the experimental finding of Gibson (1973). For this reason and because of the fact that this set of the parameters does not give the best fit, we did not choose to use these parameters. One very significant result in this paper, though, is a surprisingly good agreement between the experimental data of stripped Hb and the theoretical value which is calculated without using any adjustable parameter. A comparison between a kinetic version of the allosteric transition model of Monod *et al.* (Hopfield *et al.*, 1971) and the experimental data, on the other hand, shows not very convincing agreement. Thus, we may conclude that the sequential theory of Koshland *et al.* (1966) is probably a correct model for representing the oxygenation process of stripped Hb.

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Resonance Raman Spectra of Horseradish Peroxidase: Evidence for Anomalous Heme Structure†

Gopa Rakshit and Thomas G. Spiro*

ABSTRACT: Resonance Raman spectra are reported for native (Fe(III)) horseradish peroxidase (HRP) and its fluoro and cyano derivatives, and also for reduced (Fe(II)) HRP, and it carbonmonoxy and cyanide derivatives. As with other heme proteins, the main Raman bands are attributable to porphyrin vibrations and can be catalogued *via* their polarization properties. Several of the frequencies are sensitive to the structural concomitants of changes in spin and oxidation state of the heme group. Except in the case of native HRP itself, these frequencies classify as expected from the known spin states of the HRP derivatives. Although native HRP contains high-spin Fe(III), the Raman frequencies are less strongly shifted from the low-spin values than is the case for aquomethemoglobin. This finding suggests that doming of the porphyrin ring, which is characteristic of high-spin heme, is less pronounced in native HRP than in aquomethemoglobin. This interpretation is plausibly related to the mechanism of the peroxidase reaction, which appears to involve oxidation of the heme iron to Fe(IV). In a less domed high-spin heme, one electron would be in a relatively high energy orbital and might be subject to facile removal.

Horseradish peroxidase (HRP,[1] donor: H_2O_2 oxidoreductase EC 1.11.1.7) has been the object of extensive study both because of its similarities to and differences from myoglobin (Mb) and hemoglobin (Hb). HRP has been reviewed by Paul (1963), Saunders *et al.* (1964), and Brill (1966). Like the oxygen-carrying heme proteins, HRP contains iron-protoporphyrin IX as a noncovalently bound prosthetic group. The iron atom is accessible to binding by exogenous ligands in both the Fe(III) and Fe(II) oxidation states. These ligands produce the same sorts of alterations in spin state as in Mb and Hb. The constant axial (fifth) ligand is probably a histidine side chain, as evidenced by ultraviolet difference spectra (Brill and Sandberg, 1968), and nitrogen hyperfine splitting of the electron paramagnetic spectrum of the nitrosyl complex (Yonetani and Yamamoto, 1973). Despite these basic similarities, striking differences are also

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[1] Abbreviations used are: HRP, horseradish peroxidase; Mb, myoglobin; Hb, hemoglobin.

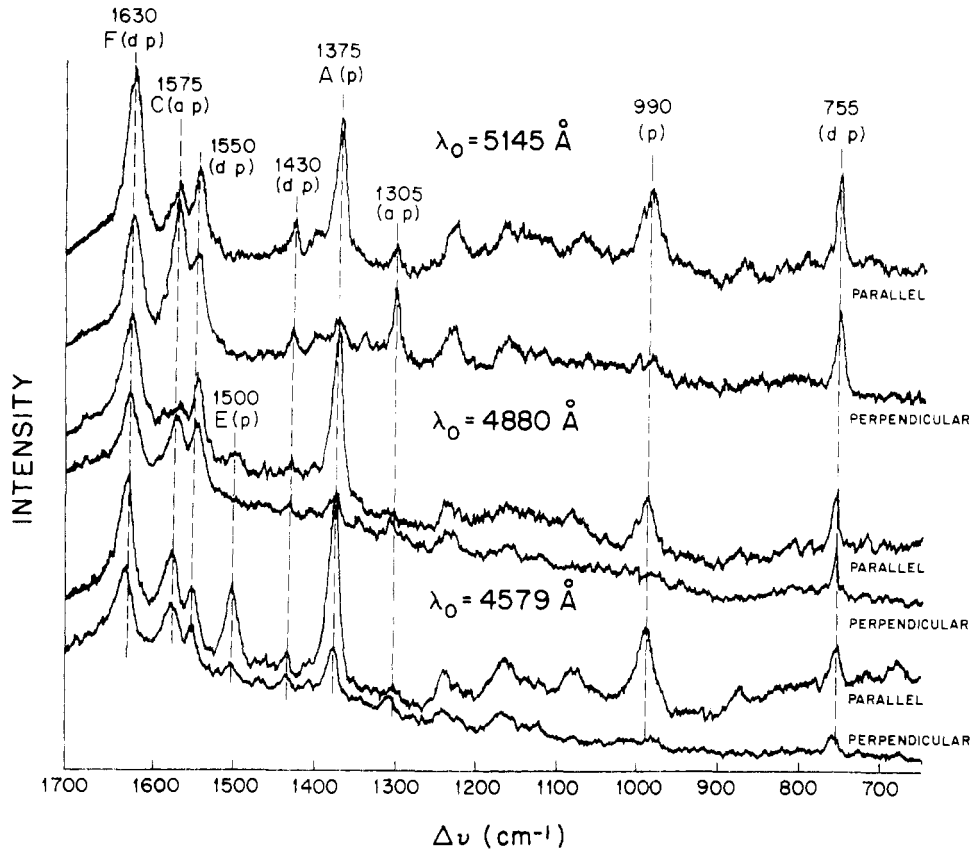

FIGURE 1: Resonance Raman spectra of 0.2 mM HRP (pH 7.0) with Ar^+ laser excitation at 5145 Å (incident power 100 mW; slit width, 6 cm^{-1}), at 4880 Å (incident power, 80 mW; slit width, 6 cm^{-1}), and at 4579 Å (incident power, 50 mW; slit width, 10 cm^{-1}) in both parallel and perpendicular polarization. Instrumental conditions: sensitivity, 10^{-9} A; time constant, 3 sec; scan rate, 50 cm^{-1}/min.

evident. The ligand binding constants are appreciably different for HRP and Mb as are the pH dependencies. Also the Fe(III) form of the protein is more difficult to reduce for HRP with $E^0 = -0.27$ V, compared with $E^0 = 0.05$ V and 0.14 V for Mb and Hb, respectively (Harbury, 1957). Instead the Fe(III) form of HRP is readily oxidized, with peroxides or other oxidizing agents, to metastable products, called compound I (Theorell, 1941) and compound II (Chance, 1952), with two and one oxidizing equivalents, respectively, with respect to the resting enzyme. These compounds are intermediates in the enzymatic oxidation of substrates by peroxides, and their characterization has been the focus of much research on HRP.

Resonance Raman spectroscopy has recently been applied to heme proteins (Strekas and Spiro, 1972a,b; Yamamoto *et al.*, 1973; Brunner *et al.*, 1972; Loehr and Loehr, 1973). In this technique the wavelength of the Raman laser source lies within an absorption band of the sample (Behringer, 1967; Koningstein, 1972). Normal Raman scattering is attenuated, but certain Raman bands, corresponding to vibrations of the chromophoric unit, are greatly enhanced. Consequently vibrational frequencies can be obtained for biological chromophores in their usual high dilution, without interference from their (nonabsorbing) matrices. In the case of heme proteins, the resonance Raman spectra, obtained with heme concentration in the range 10^{-3}–10^{-5} *M*, are dominated by porphyrin ring vibrations (Spiro and Strekas, 1972). These can be correlated with heme structure changes which accompany oxidation and spin-state changes, and several bands have been identified which are reliable indicators of heme structure (Spiro and Strekas, 1974).

In this study we apply resonance Raman spectroscopy to various derivatives of HRP in its Fe(III) and Fe(II) oxidation states. All of the derivatives examined classify as expected from their optical and magnetic properties (Brill and Williams, 1961) with the exception of the resting enzyme, for which the Raman frequencies suggest an anomalous heme structure.

Experimental Section

Horseradish peroxidase (Sigma type VI) was used as purchased. The purity value, defined as the absorptivity ratio at 403 and 275 nm, was 3.0. Solutions were prepared in 0.05 M phosphate buffer (pH 7.0) at 20°, and the concentrations (0.2–0.4 mM) were measured from the Soret absorbance, using ϵ_{430nm} 91 mM^{-1} cm^{-1} (Keilin and Hartree, 1951). The fluoride and cyanide derivatives were prepared by adding solid NaF or KCN, respectively, until the absorption spectral changes (Keilin and Hartree, 1951) were saturated. Ferro-HRP was prepared by adding solid sodium dithionite to the solution of the native protein. Its cyanide and carbonmonoxy derivatives were prepared by adding KCN and bubbling CO, respectively, in the presence of excess sodium dithionite.

Raman spectra were obtained with Ar^+ laser (Coherent CR-5) excitation at 5145, 4880, or 4579 Å. The solutions were held in 1-mm glass capillary tubes (transverse illumination) or in a glass spinning cell (Kiefer and Bernstein, 1971). The light scattered at 90° was collected in a Spex 1401 double monochromator equipped with a cooled ITT FW 130 photomultiplier and a polarization scrambler. A polaroid disk was used to analyze the parallel and perpendicular spectra.

TABLE I: Resonance Raman Vibrational Frequencies (cm^{-1}) of HRP and Its Derivatives.[a]

Fe(II)HRP (high spin)	Fe(III)HRP Fluoride (high spin)	Fe(III)HRP (pH 7.0) (high spin)	Fe(III)HRP cyanide (low spin)	Fe(II)HRP cyanide (low spin)
1605 dp, s	1608 dp, vs	1630 dp, vs	1642 dp, vs	1620 dp, m
1625 d, m	1630 p, s		1625 p, m	
1585 p, m	1580 p, w	1608 p, w		1605 p, vw
1553 ap, s	1555 ap, m	1575 ap, s	1590 ap, m	1587 ap, s
1563 p, m	1565 p, m	1575 p, m	1598 p, m	1598 p, m
1546 dp, m	1563 dp, m	1550 dp, m	1562 dp, m	1545 dp, m
			1555 dp, vw	
1472 p, m	1482 p, m	1500 p, m	1497 p, m	1498 p, m
1420 dp, w	1427 dp, w	1430 dp, w	1434 dp, w	1435 dp, w
1392 dp, w	1394 dp, w	1405 dp, w	1403 dp, w	1400 dp, w
1358 p, vs	1373 p, vs	1375 p, vs	1375 p, vs	1362 p, vs
1335 dp, w	1337 ap, w	1345 ap, w	1345 ap, w	1343 ap, w
1307 ap, w	1306 ap, w	1305 ap, m	1312 ap, w	1315 ap, w
1213 dp, w	1215 dp, vw	1230 dp, w		1235 dp, w
1178 dp, w	1177 dp, w	1170 dp, w	1167 dp, w	1172 dp, w
1136 ap, vw	1137 ap, vw	1140 ap, vw		1132 dp, vw
	990 p, w	990 p, m		
752 dp, w	753 dp, w	755 dp, m	756 dp, w	753 dp, w
674 p, w		675 p, w	680 p, w	681 p, w

[a] s = strong; m = medium; w = weak; v = very; p = polarized; dp = depolarized; ap = anomalously polarized.

Results

Resonance Raman spectra of HRP obtained by excitation with various lines of the Ar^+ laser were similar in appearance (see Figures 1-3) to those exhibited by hemoglobin and cytochrome *c* (Spiro and Strekas, 1974). As in the latter cases the various Raman bands can be distinguished by their three different states of polarization (p = polarized, $\rho_l < 3/4$; dp = depolarized, $\rho_l = 3/4$; and ap = anomalously polarized, $\rho_l > 3/4$, where ρ_l, the depolarization ratio, is the intensity ratio of the scattering components polarized perpendicular and parallel to the incident laser polarization) which are the indicators of the symmetries of the vibrations. Also p bands can be resolved from dp and ap bands by varying the excitation wavelength; the former dominate the spectrum in the vicinity of the near-uv Soret absorption band, while the latter dominate in the vicinity of the visible, α-β, absorption bands (Spiro, 1974).

The observed Raman frequencies and their states of polarization are given in Table I, which also indicates the oxidation and spin states of the various derivatives. As in the case of hemoglobin and cytochrome *c*, a slight decrease in most of the vibrational frequencies is found to accompany reduction of Fe(III) to Fe(II) heme, in the absence of a spin-state change. Much larger decreases of certain frequencies accompany conversion of heme, in a given oxidation state, from low to high spin. These latter shifts are attributable to the change in heme structure which is known to accompany spin-state changes (Spiro and Strekas, 1974). While low-spin hemes are planar, the iron atom moves out of the plane on conversion to high-spin forms, with an attendant doming of the porphyrin ring (Perutz, 1970, 1972; Hoard, 1971).

With the exception of the resting Fe(III) enzyme, to be discussed below, all the derivatives of HRP display Raman frequencies very similar to those observed for corresponding hemoglobin derivatives. In the previous study of hemoglobin and cytochrome *c* derivatives, six bands were selected as structural markers because of their dependence on oxidation and spin states. Two of these bands, however, identified as B and D by Spiro and Strekas (1974), are difficult to resolve, especially in protoheme derivatives, for which the

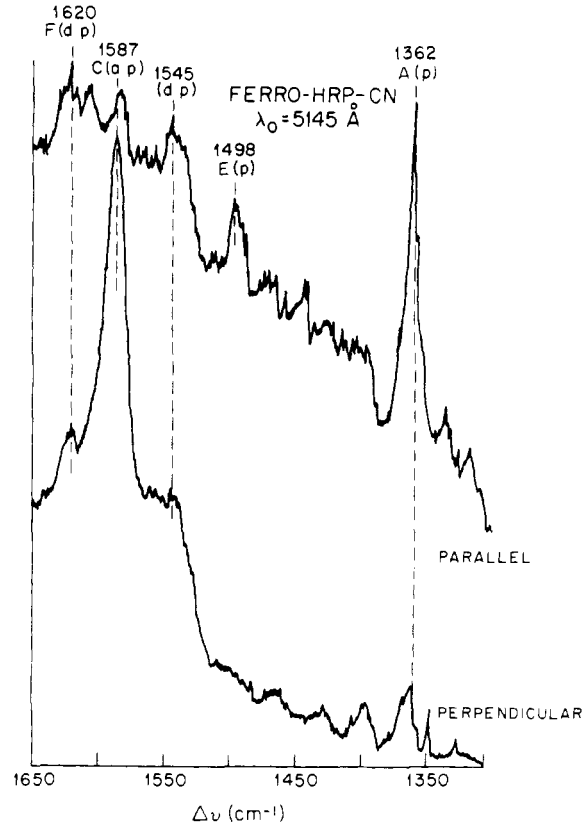

FIGURE 2: Resonance Raman spectra of Ferro-HRP cyanide in the visible region λ_0 5145 Å. The solution contained 0.3 mM heme. Incident power was 55 mW. Instrumental condition: sensitivity 10^{-9} A, time constant, 3 sec; slit width, 6 cm^{-1}, and scan rate, 50 cm^{-1}/min.

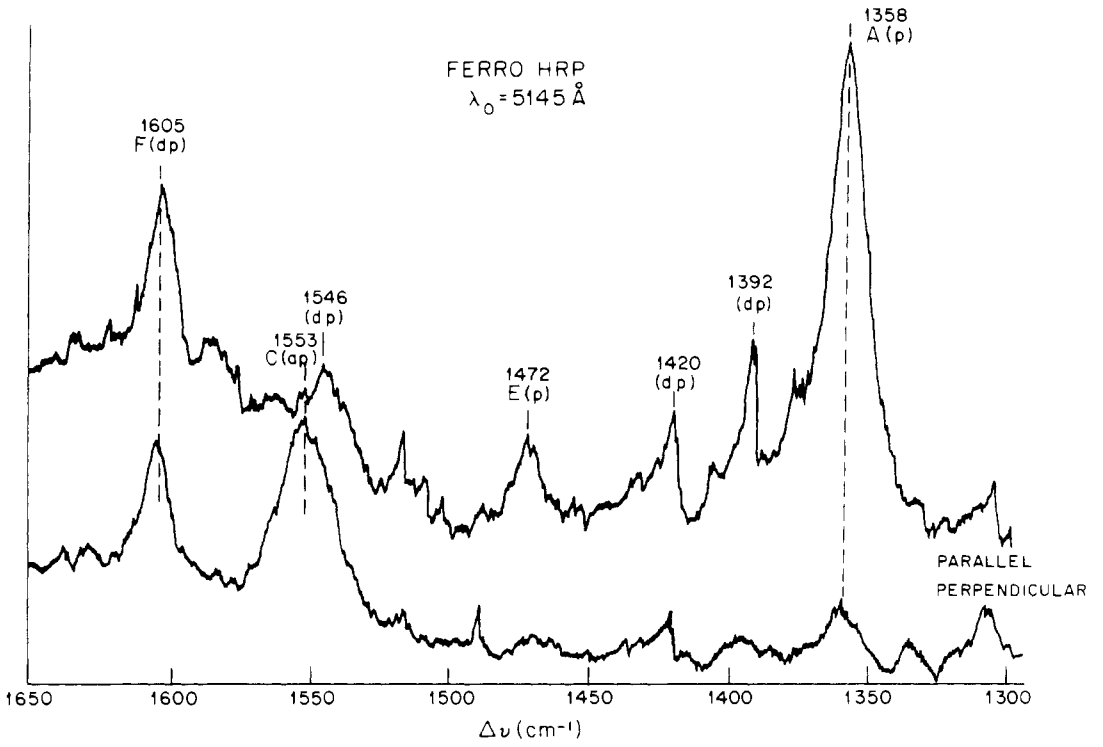

FIGURE 3: Resonance Raman spectra of Fe(II)HRP (0.3 mM in heme) with Ar^+ laser excitation at 5145 Å (incident power, 100 mW; slit width, 6 cm^{-1}). Instrumental conditions: sensitivity, 10^{-9} A, time constant, 3 sec; and scan speed, 50 cm^{-1}/min.

vinyl peripheral substituents introduce extra Raman bands. Table II lists the frequencies of the remaining four bands, A, C, E, and F, for derivatives of HRP, Hb, cytochrome *c*, and cytochrome *c'* (Strekas and Spiro, 1974). Leaving aside resting Fe(III) HRP and Fe(III) cytochrome *c'*, the classification holds up well for this extended series of derivatives. As illustrated in Figure 4, the groupings of frequencies for a given oxidation and spin state are only a few cm^{-1} wide, and are distinct from other groupings. Bands A and C are sensitive primarily to oxidation and to spin state, respectively, while bands E and F are influenced by both. As noted earlier (Spiro and Strekas, 1974), both O_2Hb and COHb classify as low-spin Fe(III) hemes, consistent with back donation of iron electrons into π^* acceptor orbitals of O_2 and CO. This interpretation is also consistent with the observed lowering of the O_2 (Barlow *et al.*, 1973) and CO (Alben and Caughey, 1968) stretching frequencies in the Hb derivatives. Spectra of CO-Fe(II)HRP were difficult to obtain because of the instability of the complex, but they reproducibly showed a strong polarized band at 1375 cm^{-1}, the characteristic Fe(III) position for the oxidation state marker band A. On the other hand, CN^--Fe(II)HRP

TABLE II: Heme-Structure Sensitive Raman Frequencies of Various Proteins.[a]

Heme Protein	Oxidation	Spin	Structure Sensitive Raman Frequencies			
			F(dp)	C(ap)	E(p)	A(p)
Fe(II)-HRP[b]	Fe(II)	High	1605	1553	1472	1358
Deoxy-Hb[c]	Fe(II)	High	1607	1552	1473	1358
Ferrocytochrome *c'*, pH 6.9[d]	Fe(II)	High	1609	1557	1475	1355
Fe(III)Hb fluoride[c]	Fe(III)	High	1608	1555	1482	1373
Fe(III)HRP fluoride[b]	Fe(III)	High	1608	1555	1482	1373
Fe(III)HRP, pH 7.0[b]	Fe(III)	High	1630	1575	1500	1375
Ferricytochrome *c'*, pH 10.3[d]	Fe(III)	Intermediate	1633	1573	1496	1373
Ferricytochrome *c'*, pH 6.9[d]	Fe(III)	Intermediate	1637	1578	1500	1372
Ferrocytochrome *c*[c]	Fe(II)	Low	1620	1584	1493	1362
Fe(II)HRP cyanide[b]	Fe(II)	Low	1620	1587	1498	1362
Ferrocytochrome *c'*, pH 12.0[d]	Fe(II)	Low		1589		1360
Ferricytochrome *c*[c]	Fe(III)	Low	1636	1582	1502	1374
Oxyhemoglobin[c]	Fe(III)	Low	1640	1586	1506	1377
Fe(III)HRP cyanide[b]	Fe(III)	Low	1642	1590	1498	1375
Fe(III)Hb cyanide[c]	Fe(III)	Low	1642	1588	1508	1374

[a] p = polarized; dp = depolarized; ap = anomalously polarized. [b] This study. [c] From Spiro and Strekas, 1974. [d] From Strekas and Spiro, 1974.

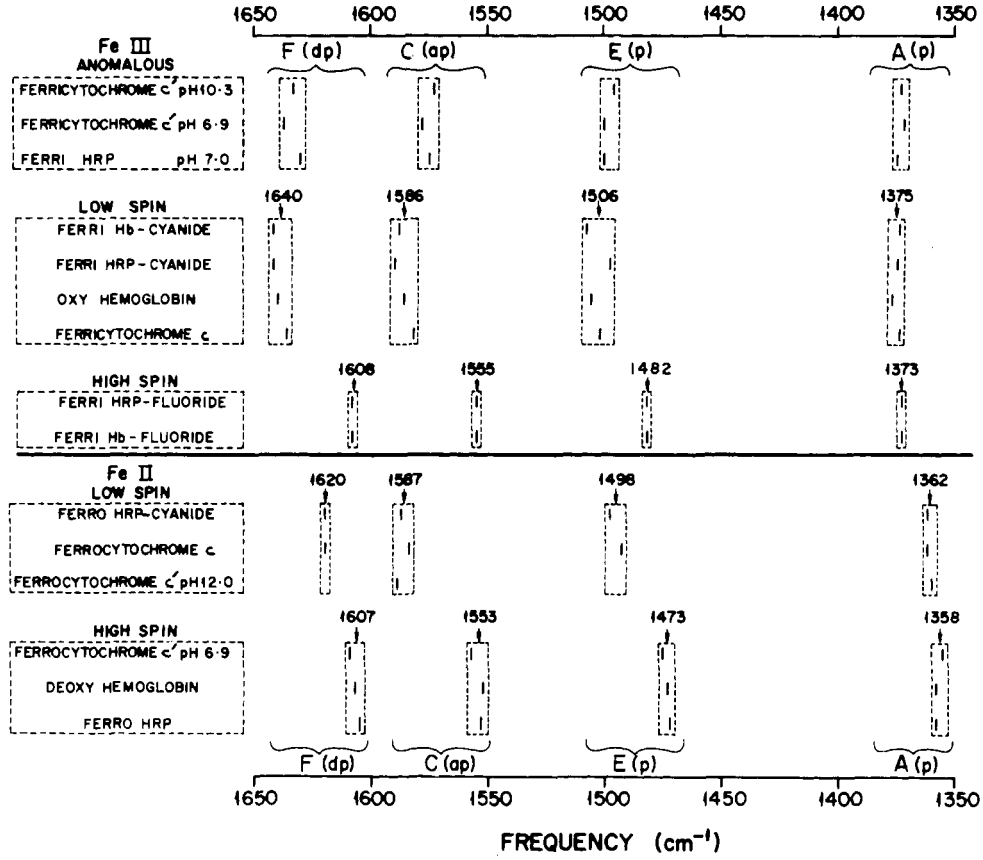

FIGURE 4: Frequency (cm^{-1}) pattern for structure-sensitive heme Raman bands, of various heme proteins. A, C, E, and F bands exhibit the predominant shifts in Raman frequencies, on changing spin and oxidation states. p = polarized; dp = depolarized; ap = anomalously polarized.

classifies as low-spin Fe(II), and provides the first Raman data (CN^--Fe(II)Hb is unstable; Keilin and Hartree, 1955) of a low-spin Fe(II) protoheme with vinyl substituents. In the previous examples, ferrocytochrome *c* and ferrocytochrome *c'* (pH 12), the vinyl groups are replaced by nonconjugated thioether links. Although isoelectronic with CO, CN^- is a poorer π acceptor, and while it serves to convert high-spin Fe(II)HRP to the low-spin state, no appreciable back donation of iron electrons is observed.

Resting Fe(III)HRP is known to be high spin in the pH range 5.0–9.0 (Brill and Williams, 1961). While the magnetic moment (Keilin and Hartree, 1951) is slightly less than that expected for five unpaired electrons $s = 5/2$, the Mössbauer results at pH 8.9 (Moss *et al.*, 1969) and the optical and electron paramagnetic resonance (epr) spectra measured by Blumberg *et al.* (1968), between pH 7.0 and 8.4, clearly indicate a single high-spin Fe(III) species. However, the resonance Raman spectra of Fe(III)HRP, which remain unchanged in the pH range 5.0–8.0, reveal that the Raman marker frequencies do not fall into the high-spin Fe(III) groupings, as shown in Table II and Figure 4. Rather they are closer to the low-spin Fe(III) groupings. A similar situation was encountered for ferricytochrome *c'* (Strekas and Spiro, 1974), but in that case the Raman bands could be assigned to an intermediate-spin heme, consistent with observed intermediate magnetic moments (a spin state mixture was ruled out by the observation of only one set of vibrational frequencies, rather than two) and with a recent epr analysis (Maltempo *et al.*, 1974).

Discussion

The spin-state associated structural feature, to which the Raman marker bands are presumably sensitive (Spiro and Strekas, 1974), is thought to be the doming of the porphyrin ring upon the out-of-plane displacement of the iron atom (Perutz, 1970; Hoard, 1971) accompanying the transition from low-to-high-spin hemes. Preliminary results of normal coordinate calculations (Stein *et al.*, 1974) confirm that the observed lowering of the Raman marker bands is a reasonable consequence of kinematic and force constant changes associated with doming of the porphyrin ring. Accordingly the anomaly in the marker band frequencies of resting Fe(III)HRP can be associated with a high-spin Fe(III) heme which is closer to being planar than is the case for the other high-spin Fe(III) heme proteins so far examined, *i.e.*, F^--Fe(III)Hb, H_2O-Fe(III)Hb, and F^--Fe(III)HRP.

While heme structure is often viewed as a function of spin state, it is perhaps more instructive to consider the spin state as a function of heme structure, especially for heme proteins, where the polypeptide chain imposes structural constraints of its own. These constraints have been emphasized by Perutz (1970, 1972), Hoard (1971), and Hopfield (1973) for hemoglobin, for which it appears that tension in the polypeptide-heme complex is responsible for the conformation changes that produce reversible and cooperative binding of oxygen. It is conceivable that polypeptide constraints in different proteins could produce different heme structures, *e.g.* different degrees of doming for nominally out-of-plane structures. These differences might not affect the spin state in a linear fashion, since the number of unpaired electrons depends on a complex balance between the orbital energies and the pairing energy.

A simplified view of this balance for various heme struc-

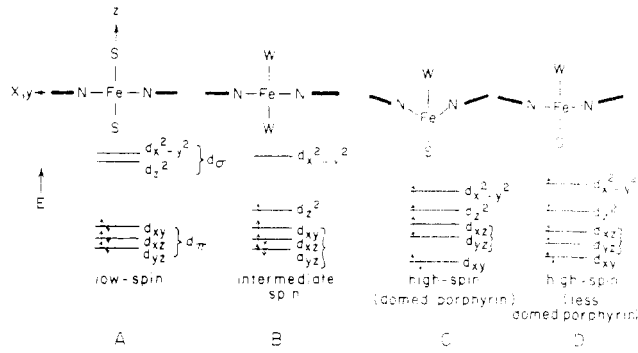

FIGURE 5: Heme ligation, geometry, and spin state. Energy level schemes of heme iron d orbitals for d^5 and d^6 configurations, representing the effect of different ligation and concomitant structural changes on the spin state.

tures is given in Figure 5. The first structure is that of a typical low-spin heme, with strong-field axial ligands and a planar porphyrin ring. The d_σ antibonding orbitals, $d_{x^2-y^2}$ and d_{z^2}, are raised in energy by interaction with strong planar and axial ligand fields. The energy gap with respect to the d_π orbitals, d_{xy}, d_{xz}, and d_{yz}, is larger than the pairing energy, so the five or six d electrons occupy the d_π orbitals, leaving $s = \frac{1}{2}$ and 0 for Fe(III) and Fe(II), respectively. In the second structure the stong-field axial ligands are replaced by weak field ligands, but the porphyrin ring remains planar. The two d_σ orbitals are now split: $d_{x^2-y^2}$ remains at about the same energy, since its interaction with the pyrrole nitrogen atoms remains the same, but the d_{z^2} orbital energy is lowered. If it is lowered far enough then the gap between $d_{x^2-y^2}$ and d_{z^2} may exceed the pairing energy while the latter exceeds any of the other orbital energy separations. Consequently, only the $d_{x^2-y^2}$ orbital remains unoccupied and the spin state is intermediate, $s = \frac{3}{2}$ and 1 for Fe(III) and Fe(II), respectively. This situation obtains for the recently prepared unligated ferrotetraphenylporphyrin (Collman and Reed, 1973), and may obtain for the ferricytochrome *c'*, described above (Strekas and Spiro, 1974).

The third structure in Figure 5 is the one found in aquomethemoglobin. Here one axial ligand (imidazole) is strong field, while the other (water) is weak field. The iron is displaced from the mean heme plane in the direction of the strong-field ligand, and the porphyrin ring is domed, allowing the pyrrole nitrogen atoms to point toward the iron atom. The d_{z^2} energy is lowered, because one of the axial ligands is weak field, while the $d_{x^2-y^2}$ energy is lowered because of the out-of-plane geometry (Griffith, 1964). None of the energy separations exceed the pairing energy and all orbitals are occupied, giving $s = \frac{5}{2}$ and $\frac{4}{2}$ for Fe(III) and Fe(II), respectively. The final structure in Figure 5 represents our model for the heme structure in resting Fe(III)HRP. The only difference from H_2O-Fe(III)Hb is that the porphyrin ring is less domed. Accordingly the $d_{x^2-y^2}$ orbital is raised in energy, but not by enough to exceed the pairing energy, so that the spin state remains $s = \frac{5}{2}$.

This picture is greatly oversimplified, particularly because the actual many-electron states of the heme group are not adequately represented by a set of one-electron orbitals. Electron correlation alters the energy levels appreciably. Indeed the $s = \frac{5}{2}$ and $s = \frac{3}{2}$ states can mix quantum mechanically, so that no sharp distinction between high and intermediate spin states is possible (Maltempo *et al.*, 1974). Nevertheless the qualitative relationships implied in Figure 5 may prove useful in understanding the properties of heme proteins.

In particular the structural difference suggested between H_2O-Fe(III)Hb and resting Fe(III)HRP would leave the latter with at least one electron in a relatively high-energy orbital, which would be easy to remove. This could help to account for the ready formation of compounds I and II, with two and one oxidizing equivalents, respectively, above the resting enzyme. Spectroscopic evidence (Chance, 1949; Blumberg *et al.*, 1968; Moss *et al.*, 1969) strongly indicates that in compound II the iron atom is in oxidation state Fe(IV). There is evidence that compound I also contains Fe(IV), with the extra oxidizing equivalent present as a free radical, either on the porphyrin ring (Dolphin and Felton, 1974) or on a nearby protein residue.

It is possible, then, that the HRP polypeptide chain arranges for the facile oxidation of Fe(III) to Fe(IV) by constraining the porphyrin ring to a more planar conformation, thereby raising the $d_{x^2-y^2}$ orbital energy. This would be another illustration of the apparently widespread propensity of enzymes to build unusual conformations into their active sites to facilitate reactions, which Vallee and Williams have discussed as the "entatic state" (Vallee and Williams, 1968). Thus while hemoglobin appears to apply tension to the protein–heme linkage, in order to trigger conformation change on reversible oxygenation, HRP may instead apply compression to this linkage, in order to facilitate oxidation.

The proposed heme conformation of native HRP might be maintained through constraints between the histidine bound to the iron atom and the groups which interact with the porphyrin ring. Alternatively it may result from constraints on the *distal* side of the heme. There is no firm evidence on the identity of the sixth ligand in native HRP. If, as is often supposed, it is a water molecule, it must be rather firmly bound to the protein, presumably through hydrogen bonding, since the transition to an alkaline low-spin form is a full 2 p*K* units higher (11.0 *vs.* 9.0) for HRP (Keilin and Hartree, 1951) than for metHb (George and Hanania, 1953), and since the binding of exogenous ligands seems to be accompanied by binding of a proton (Brill, 1966). Alternatively the sixth ligand might be a weak-field protein side chain, *e.g.*, carboxylate. In either case a role for the sixth ligand in maintaining the special conformation of native HRP is strongly supported by the observation that its replacement by fluoride apparently returns the heme to the more domed conformation characteristic of F^--Fe(III)Hb, as shown by the resonance Raman frequencies of F^--Fe(III)HRP.

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The Kinetics of Oxidation of Reduced Cytochrome *c* by Ferricyanide Derivatives†

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ABSTRACT: The kinetics of oxidation of reduced horse heart cytochrome *c* by ferricyanide derivatives, $Fe(CN)_5X^{n-}$, where X = PPh_3, SCN^-, CN^-, NH_3, and N_3^-, were measured by stopped-flow techniques. The reactions were found to be first order in the concentration of both reduced cytochrome *c* and oxidizing agent. The rate constant for the oxidation increased as the reduction potential increased. The values were 3.0×10^7 M^{-1} sec^{-1} for $Fe(CN)_5PPh_3^{2-}$, 1.0×10^7 M^{-1} sec^{-1} for $Fe(CN)_5CNS^{3-}$, 8.0×10^6 M^{-1} sec^{-1} for $Fe(CN)_6^{3-}$, 2.5×10^6 M^{-1} sec^{-1} for $Fe(CN)_5NH_3^{2-}$, and 9.0×10^5 M^{-1} sec^{-1} for $Fe(CN)_5N_3^{3-}$. The rate of oxidation decreased markedly as the ionic strength increased and also decreased somewhat in 10% 1-propanol. The entropy of activation of about −20 eu was found to be nearly independent of the oxidizing agent but the energy of activation was found to decrease with increasing rate constant from a value of 3.5 kcal/mol for $Fe(CN)_5N_3^{3-}$ to an unusually low value of about 1 kcal/mol. The abnormally low value for the energy of activation was explained in terms of two mechanisms: one in which the oxidizing agent was first bound to the cytochrome *c* followed by slow electron transfer, and the other in which the cytochrome *c* exists in a labile equilibrium with another more easily oxidizable conformation.

Numerous investigations of the kinetics of oxidation of reduced cytochrome *c* by various oxidizing agents have previously been reported. Among these are several studies on the rate of oxidation of reduced cytochrome *c* by potassium ferricyanide over a wide range of pH, ferricyanide concentration, temperature, and ionic strength (Brandt *et al.*, 1966; Havsteen, 1965; Sutin and Christman, 1961; Morton *et al.*, 1970). There are, however, no data available on how the redox potential of the oxidizing agent affects the rate of oxidation. To study this effect of redox potential we have selected a series of ferricyanide derivatives, $Fe(CN)_5X^{n-}$, where X = azide, cyanide, triphenylphosphine, ammonia, and thiocyanate. Also included in the study are the oxidizing agents $Fe(dipy)(CN)_4^-$ and $Fe(dipy)_2(CN)_2^+$.[1] This

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[1] Abbreviations used are: dipy, α,α'-dipyridyl; PPh_3, triphenylphosphine.