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Conformational Transitions and Vibronic Couplings in Acid Ferricytochrome *c*: a Resonance Raman Study†

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ABSTRACT: Resonance Raman spectral changes in ferricytochrome *c* as a function of pH between 6.7 and 1.0 are reported and the structural implication is discussed in terms of the "core-expansion" model advanced by L. D. Spaulding et al. [(1975) *J. Am. Chem. Soc. 97*, 2517]. The data are interpreted as indicating that the iron in high-spin acid ferricytochrome *c* (at pH 2.0) with two water molecules as axial ligands lies in the plane of the porphyrin ring. At pH 1.0 there is a different high-spin form of cytochrome *c* which has an estimated iron out-of-plane distance of ~0.46 Å. The effect of a monovalent anion at pH 2.0 is to produce a thermal spin mixture with predominant low-spin species. Excitation at ~620 nm in acid cytochrome *c* (pH 2.0) enhances only three depolarized ring vibrations at 1623, 1555, and 764 cm^{-1}. Marked enhancement of depolarized modes relative to polarized and anomalously polarized modes is attributed to the vibronic coupling between porphyrin $\pi \rightarrow \pi^*$ and porphyrin $\pi \rightarrow$iron(d_π) charge-transfer states.

Cytochrome *c* undergoes an interesting structural transition at pH 2.5 (Boeri et al., 1953; Gupta & Koenig, 1971; Fung & Vinogradov, 1968; Lanir & Aviram, 1975) which converts the native low-spin heme to a high-spin form with a concomitant displacement of the axial ligands by two water molecules (Lanir & Aviram, 1975). Addition of anions such as Cl^- or ClO_4^- causes some reversal in the spectral and magnetic changes which accompany the titration (Boeri et al., 1953; Aviram, 1973). The question of whether the iron in acid, high-spin ferricytochrome *c* is in-plane or out-of-plane is of

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considerable importance in view of an earlier controversy between the "doming" model (Spiro & Strekas, 1974; Stein et al., 1975) and the "core-expansion" mechanism (Spaulding et al., 1975; Felton & Yu, 1978; Yu, 1977) of Raman shifts in porphyrins. Since the "doming" model assumed that the out-of-plane displacement of the iron was proportional to the magnetic moment, an *in-plane high-spin* iron would have never been predicted. However, recent X-ray studies demonstrated that the high-spin iron in bisaquo- or bis(tetramethylene sulfoxide)(tetraphenylporphinato)iron(III) perchlorate is located precisely in the plane of the porphyrin ring (Kastner et al., 1978; Mashiko et al., 1978).

In this work, we report resonance Raman studies of cytochrome *c* at acidic pH values and the effect of anions on the Raman spectrum. The "core-expansion" correlation first proposed by Spaulding et al. (1975) will be used to argue the existence of an *in-plane high-spin* iron in a heme with two weak field axial ligands. In addition, Raman spectra of acid cytochrome *c* excited near 620 nm will be presented and the nature of the electronic transition responsible for the 620-nm absorption will be discussed.

Materials and Methods

Horse heart cytochrome *c* (Sigma type VI) was further purified on a DEAE-Sephadex column. The pH of the solution was adjusted by adding HCl or NaOH.

Raman spectra were determined using a Spex 1401 double monochromator, Coherent Radiation Model CR-5 argon ion laser or krypton ion laser, Model CR-500K, and photon-counting electronics. The scattered light intensity at 90° was analyzed with a polarizer oriented parallel (||) or perpendicular ($\perp$) to the incident polarization followed by a polarization scrambler. A CMX-4 xenon flashlamp pumped dye laser (Chromatix Corp.) was used for excitation in the 610–630-nm region.

Results

The observed resonance Raman spectra of ferricytochrome *c* at neutral and alkaline pH values are very similar to those obtained in previous studies (Kitagawa et al., 1977). Some minor changes in the relative intensities are noticed, however. In particular the 1585, 1316 and 1234 cm^{-1} lines (see Figure 1a) are more intense than the corresponding lines shown by Kitagawa et al. (1977a,b). Figure 1 shows part of the resonance Raman scattering spectra of ferricytochrome *c* at different pH. The polarization of the Raman lines is given besides the peak frequency (dp indicates depolarized; p, polarized; ap, anomalously polarized). The marked changes in the scattering of the heme chromophore upon lowering the pH reflect structural and environmental changes around the heme. Acidification of the solution of cytochrome *c* causes the heme crevice to open. This transition has a p*K* of 2.5 and is accompanied by displacement of methionine and histidine from the iron by two solvent molecules (Lanir & Aviram, 1975) as well as conversion of the native low-spin hemoprotein to a high-spin form (Boeri et al., 1953). However, some significant changes in the Raman spectrum were observed already at pH 3 (Figure 1b) where the changes in the absorbance and other physical properties of the heme are very small. The Raman changes consist of an intensity decrease and shift of the 1316 line to 1320, a decrease in the 1251 and 1234 lines and finally some increase at 1563 cm^{-1}, a process that starts already at pH 4.6. The results suggest that the resonance Raman technique is sensitive to small changes around the heme which are not detected by other spectroscopic methods. Note, however, that proton resonance assigned to the heme methyl groups undergoes significant changes between pH 3 and 4,

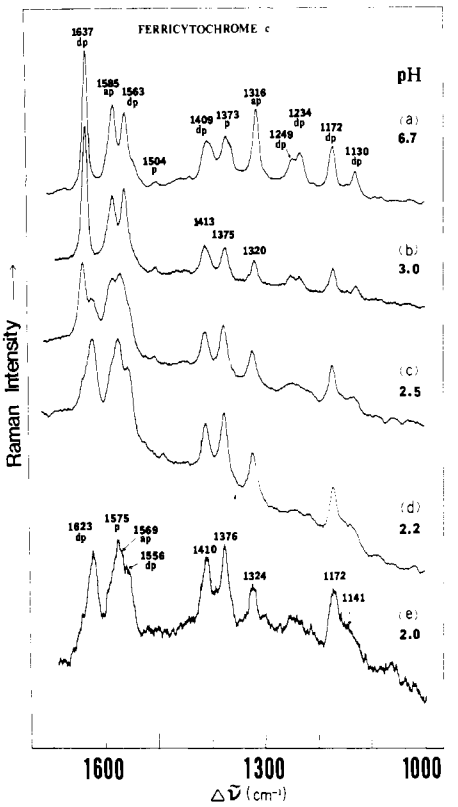

FIGURE 1: Resonance Raman spectra of ferricytochrome *c* at different pHs obtained with 514.5-nm excitation. Experimental conditions: (spectra a–d) 200-mW incident power, 300-μm slit width, 1-cm slit height, 0.5 cm^{-1}/s scan rate, 5-s time constant; (spectrum e) time constant 2.5 s. The identification of the ap line at 1569 cm^{-1} was based on polarized Raman spectra (not shown).

although the denaturation process in cytochrome *c* starts only below pH 3.0 (Cohen et al., 1974). At pH 2.5 there are equivalent amounts of high-spin ($S = 5/2$) and low-spin ($S = 1/2$) species. Accordingly, the Raman spectrum (Figure 1c) consists of two sets of lines which are superimposed. Considering the fact that the absorbance ratio at 514.5 nm of the low spin (pH 7.0) to the high spin (pH 1.8) is 1.1, it is evident that low-spin ferric hemoproteins scatter more strongly, by at least a factor of 3, than the corresponding high-spin species. At pH 2.5 the 1249 and 1234 lines decrease further and are broadened beyond resolution. The 1130 line shifts to higher energies as does the 1316 cm^{-1} line to 1323 cm^{-1}. In particular, a new set of lines, due to the high-spin acid cytochrome is observed at high frequencies with prominent lines at 1623 cm^{-1} (dp), 1575 (p), 1569 (ap), and 1556 (dp). The line positions are comfirmed on further acidification of the solution of cytochrome *c* to pH 2.2 (~90% high spin) and pH 2.0 (~100% high spin). The line positions are summarized in Table I.

Acidification of the solution to pH 1.0 is reported to partly convert the cytochrome into a different high-spin form that shows a lower rhombicity in the EPR spectrum which now resembles that of hemin chloride (Peisach et al., 1971). The nature of this high-spin species is uncertain. Its Raman lines at high frequencies are given in Table I, and of particular interest are the depolarized lines at 1634 and 1558 cm^{-1} and the ap line at 1572 ± 2 cm^{-1}. These frequencies are similar to the Raman lines obtained for chlorohemin (Spaulding et al., 1975; Kitagawa et al., 1977a) and in cytochrome *c'* at pH 10.3 (Kitagawa et al., 1977a; Strekas & Spiro, 1974). Both hemes are considered to have five-coordinate geometry. It is thus suggested that acid cytochrome at pH 1.0 is a five-co-

Table 1: Raman High Frequencies (cm^{-1}) for Oxidized Cytochrome *c* under Various Conditions

pH 7.0, low spin	pH 2.0, high spin	pH 1.0, high spin	pH 2.0 + anion thermal mixture	
			low spin	high spin
1637 (dp)	1623	1634	1640	1614
	1575 (p)	1582 (p)		
1585 (ap)	1569	1572	1589	
1563 (dp)	1556	1558	1566	
1506 (p)	1490		1507	
1409 (dp)	1410	1410	1412	
1373 (p)	1376	1376	1375	
1316 (ap)	1324	1320	1321	
1249 (ap)	~1250	~1250	~1250	
1234 (dp)	~1235	~1235	~1238	
1172 (dp)	1172	1172	1172	
1130 (dp)	~1144		1130	

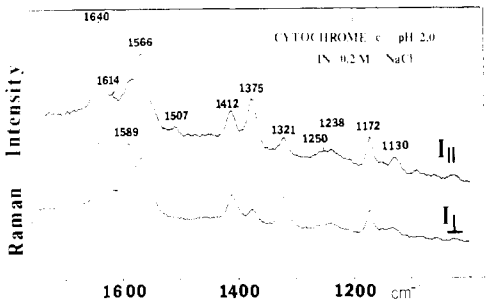

FIGURE 2: Resonance Raman of acid ferricytochrome *c*, pH 2.0, in 0.2 M NaCl solution obtained with 514.5-nm excitation. Experimental conditions: 220-mW incident power, 240-μm slit width; 1-cm slit height; 0.5 cm^{-1}/s scan rate, 5-s time constant.

ordinated heme protein in which the fifth ligand is either chloride or a water molecule. Considering the value for the proton relaxation rate of water in solution of cytochrome *c* at pH 1.0 (Lanir & Aviram, 1975), it is reasonable to assume that the only ligand on the iron is a water molecule.

The broad Raman spectrum at pH 1.0 clearly demonstrates the presence of two minor components in addition to the five-coordinate species. The first is some residual fraction of the high-spin component that predominates around pH 2.0. The second minor species is formed as a result of addition of Cl^- as a counterion to the solution (see below).

Addition of anions to acid ferricytochrome *c* produces a dramatic reversal in the spectroscopic and magnetic properties of the product (Boeri et al., 1953; Aviram, 1973). The protein exists in a mixed-spin form, which can be either a thermal mixture of high-spin and low-spin forms or a quantum mechanical admixture (Maltempo et al., 1974).

The resonance spectrum (Figure 2) strongly indicates that anions produce thermal mixed-spin forms, in which the predominant component has a low-spin iron. A depolarized line derived from the small fraction of the high-spin derivatives is clearly seen at 1614 cm^{-1}. Crude calculations based on the relative peak intensities and sensitivity of the two spin state forms showed the presence of ~70% low-spin form.

These results are compatible with NMR relaxation results that indicated the presence of 30% high-spin form, in which one water molecule is bound to the high-spin iron (Lanir & Aviram, 1975). More recently, EPR studies by Ripsin & May (unpublished results) showed also a mixture of high-spin and low-spin species in acid cytochrome *c* in the presence of anions. The new low-spin heme protein has Raman lines at 1640 (dp), 1589 (ap), and 1566 (dp) which differ significantly from the corresponding lines of the low-spin native cytochrome *c* (see Table I).

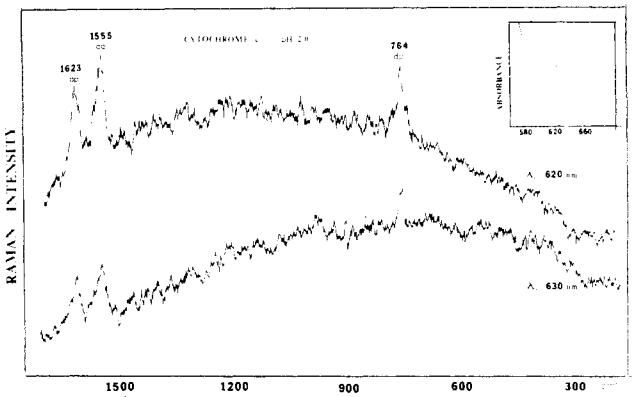

FIGURE 3: Raman spectra of acid cytochrome *c*, pH 2.0, obtained in the charge-transfer excitation region. At 630 nm Kiton red is the pumped dye. Pulse repetition rate, 15 Hz; average power, 50 mW; slit width, 400 μm. At 620 nm, Rhodamine 6 G is the pumped dye. Pulse repetition rate, 20 Hz; average incident power, 40 mW.

Acid cytochrome *c* exhibits a strong absorption band (ϵ ~3500 M^{-1} cm^{-1}) around 620 nm (Aviram, 1973). The exact nature of this absorption in cytochrome *c* is not known, although absorption bands near 16×10^3 cm^{-1} in other high-spin ferric heme proteins were assigned (Smith & Williams, 1970; Gouterman et al., 1975; Brill & Williams, 1961) to the $a_{2u}(\pi)$, $a_{1u}(\pi) \rightarrow e_g(d_\pi)$ charge-transfer transitions. Excitation within this absorption band (see Figure 3) enhances only depolarized ring modes (Shelnutt et al., 1976) at 1623, 1555, and 764 cm^{-1} without discernible low frequency vibrations involving iron–ligand or iron–pyrrole nitrogen stretches (200–700 cm^{-1}). The iron–axial ligand vibration has been observed (Asher et al., 1977) for methemoglobin azide and methemoglobin hydroxide when the exciting wavelength is within the ligand-to-iron charge-transfer bands. The selective enhancement of depolarized ring modes also was observed upon excitation within the 16×10^3 cm^{-1} region in other heme proteins such as methemoglobin, formate–hemoglobin, and horseradish peroxidase. Excitation at 620 nm of cytochrome *c* at pH 2.5, in which equal amounts of low-spin and high-spin species coexist, gave exclusively the enhanced dp lines due to the high-spin component. The lack of low frequency enhancement in acid cytochrome *c* appears to indicate that the 620-nm charge-transfer band involves heme macrocycle to iron transition rather than axial ligand to metal charge transfer.

Discussion

Lowering the pH value below 3.0 in cytochrome *c* induces a spin transition of the heme iron as well as a conformational change in which the two axial ligands (His-18 and Met-80) are replaced by two water molecules (Lanir & Aviram, 1975). The Raman spectrum in the high frequency region of ferricytochrome *c* at pH 2.0 is very similar to that of catalase (Felton et al., 1976) and is typical of high-spin heme proteins and heme models (Felton & Yu, 1978; Spiro et al., 1979) in which two weak ligands coordinate the iron. It was well documented (Spaulding et al., 1975) that the position of an ap line around 1610–1550 cm^{-1} is correlated with the distance between the center of the porphyrin core and the pyrrole nitrogen, designated as d(Ct–N). According to the "core expansion" theory, an increase in the Ct–N distance corresponds to a linear decrease in this a_{2g} frequency. The importance of this basic relationship was reemphasized recently by Huong & Pommier (1977), Spiro et al. (1979), and Scholler & Hoffman (1978). In Figure 4 we plot the correlation of band IV (ap line around 1610–1550 cm^{-1}) and band V (dp line near 1640–1610 cm^{-1}) with the size of the core expressed

as d(Ct–N). It includes our previous data along with several new data (Spiro et al., 1979). The correlation for band V is less satisfactory and the points scatter beyond 0.01 Å. This is understandable in terms of the normal coordinates assignable to these two types of vibrations. The specific nature of the a_{2g} mode at $\sim$1590 cm^{-1} involves dominant stretching contributions from bonds constituting the inner 16-membered ring of the porphinato core, while the vibration responsible for band V contains, in addition, stretching contributions from the pyrrole ring (Sunder & Bernstein, 1976). Nevertheless, in evaluating core sizes, we used both bands. On the basis of the correlations, the ap line at 1569 ± 1 cm^{-1} and the dp line at 1623 cm^{-1} in acid cytochrome c imply a d(Ct–N) = 2.033 ± 0.01 Å. If a traditional Fe–N bond length of 2.065 ± 0.01 Å for high-spin ferric porphyrin is assumed (Hoard et al., 1965; Koenig, 1965), the out-of-plane displacement of the iron is inferred to be 0.35–04 Å. However, very recently Kastner et al. (1978) reported the X-ray structure of diaquo($\alpha,\beta,\gamma,\delta$-tetraphenylporphinato)iron(III) perchlorate, an iron porphyrin with two weak field axial ligands. The significant finding is that the high-spin iron(III) is precisely centered in the plane of the porphyrin ring. The average Fe–N bond distance in this heme model is only 2.040 Å, much shorter than the Fe–N bond length found in high-spin pentacoordinated model compounds (Koenig, 1965) or six-coordinate porphyrins in which one of the axial ligands is weak and the other is a nitrogen base (Hoard et al., 1965). The same Fe–N distance is reported in bis(tetramethylene sulfoxide)(tetraphenylporphinato)iron(III) perchlorate (Mashiko et al., 1978). Such a short Fe–N distance in high-spin hemes with two weak axial ligands can argue for an in-plane position of the iron(III) atom in acid ferricytochrome c. It is also possible that catalase which has two weak ligands, one of which is a water molecule (Lanir & Schejter, 1976), may also have an in-plane iron because it exhibits an ap line at 1566 cm^{-1} (Felton et al., 1976).

At pH 1.0 a different high-spin form of cytochrome c is formed as indicated by the EPR spectrum (Peisach et al., 1971). This species is characterized by Raman lines (Table I) similar to those in cytochrome c' and chlorohemin (Strekas & Spiro, 1974; Kitagawa et al., 1977a), which have a five-coordinate geometry. The ap line at 1572 and dp line at 1634 cm^{-1} yield a d(Ct–N) of 2.02 and 2.01 Å, respectively. The core size is much too small to accommodate the high-spin iron(III) with d(Fe–N) = 2.07 Å (Koenig, 1965). Triangulation results in a 0.46 ± 0.03 Å estimated distance for the iron out-of-plane displacement in cytochrome c at pH 1.0. When an excess of monovalent anion is added to cytochrome c at pH 2.0, a dramatic change occurs in its physical properties. The magnetic susceptibility drops significantly and is compatible with either a species which has a spin of 3/2 or a thermal mixture of two species with a spin of 5/2 and 1/2. The Raman spectrum reveals that the second possibility prevails and that the low-spin species predominates in the mixture. Bands IV and V (Table I) and Figure 4 can be used again, and d(Ct–N) = 1.995 Å is found for this low-spin species. The central hole is large enough for an in-plane geometry. It is slightly smaller than the d(Ct–N) in native cytochrome c in which the value for this distance is 2.002 Å.

The charge-transfer bands in heme proteins under consideration may arise from either axial ligand-to-metal or porphyrin-to-metal d_π transition (Gouterman et al., 1975; Eaton & Hochstrasser, 1968). The former is z-polarized while the latter are x,y-polarized. The axial ligand-to-metal stretch in methemoglobin azide was observed (Asher et al., 1977) at 413 cm^{-1} when excited near the 640-nm absorption band,

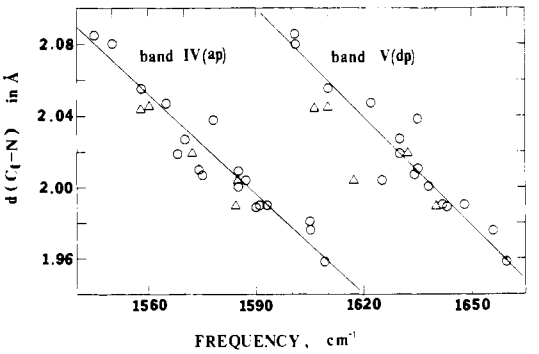

FIGURE 4: A plot of the frequency of RR bands IV (ap) and V (dp) vs. d(Ct–N). Open circles represent correlation that is based on our data (Spaulding et al., 1975). The triangles represent recent data of Spiro et al. (1979).

which contains an unresolved z-polarized electronic transition (Kabat, 1967; Eaton & Hochstrasser, 1968). The situation with the x,y-polarized charge transfer is somewhat more complex. Promotions of a_{2u}, $a_{1u} \rightarrow e_g(\pi)$ yield two $\pi\rightarrow\pi^*$ states of E_u symmetry (under the D_{4h} point group). After configuration interaction these become the Q and B (Soret) excited states. Charge-transfer states characterized by promotions from a_{2u} and a_{1u} porphyrin orbitals to the partially occupied d_π levels of high-spin ferric complexes yield two additional E_u states. In ferric porphyrins the charge-transfer states have an energy close to that of the Q state. Eaton & Hochstrasser (1968) observed four visible absorption maxima in single crystal spectra of high-spin ferrimyoglobin complexes. The two highest energy transitions are assigned to Q_0 and Q_v, the 0–0 and 0–v vibronic transitions. The two lowest energy states, denoted as I and II are x,y-polarized charge-transfer states. In Figure 3, excitation into band I enhances depolarized modes of acid cytochrome c, but not totally symmetric modes. A similar phenomenon is observed (Asher et al., 1977) in methemoglobin fluoride. The explanation of depolarized mode enhancement is found in earlier studies of model porphyrin resonance Raman scattering (Shelnutt et al., 1976, 1977).

Dipole intensity in the Q band is due to admixture of the B state into the lower energy Q state. The interactions between Q and B states are either vibronic or electronic in origin. If charge-transfer states have energies near those of the Q state, then these also will interact with the $\pi\rightarrow\pi^*$ states by either configuration interaction or vibronic coupling. Following interaction, all excited states will be *vibronic* in nature. One may no longer separate electronic and vibrational motion. The Raman scattering intensity will depend upon the amount of B state with no or one quantum of vibrational energy in the vibronic wave functions of band I. The contribution of the B state with one vibrational quantum excited, in turn, depends upon the vibronic coupling, V^K, of the Kth normal mode Q_K between the charge-transfer state and B state. In earlier work on the theory of Raman scattering intensity in manganese etioporphyrin (Shelnutt et al., 1976), we showed that depolarized modes uniquely couple the two states. The form of the coupling operator for e.g., y-polarized states, is

$$V^K = \left\langle B_y \middle| \left(\frac{\partial V}{\partial Q_K}\right)_0 \middle| I_y \right\rangle \tag{1}$$

where V is the nuclear-electron attractive potential. Evaluation of eq 1 in terms of moleuclar orbitals yields

$$V^K = (1/2)\left[\left\langle e_{gy} \middle| \left(\frac{\partial V}{\partial Q_K}\right)_0 \middle| d_{yz} \right\rangle - \left\langle e_{gx} \middle| \left(\frac{\partial V}{\partial Q_K}\right)_0 \middle| d_{xz} \right\rangle \right] \tag{2}$$

where e_g is the π^* porphyrin molecular orbital and d_{xz} and d_{yz} are the partially filled d levels.

Generally, one expects the matrix elements in eq 2 to be small in magnitude, since movement of nuclei on the porphyrin periphery will not affect the metal orbitals. However, there is appreciable mixing between pure $d_\pi°$ and porphyrin $e_g°$ orbitals in ferric porphyrins (Zerner et al., 1966), and one has

$$|e_{gy}\rangle = |e_{gy}°\rangle + \lambda|d_{yz}°\rangle$$

$$|d_{yz}\rangle = |d_{yz}°\rangle - \lambda|e_{gy}°\rangle$$

With this representation, V^K has an important contribution from

$$\lambda \left\langle e_{gy}° \left| \left(\frac{\partial V}{\partial Q_K} \right)_0 \right| e_{gy}° \right\rangle$$

The matrix element involves only porphyrin atomic orbitals, and so vibrations of the macrocycle are enhanced. Relative to the total scattering intensities in the Q band, Raman scattering in the charge-transfer band is reduced by λ.[2]

The lack of low frequency enhancement is consistent with an in-plane, high-spin ferric ion in acid cytochrome *c*. The Fe–axial ligand stretch is orthogonal to the heme and cannot be enhanced via the in-plane electronic transitions. With an out-of-plane iron such as found in methemoglobin fluoride (Asher et al., 1977) and horseradish peroxidase fluoride (our unpublished results) the Fe–axial ligand vibration has been observed when excited at the charge-transfer maximum (600 nm). Laser irradiation at higher energies may now be exciting band II, the other degenerate charge-transfer state. Under this condition totally symmetric modes are enhanced by coupling to the B state. The symmetry arguments evoked here to explain Raman intensity will not change markedly if there is a small *x,y* inequivalence in the same heme electronic states.

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