant excited state; and (B) modes which mix the resonant electronic transition to another one of higher energy. Type A vibrational modes are expected to be totally symmetric, while type B modes may have any symmetry which is contained in the direct product of the two electronic transition representations.

This theoretical framework serves admirably to explain the resonance Raman scattering observed for heme proteins. The visible and near ultraviolet ab-

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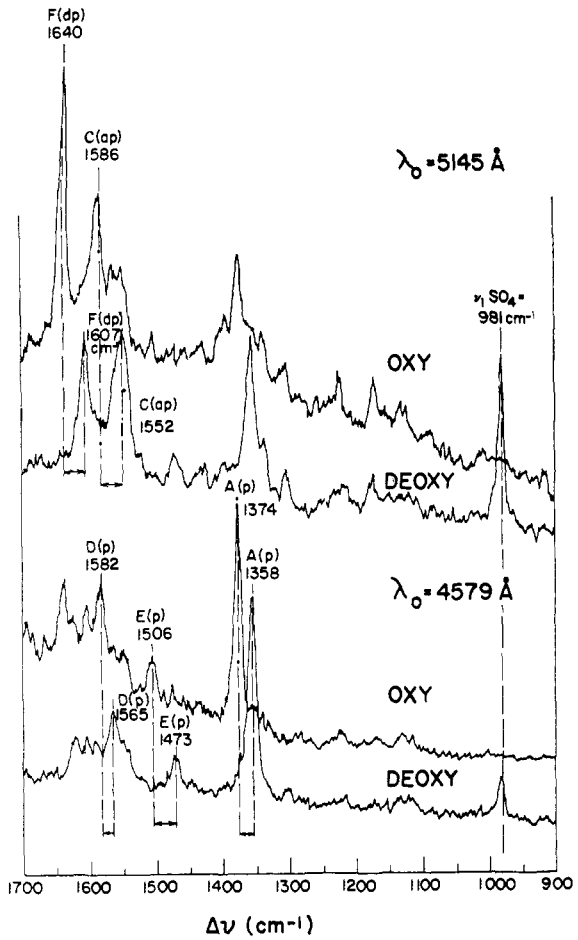

Figure 1. Resonance Raman spectra of oxy- and deoxyhemoglobin in the visible (λ_0 5145 Å) and Soret (λ_0 4579 Å) scattering regions. The deoxyhemoglobin solution was 0.34 mM in heme and contained 0.4 M $(NH_4)_2SO_4$, the ν_1 (981 cm^{-1}) band of which is indicated. The oxyhemoglobin solution was 0.68 mM in heme. Some evidence of photodissociation (*e.g.*, the shoulder at 1358 cm^{-1}) can be seen in the λ_0 5145 Å spectrum, which was obtained with the sample in a capillary tube. The effect is more pronounced at 4579 Å but was minimized, in the spectrum shown, by use of a spinning cell.[43] Heme marker bands are indicated by the designations given in Table III. The B(dp) band has been omitted, since it is resolved only in polarization spectra for the deoxy species. Instrumental conditions: slit width, 6 cm^{-1}; sensitivity, 10^{-9} Å; time constant, 3.0 sec; scan speed, 50 cm^{-1}/min. From 25 to 50 mW of incident power was used for the various spectra.

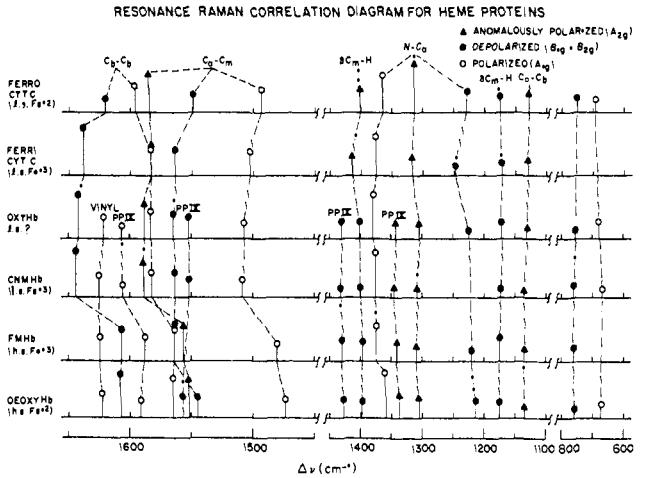

Figure 2. Correlation diagram for resonance Raman bands of hemoglobin and cytochrome c. Spin and oxidation states of the various species are indicated. See text for discussion of oxyhemoglobin oxidation state. The lengths of the lines are roughly proportional to the observed relative intensities at 5145 Å excitation (4965 Å for FMHb) for anomalously polarized (▲) and depolarized (●) bands and at 4579 Å excitation for polarized (○) bands. See text for assignments to various heme internal coordinates, which are indicated at the top. The bands marked PPIX (protoporphyrin IX) and VINYL are observed for hemoglobin derivatives only.

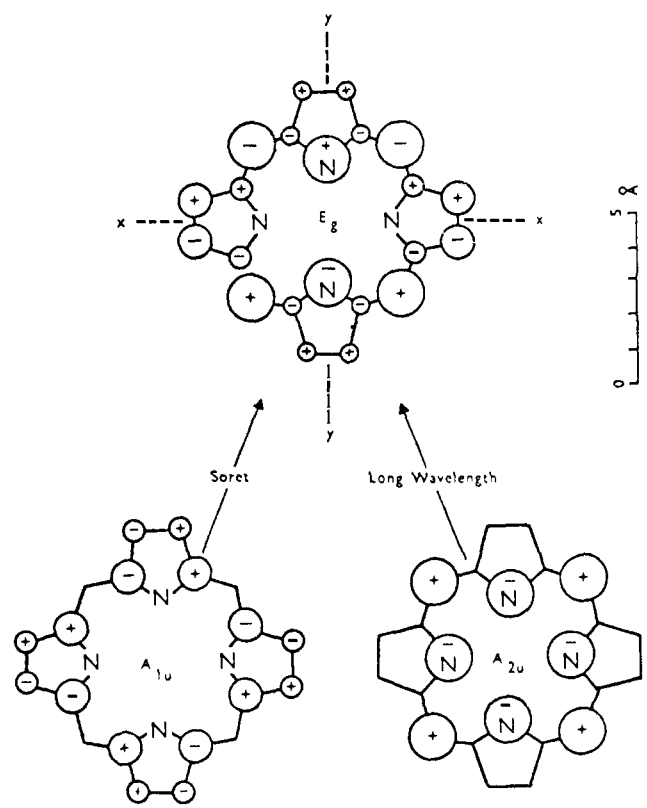

Figure 3. Representation of the electron density distribution for the orbitals giving rise to the visible and Soret porphyrin transitions (from ref 28, p 92).

sorption spectra of metalloporphyrins are dominated by two allowed π–π^* electronic transitions.[27] In molecular orbital terms these originate in the two highest filled molecular orbitals of A_{2u} and A_{1u} symmetry under the D_{4h} molecular point group and terminate in the lowest empty molecular orbital of E_g symmetry. Figure 3 is a diagrammatic representation of the wave functions for these orbitals.[28] The two electronic transitions are both of E_u symmetry and are subject to strong configuration interaction,[27] with the result that the transition dipoles add for the higher energy transition and largely cancel for the lower energy one. The higher energy transition is assigned to the very intense ($\epsilon \sim 10^5$ M^{-1} cm^{-1}) absorption band, called the Soret or γ band, shown by all metalloporphyrins at ~400 nm. The lower energy transition is assigned to what is normally the first strong absorption band, called the α band, which is found at ~550 nm, and is an order of magnitude weaker than the Soret band. The lower energy transition can "borrow" back some of the intensity of the higher energy transitions through appropriate vibrations. These are of rela-

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(28) J. E. Falk, "Porphyrins and Metalloporphyrins," Elsevier, Amsterdam, 1964.

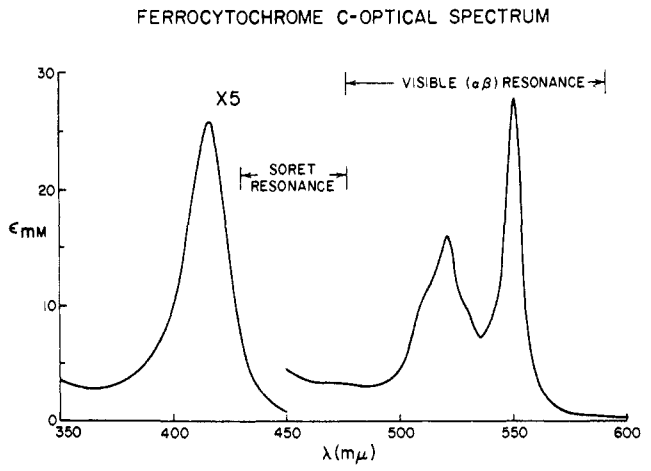

Figure 4. The near uv (Soret) and visible (α–β) absorption spectrum of ferrocytochrome c. Arrows span the approximate regions in which resonance with each of the two kinds of optical transition dominates.

tively high frequency and produce a vibronic side band, called the β band, which peaks at an energy $\sim$1300 cm^{-1} higher than the α band and sometimes shows vibrational structure, especially at low temperatures. A typical spectrum, that of ferrocytochrome c, is shown in Figure 4.

It has been demonstrated[14] that heme protein Raman spectra contain a set of bands which are in resonance with the Soret band. These are of type A,[25] as shown by their frequency dependence,[14] and are polarized (totally symmetric) as expected. Resonance with the α band might also be expected to enhance type A modes, but these have not been observed, probably because their intensity is too low, in consonance with the diminished intensity of the α band itself. Rather, nontotally symmetric bands are observed, whose intensities reach maxima at the α peak,[15] and also within the β band,[13] at positions accurately predicted by the corresponding 0–1 frequency, *i.e.*, the frequency of the α peak plus the vibrational frequency of the particular Raman band. These are type B modes, which mix the two electronic transitions and give rise to the β band. Their allowed symmetries are $E_u \times E_u = A_{1g} + A_{2g} + B_{1g} + B_{2g}$. The A_{1g} modes, however, have been shown to be ineffective in vibronic mixing,[29] and no polarized Raman bands are found to be in resonance with the α and β bands. The B_{1g} and B_{2g} modes give rise to depolarized Raman bands, which are observed. The A_{2g} modes are inactive in normal Raman scattering, since they are associated with *antisymmetric* scattering tensors,[30] *i.e.*, $\alpha'_{xy} = -\alpha'_{yx}$. Antisymmetric scattering becomes allowed in the resonance region, however, and gives rise to bands with *inverse* polarization.[13] The plane of polarization of the incident light is rotated through 90° on scattering, and $\rho_l = \infty$. The appearance of intense bands with inverse polarization is the most remarkable characteristic of heme protein resonance Raman spectra. Their observation[13] was the first experimental confirmation of antisymmetric vibrational scattering, although the phenomenon had been predicted by Placzek[22] nearly 40 years earlier.

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(30) W. M. McClain, *J. Chem. Phys.*, **55**, 2789 (1971).

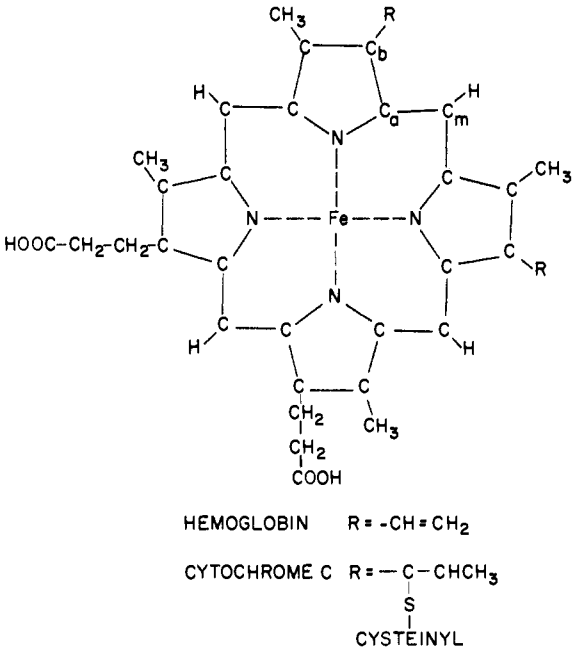

Figure 5. Structure of heme, indicating pyrrole substituents which occur in hemoglobin and cytochrome c. The different carbon atoms of the porphyrin skeletal framework are designated by the subscripts a, b, and m.

Some of the anomalous Raman bands have $\rho_l = \infty$ within experimental error; *i.e.*, no scattering intensity is observable in parallel polarization. But others have appreciable intensity in parallel polarization and $^3/_4 < \rho_l < \infty$. We characterize these as anomalously polarized, reserving the term *inverse* polarization for $\rho_l = \infty$. If the molecule has D_{4h} symmetry, then A_{2g} modes should have $\rho_l = \infty$, since their scattering tensors are strictly antisymmetric.[30] There are two possible explanations for a significant parallel component of anomalously polarized modes. The effective symmetry of the molecule may be less than D_{4h}, in which case the formerly A_{2g} modes may acquire symmetric as well as antisymmetric components of the scattering tensor.[30] Alternatively, there may be accidental degeneracies between A_{2g} modes and modes of other symmetries, which are polarized (A_{1g}) or depolarized (B_{1g} or B_{2g}), giving an overall depolarization ratio which is anomalous. Initially, we preferred the former explanation,[13] but in view of the many opportunities for accidental degeneracy (see the next section) we now favor the latter.

Vibrational Assignments for Cytochrome c. In analyzing the present vibrational data, it is useful to start with cytochrome c. Its heme chromophore, shown in Figure 5, has peripheral substituents, all of which are saturated and therefore not involved in the π conjugation of the porphyrin ring. The optical spectra are those of a planar chromophore with essentially fourfold (D_{4h}) symmetry.[31] If we ignore the peripheral substituents, then the chromophore has 81 normal modes of vibration classifiable as

$$\Gamma_{\text{in-plane}} = 7A_{1g} + 6A_{2g} + 7B_{1g} + 7B_{2g} + 14E_u$$

and

$$\Gamma_{\text{out-of-plane}} = 2A_{1u} + 5A_{2u} + 4B_{1u} + 3B_{2u} + 6E_g$$

(31) W. A. Eaton and R. M. Hochstrasser, *J. Chem. Phys.*, **46**, 2533 (1967).

Among the in-plane modes, the E_u vibrations are inactive in Raman scattering but might contribute to the observed spectrum to the extent that substituents destroy the effective symmetry center of the chromophore (see the next section). The resonance Raman spectrum is dominated by in-plane modes, inasmuch as the dominant electronic transitions are π–π^* transitions polarized in the plane.

Most of the strong resonance Raman bands lie between 1000 and 1650 cm^{-1} and are expected to involve[32] primarily stretching of the various C–C and C–N bonds in the porphyrin ring, as well as bending of the methine protons. Carbon–carbon double and single bond stretching vibrations are generally found at the higher and lower end of this range, respectively. The general order of the bond stretching frequencies is expected to be that of the bond orders, which porphyrin structures[33] show to be

$$C_b\text{–}C_b > C_a\text{–}C_m > C_a\text{–}N > C_a\text{–}C_b$$

The atom labels are those of Figure 5. The vibrations to which these internal coordinates, as well as C_m–H bending, contribute are given in Table II. While

Table II. Symmetries of Porphyrin Skeletal Stretching and Methine Proton Bending In-Plane Modes

Internal coordinate	Number of contributions to each symmetry class				
	A_{1g}	A_{2g}	B_{1g}	B_{2g}	E_u
$N\text{–}C_a$	1	1	1	1	2
$C_a\text{–}C_m$	1	1	1	1	2
$C_a\text{–}C_b$	1	1	1	1	2
$C_b\text{–}C_b$	1	0	1	0	1
$\delta C_m\text{–}H$	0	1	1	1	2
Total	4	4	5	4	9

vibrations of the peripheral substituents on the pyrrole rings also occur in this frequency region, they are not expected to exhibit resonance enhancement, since they are not conjugated to the chromophore. All of the observed bands can be accounted for without reference to these substituents.

In the region under consideration, the resonance Raman spectra of ferrocytochrome c show four anomalously polarized bands, associated with A_{2g} vibrations, which is just the number expected. They also show three polarized, A_{1g}, bands, one fewer than expected. The observed A_{1g} bands are all >1350 cm^{-1}. The missing band is attributed to C_a–C_b stretching, since the C_a–C_b bond is close to a single bond. The reason for its absence is uncertain. Four depolarized bands are observed, while four B_{2g} and five B_{1g} modes are expected. Accidental degeneracies between these two symmetry classes are quite likely, however, since some pairs of vibrational modes differ only with respect to the phases of nonadjacent bond stretching coordinates. Accidental degeneracies with the A_{1g} and A_{2g} symmetry classes are also possible. Indeed three of the four A_{2g} vibrations, 1129, 1312, and 1399 cm^{-1}, show significant intensity in parallel polarizaton, which could be explained by degenerate modes of B_{1g} or B_{2g} symmetry.

In summary, the observed resonance Raman spectra of ferrocytochrome c are well explained on the basis of contributions from all of the bond stretching coordinates of the porphyrin ring as well as from methine proton bending. All four expected A_{2g} modes and three of the four expected A_{1g} modes are observed, while only four of nine B_{1g} and B_{2g} modes are seen; accidental degeneracies can plausibly account for the rest of them.

Figure 2 allows comparison of the various heme protein spectra. The correlations are based on the different band polarizations. The indicated assignments to internal coordinates are mainly for accounting purposes. Undoubtedly these coordinates mix quite heavily in the various normal modes.[32] The spectra of ferricytochrome c show all of the features observed for ferrocytochrome c although there are small frequency shifts, whose significance will be discussed later.

Vibrational Assignments for Hemoglobin. Figure 2 shows that cyanomethemoglobin has resonance Raman bands corresponding closely to those of ferricytochrome c with respect to frequency and polarization. The single exception is the depolarized band at 1400 cm^{-1} which correlates with an anomalously polarized band at 1412 cm^{-1} in ferricytochrome c. However, the latter band has substantial intensity in parallel polarization, and the depolarization ratio is only moderately in excess of the depolarized value, 0.75. We feel that this band definitely contains two vibrational modes, one of A_{2g} ($\rho_l = \infty$) and one of B_{1g} or B_{2g} ($\rho_l = 0.75$) symmetry, and that in cyanomethemoglobin (as well as in the other hemoglobin derivatives) the A_{2g} intensity is lowered sufficiently that no significant anomalous polarization can be detected.

While the ferricytyochrome c spectrum corresponds closely, band for band, with that of cyanomethemoglobin, the latter has additional features which are missing in the former: two polarized bands, at 1625 and 1605 cm^{-1}, two depolarized bands, at 1552 and 1432 cm^{-1}, and an anomalously polarized band, at 1345 cm^{-1}. Similar extra features are observed in all the hemoglobin spectra and are logically attributed to the influence of the two vinyl substituents at the periphery of the protoheme prosthetic groups (Figure 5). These double bonds, which are saturated in cytochrome c, are in a position to conjugate with the π system of the porphyrin ring, and indeed the electronic spectra of protoheme derivatives are red-shifted with respect to their saturated (mesoheme) analogs by ~10 nm.[28] These additional double bonds might show up directly in the resonance Raman spectrum, and it is reasonable to assign at least one of the two additional polarized bands in the hemoglobin spectra, both of which occur in the double bond stretching region, to this source. In addition, the vinyl substituents may have an indirect effect on the Raman spectrum by lowering the effective symmetry of the chromophore. Even if the nuclear framework of the porphyrin ring retains effective D_{4h} symmetry, removal of the electronic symmetry center by the conjugated vinyl groups (both of which are attached to the same side of the porphyrin ring) could induce Raman activity into the infrared vibrational modes. Inasmuch as the high-frequency

(32) H. Ogoshi, Y. Saito, and K. Nakamoto, *J. Chem. Phys.*, 57, 4194 (1972).

(33) J. L. Hoard, "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Ed., W. H. Freeman, San Francisco, Calif., 1968.

Table III. Heme Protein Resonance Raman Bands (cm^{-1}) Which Are Sensitive to Oxidation and or Spin State

Spin[19] state[a]	Oxidation state	Molecule	Oxidation state markers A(p)	B(dp)	Spin state markers C(ap)	D(p)	Oxidation and spin state markers E(p)	F(dp)
ls	Fe(III)	Ferricyt c	1374	1562	1582	1582	1502	1636
ls	Fe(III)	CNMHb[b]	1374	1564	1588	1583	1508	1642
hs	Fe(III)	FMHb[b]	1373	1565	1555	1565	1482	1608
hs	Fe(II)	Deoxy Hb[b]	1358	1546	1552	1565	1473	1607
ls	Fe(II)	Ferrocyt c	1362	1548	1584	1594	1493	1620
ls	?	Oxy Hb[b]	1377	1564	1586	1582	1506	1640

[a] Low spin, ls; high spin, hs. [b] Hb, hemoglobin; MHb, methemoglobin.

porphyrin coordinates contribute to nine in-plane infrared (E_u) modes (Table II), this mechanism could easily account for the additional hemoglobin bands observed. The states of polarization of such bands are difficult to predict. The effective symmetry of the protoheme chromophore, with the vinyl groups included, is C_s. Under this point group all vibrations have scattering tensors which can have both symmetric and antisymmetric components,[30] so there are no symmetry restrictions on the depolarization ratio.

Electronic and Geometric Effects on the Vibrational Frequencies. The resonance Raman spectra in the high-frequency region are similar for ferri- and ferrocytochrome c, but reduction of the oxidized protein leads to perceptible lowering of most of the vibrational frequencies. Similarly, the frequencies of deoxyhemoglobin (high-spin Fe(II)) are lower than those of fluoromethemoglobin (high-spin Fe(III)). These frequency shifts can be understood in terms of changes in π back donation of electrons from the central iron atom to the porphyrin ring.[34] The iron d orbitals are more extended for Fe(II) than for Fe(III), because of the lower effective charge, and overlap with porphyrin π^* orbitals is greater. Populating the π^* orbitals results in weakening the porphyrin ring bonds and lowering their force constants. The observed lowering of vibrational frequencies associated with the stretching of porphyrin ring bonds can be understood on this basis.

It is also possible to weaken the porphyrin bonds by transferring electrons from the bonding π orbitals on porphyrin to empty π orbitals on Fe(III) (high or low spin) or high-spin Fe(II). Molecular orbital calculations by Zerner, Gouterman, and Kobayashi[35] indicate that low- and high-spin Fe(III) and high-spin Fe(II) are indeed net π acceptors in heme proteins, by about 0.4, 0.3, and 0.3 electron, respectively, while low-spin Fe(II), as expected, is a net π donor, by about 0.1 electron. These numbers suggest that the porphyrin bonds would be weakened more by π acceptance in ferricytochrome c than by π donation in ferrocytochrome c, but it is difficult to evaluate quantitatively the competing effects of filling antibonding and depleting bonding orbitals. The vibrational frequencies show the opposite trend, but the shifts are small.

On the basis of these electronic arguments very little change in the vibrational frequencies would be expected on changing the spin state for either Fe(III) or Fe(II). In their calculations, Zerner, *et al.*, suggest that high- and low-spin Fe(III) are almost equally good π acceptors, while conversion of low- to high-spin Fe(II) is similar to oxidation of low-spin Fe(II) in that π acceptance by high-spin Fe(II) is expected to counterbalance π donation by low-spin Fe(II) in its effect on the porphyrin bonds. Yet substantial frequency shifts do accompany spin state changes, as shown in the correlation diagram, Figure 2. The frequencies for high-spin fluoromethemoglobin are lower than the corresponding frequencies of low-spin cyanomethemoglobin, and the frequencies for high-spin deoxyhemoglobin are lower than those of low-spin ferrocytochrome c. The shifts are much larger than those associated with the reduction of ferricytochrome c and they are in the opposite direction, on the basis of the electronic considerations.

We attribute these shifts to *structure changes* which are associated with changes in spin state. It is well established[33-37] that high-spin iron(II) and -(III) are both too large to fit into the central hole of the porphyrin ring. Consequently, they are forced out of the plane, by ~0.8 Å for Fe(II) and ~0.3 Å for Fe(III). It is also known[36] that the out-of-plane structures exhibit significant doming and ruffling of the porphyrin ring. We feel that these changes in ring conformation are primarily responsible for the frequency shifts accompanying the spin state changes. Both force constants and the kinematic coupling among the internal coordinates would be affected. Another contributor would be the out-of-plane displacement of the iron itself, inasmuch as coupling would be decreased between porphyrin ring vibrations and the lower frequency modes associated with Fe–N stretching (not observed in this study but certainly present in the molecules), and the ring frequencies would thereby be somewhat decreased.

Frequencies which shift appreciably on changing oxidation or spin state are listed in Table III. Band A, which is polarized and is the most intense band in the Soret scattering region (see Figure 1), occurs at ~1375 cm^{-1} for Fe(III) hemes and ~1360 cm^{-1} for Fe(II) hemes, independent of spin state. Both Brunner, *et al.*,[9,12] and Rimai and coworkers[10] have called attention to this band and suggested its use as an oxidation state marker. Band B, which is depolarized and best observed in the α–β scattering region, shifts as much as band A, ~1564 cm^{-1} for Fe(III) to ~1547 cm^{-1} for Fe(II), but it is less useful as an oxidation state marker because of the presence of another depolarized band nearby (~1555 cm^{-1}) in protoheme derivatives.

Bands C (inverse polarized) and D (polarized) ap-

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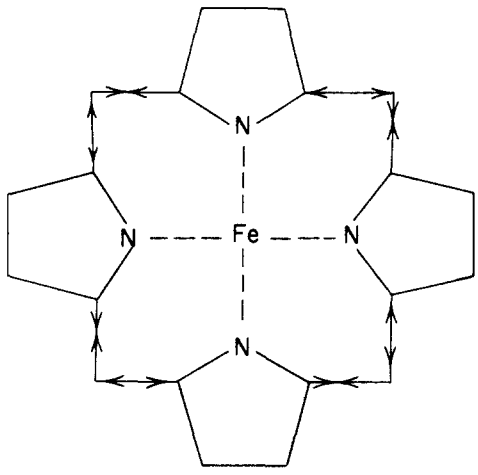

Figure 6. Suggested form of the ~1590-cm^{-1} A_{2g} vibration involving the porphyrin–methine bridges. A mode of this symmetry is formally equivalent to a rotation about the central z axis, perpendicular to the heme plane.

pear to be reliable spin state markers. The former shifts from ~1555 to ~1585 cm^{-1} on converting from high- to low-spin heme, independent of oxidation state, while the latter shifts correspondingly from ~1565 to ~1585 cm^{-1}. (The high value, 1594 cm^{-1}, for band D shown by ferrocytochrome c is anomalous and bears further investigation. This is the only frequency which *increases* on oxidation.) Band C is the most intense feature of low-spin heme Raman spectra in the α–β reasonance region. This was noted in the first report of heme protein resonance Raman spectra,[6] along with its apparent disappearance in high-spin hemes. The intensity of band C was suggested as an indicator of spin state, although no physical basis for the connection could be offered. We now know that band C is present in high-spin hemes, markedly shifted in frequency, but the intensity is greatly reduced. The *inverse* polarization (no parallel polarized remnant is observed for this band) establishes that band C arises from an A_{2g} mode, and its high frequency indicates that it is mainly C_a–C_m stretching in character. The A_{2g} vibrations have rotational symmetry around the fourfold axis, and the suggested form of the band C mode is drawn in Figure 6. From the form of the porphyrin molecular orbitals shown in Figure 3, it is clear that the methine bridge A_{2g} mode could be especially effective in mixing the two electronic transitions, and its intensity would be correspondingly enhanced. The doming of the porphyrin ring, which accompanies the out-of-plane displacement of high-spin iron, involves significant buckling of the formerly planar methine bridge[36] and therefore a significant disruption of the π delocalization at the methine carbon atoms. The methine bridge A_{2g} mode would then become much less effective in mixing the electronic transitions, and its loss of intensity in high-spin hemes can be understood on this basis.

Rimai and coworkers[10] have suggested that the relative Raman intensity at ~1584 and ~1566 cm^{-1} is diagnostic for spin state, the ratio being between 2 and 5 for low-spin hemes and ~0.4 for high-spin hemes.[10]

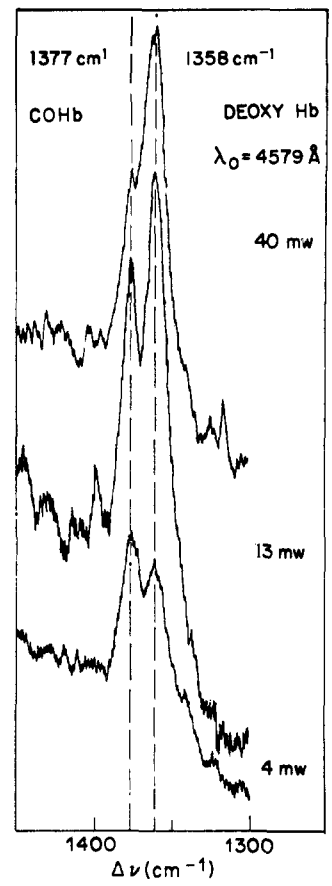

Figure 7. Portion of the Raman spectrum of carboxyhemoglobin (1377 cm^{-1} peak), showing reversible photodissociation to deoxyhemoglobin (1358 cm^{-1} peak). The sample, 0.66 mM COHb with sodium dithionite added, was placed under argon and run in a (sealed) rotating cell, at ~2000 rpm. The changing peak heights correspond to changing ratios of COHb and deoxyHb at the indicated power levels (measured at the sample) of 4579 Å Ar^+ laser radiation. The spectra were recorded in order of *decreasing* power levels, and the absorption spectrum of the solution at the end of the experiment corresponded to that of pure COHb. Photodissociation is less pronounced with longer wavelength excitation.

They were limited to excitation in the Soret region (441.6 nm) and were apparently observing mainly the *shift* in band D (see Table III). For high-spin protohemes there is a weak polarized band at ~1590 cm^{-1} (see Table II) which would account for their observed intensity ratio. For low-spin hemes the intensity ratio apparently rests on residual intensity from band B which, although in resonance with the α–β bands, is strong enough to show some intensity at 441.6 nm. The residual intensity would depend on the position of the α–β bands as well as on oxidaton state (see Table III), and this might account for the range of ratios which Rimai and coworkers observed.

Bands E (polarized) and F (depolarized) are sensitive to both oxidation and spin state. The spin state shift is ~20 cm^{-1} in both cases, but while reduction lowers the frequency by 10–20 cm^{-1} for low-spin hemes, the shift is less pronounced for high-spin hemes. It seems evident that the best single oxidation state marker is band A, which stands out clearly in the Soret resonance region, while the best single spin state marker is band C, which stands out clearly in the α–β resonance region. However, use of all six bands in Table III would obviously lend greater confidence to the analysis of spin and oxidation states.

We emphasize that the observed frequency shifts reflect both electronic changes, which influence bond strength and therefore force constants, and geometric changes, which alter both force constants and kinematic coupling. It is to be anticipated that cases will be found which do not fit the present correlations for oxidation and spin state, because of structural influences of protein conformation or axial ligands.

Oxidation State of Oxyhemoglobin. While oxyhemoglobin is known to be diamagnetic, a considerable controversy has developed over the years as to whether the iron–oxygen unit should properly be considered Fe^{2+}–O_2[38,39] (low spin) or Fe^{3+}–O_2^-,[40] with the superoxide radical antiferromagnetically coupled to low-spin Fe^{3+}, or even as Fe^{4+}–O_2^{2-}.[41] It is therefore of interest to observe that with respect to the oxidation and spin state Raman marker bands, oxyhemoglobin clearly classifies as low-spin Fe(III) (see Table III). Indeed, the resonance Raman spectrum of oxyhemoglobin is essentially identical with that of cyanomethemoglobin, as can be seen in Figure 2, and it is much closer to the spectrum of ferri- than ferrocytochrome c. From the standpoint of the porphyrin vibrational frequencies, which, as we have seen, are quite sensitive to oxidation state, the best formulation is clearly Fe^{3+}–O_2^-. This conclusion has been reached earlier by Rimai and coworkers[10] on the basis of the position of the ~1370 cm^{-1} band for a series of heme proteins, but the present study allows a much more detailed comparison.

On the other hand, it is instructive to note that carbonmonoxyhemoglobin, in which O_2 is replaced by CO, gives resonance Raman spectra identical with those of oxyhemoglobin. We have studied the carboxyhemoglobin spectrum in some detail, taking care to correct for photodissociation in the laser beam (see Figure 7), and can find no differences, at least in the high-frequency region, with respect to oxyhemoglobin. The formulation Fe^{3+}–CO^- is much less congenial than Fe^{3+}–O_2^-, in view of the instability of the CO^- radical. Inorganic chemists are, however, quite accustomed to the notion of relief of charge on a metal atom by back bonding to π acceptor ligands like CO or O_2. Indeed, the C–O stretching frequency in carbonmonoxyhemoglobin,[42,43] ~1950 cm^{-1}, is ~200 cm^{-1} lower than that of free CO and is typical of transition metal carbonyls. It is reasonable to conclude that π back donation occurs to about the same extent in oxy- and carboxyhemoglobin, and that the resultant charge on the iron atom is about the same as for low-spin ferric heme derivatives.

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Temperature Dependence of the Line-Narrowed ^{19}F Nmr Spectrum of Solid Perfluorocyclohexane[1]

J. D. Ellett, Jr., R. G. Griffin, and J. S. Waugh*

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
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Abstract: The rate of ring inversion in solid perfluorocyclohexane was measured as a function of temperature by analyzing line-narrowed ^{19}F nmr spectra. The activation parameters describing the inversion depend strongly on a parameter used in the analysis, the residual line width, which could not be measured precisely. It is nonetheless possible to conclude that the enthalpies and entropies of activation for chair-to-chair inversion in the liquid and solid states do not differ radically. The free energy of activation in the solid at the coalescence temperature of 262°K is 11.5 kcal/mol, roughly 1 kcal/mol higher than the liquid state value. In addition, we present a line-narrowed nmr spectrum of perfluorocyclohexane taken at a sufficiently low temperature to permit an estimation of the anisotropy of the chemical shift tensors of the ^{19}F nuclei. This spectrum resembles the line-narrowed ^{19}F nmr spectrum of Teflon.

The rate of interconversion of cyclohexane and related molecules between alternate chair conformations has been studied by many workers.[2] Perfluorocyclohexane is of particular interest because, unlike cyclohexane, it exhibits a negative entropy of activation.[3] A possible interpretation of this is that perfluorocyclohexane passes through a planar rather than a "skew-boat" intermediate conformation.[4] Multiple-pulse line-narrowing experiments previously made in this laboratory[5] showed that the "high-resolution" nmr spectrum of solid perfluorocyclohexane has qualita-

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(2) For an introduction to the literature on this topic, see the review article by W. A. Thomas in "Annual Review of NMR Spectroscopy," Vol. 1, E. F. Mooney, Ed., Academic Press, New York, N. Y., 1968, p 44 ff.
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