4. The holoazurin Trp ^1L_b absorption band red shifts ca. 1 nm compared to apoazurin, while the ^1B_b transition red shifts ca. 2 nm. The hydrophobicity of the Trp environment appears little changed between Cu(I) azurin, Cu(II) azurin, and apoazurin as evidenced by the narrowness of the Trp ^1L_b 0-0 bands and from the Raman band shape for the Trp 1354-cm^{-1} band. The Trp 0-0 ^1L_b absorption spectral shifts most likely derive from subtle environmental changes, possibly alterations at sites somewhat distant from the Trp ring, such as alterations in electrostatic interaction from the Cu site.

5. The absorption difference spectra (oxidized minus reduced as well as holoazurin minus apoazurin) show large changes in the 200-300-nm spectral region which presumably derive from alterations in sulfur and imidazole → Cu(II) charge-transfer transitions. Numerous charge-transfer transitions can occur since the Cu is ligated to two histidines, one cysteine, and one methionine; simple Cu(II)-(imidazole)$_4$ complexes show increased absorbances throughout the 200-300-nm spectral region (Figure 6).

We thus conclude that facile energy transfer between the Trp and the Cu-ligand complex is responsible for the fluorescence quenching in Trp. Presumably this quenching occurs between the Trp $^1L_{a,b}$ states and a Cu-ligand charge-transfer state. In addition, the Trp absorption spectral shifts between the Cu(I) azurin, Cu(II) azurin, and apoazurin suggest intimate coupling between the electronic transitions of the Cu-ligand complex and those of Trp. It is possible that strong excitonic interactions are present.

Registry No. Trp, 73-22-3; His, 71-00-1; Cu, 7440-50-8.

A Molecular Model for the Major Conformational Substates in Heme Proteins†

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Abstract: We present a molecular model of the major "conformational substates" observed by infrared spectroscopy in carbonmonoxyhemoglobins, myoglobins, and peroxidases, in terms of an electrical perturbation of the CO fundamental vibrational frequencies due to the possibility of $H^{\epsilon 2} \leftrightarrow H^{\delta 1}$ tautomerism, and 180° C^{β}-C^{γ} ring flips, of distal histidine residues. The model supports a previous interpretation of vibrational frequencies and nuclear magnetic resonance chemical shifts of CO ligands in heme proteins as originating in a weak electric field (dipole and quadrupole interactions with permanent dipole moments, dipole polarizabilities, and shielding polarizabilities), and opens up the possibility of future detailed molecular interpretations of both NMR chemical shifts (of ^1H, ^{13}C, and ^{15}N), as well as IR data, on proteins and other macromolecules.

Conformational substates (CS) in heme proteins, as evidenced by both infrared (IR) spectroscopy[1,2] and X-ray crystallography,[3] have been a topic of considerable interest for many years, and great emphasis has been placed on investigating the heme proteins carbonmonoxyhemoglobin and carbonmonoxymyoglobin. Much valuable information has been obtained from such studies, but no entirely satisfactory molecular model for the individual CSs has been forthcoming. We propose in this paper a molecular model that appears to rationalize the observation of numerous substates in a wide variety of heme proteins, including the hemoglobins, myoglobins, and several peroxidases. Our model is extremely simple, and involves electric field induced frequency shifts due to 180° ring flips of the $H^{\delta 1}$ and $H^{\epsilon 2}$ tautomers of distal histidine residues.

Recently, we have been investigating the ^{13}C and ^{17}O nuclear magnetic resonance (NMR) spectra of ^{13}CO and $C^{17}O$ ligands in a series of carbonmonoxyheme proteins, as a prelude to an investigation of the long-standing general problem of the nature of the chemical shift nonequivalences seen in proteins, and we have found an excellent correlation between the isotropic chemical shieldings, $\delta_i(^{13}C)$ and $\delta_i(^{17}O)$, and the mean fundamental vibration frequency, ν_{CO},[4] of the heme's CO ligand. Our observations, and additional correlations with ν_{FeC}, and with the $C^{17}O$ nuclear quadrupole coupling constant, e^2qQ/h, were explained in terms of a "back-bonding" model.[4] However, there seems to be a more fundamental and quantitative basis for the correlations between the chemical shifts, CO vibrational frequencies, and the nuclear quadrupole coupling constants.

Calculations we have carried out suggest that the perturbation of heme-CO by other ligands is largely electrical in nature.[5] This is consistent with the idea that the primary electronic structure change in a molecule from perturbation of a nearby, but not chemically bonded, molecule is charge polarization.[6] This idea has provided a basis for a general, weak interaction model,[7] and it was employed in our recent study[5] of carbonmonoxyheme proteins. We calculated the effect on the vibrational potential and the chemical shielding tensors due to a range of electrical potentials that arise from a nearby point dipole and point quadrupole. The result was that we were able to suggest an explanation for the general trends seen in the heme proteins: a $\delta_i(^{13}C)$ increase, $\delta_i(^{17}O)$ decrease, $e^2qQ/h(^{17}O)$ decrease, and ν_{CO} decrease, in terms of the interaction of CO with an electric field. Although the

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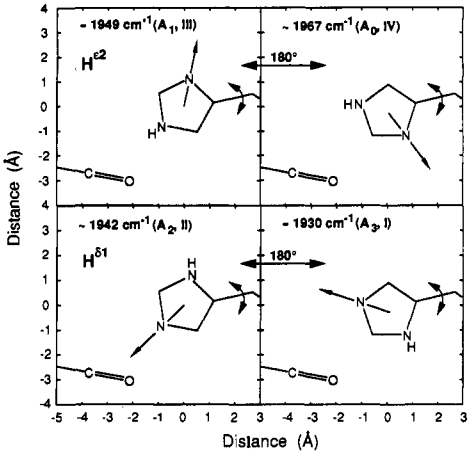

Figure 1. Schematic diagram illustrating the $H^{\delta 1}$ and $H^{\epsilon 2}$ tautomers of the C^{β}–C^{γ} ring-flip isomers of the distal histidine in HbCO or MbCO, together with the FeCO fragment of the heme.

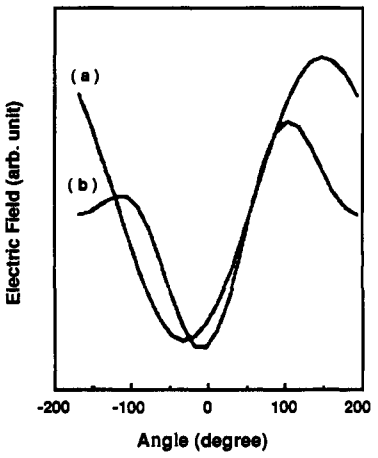

Figure 2. Electric field strengths at CO, given in arbitrary units, as a function of the orientation angle, in degrees, due to (a) the dipole moment of methylimidazole and (b) the dipole plus quadrupole.

electric properties calculated for the CO group did not reflect the Fe–CO bonding, the results are meaningful in showing the relative effect of an external field. This has now led to the idea that the conformational substates in many heme proteins could be due primarily to the nature of the local electric potential.

It is clear from Figure 1 that four different electrical fields can be generated from a distal histidine residue by the simple processes of $H^{\delta 1} \leftrightarrow H^{\epsilon 2}$ tautomerism, combined with a 180° C^{β}–C^{γ} ring flip. The CO is close enough to be influenced by the histidines' dipole and quadrupole moments. We have carried out ab initio electronic structure calculations on methylimidazole, and find that, at the four orientations shown in Figure, the field due to the quadrupole happens to be essentially proportional to the field due to the dipole (see Figure 2). Thus, it is reasonable for the cosine dependence of just a dipole interaction to represent the deviation of the fundamental vibrational frequency, $\Delta\bar{\nu}$, on θ, the angle between E_x and the CO bond axis. For simplicity, we treat the problem in this paper in an empirical fashion, and write the fundamental vibrational frequency, $\bar{\nu}$, in the carbonmonoxyheme proteins as

$$\bar{\nu} = \bar{\nu}_0 + a \cos\left[\frac{n360}{5} + b\right] \qquad (1)$$

where $\bar{\nu}_0$ and a are variables that describe the field-free fundamental vibrational frequency and the histidines local charge field, which on the basis of the dipole moment and other considerations is expected to be $\sim 4 \times 10^7$ V cm^{-1}. n takes the values −1, 0, 1, 2 by symmetry, and b reflects in a simple way the orientation of the CO bond axis with respect to the imidazole, as shown in Figure 1.

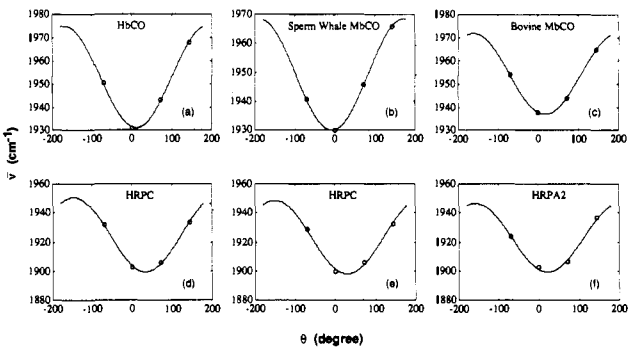

Figure 3. Comparison between experimental data (circles) and eq 1 (solid curve) for (a) HbCO, average values from Potter et al. (ref 2); (b) sperm whale MbCO (ref 1); (c) bovine heart MbCO (ref 8); (d) horsedish peroxidase (mainly C isoenzyme, Uno et al., ref 14); (e) horseradish peroxidase, C isoenzyme (ref 15); and (f) horseradish peroxidase, A_2 isoenzyme (from ref 15).

We obtain excellent agreement with the experimental observations[2] on HbCO using the following parameters: $\bar{\nu}_0 = 1954$ cm^{-1}, $a = -23$ cm^{-1}, $b = -11°$. Our results are shown in Table I and graphically in Figure 3a. Thus, the electric field generated by the distal histidine, which is immediately adjacent to the CO ligand, has four projections (cos −82°, cos −10°, cos 62°, cos 134°) due to the presence of a large dipole moment vector[28] that rotates by 360/5 = 72° in the $H^{\delta 1}$ and $H^{\epsilon 2}$ tautomers of the two, 180° ring-flip isomers. The resultant fields are then $\sim 5.5 \times 10^6$, 3.9×10^7, 1.9×10^7, and -2.8×10^7 V cm^{-1}. Fields of this size significantly affect vibrational stretching frequencies.[5,6]

Since we have used three adjustable parameters to fit four vibrational frequencies, the question of the adequacy of this fitting procedure needs to be addressed. Can we fit anything?

There are 4!/2 = 12 possible combinations of the four dipole moment vector orientations for the distal histidine residue. We have investigated several different proteins (Table I) to determine whether all of these combinations can be fit by judicious choice of ν_0, a, and b. For example, for mouse HbCO, the 12 solutions yield the following rms deviations between theory and experiment: 0.1, 0.8, 3.2, 4.1, 4.1, 6.1, 6.9, 8.8, 8.9, 9.9., 10.7, and 11.8 cm^{-1}. Clearly, there are at most two solutions, having $b = -11°$ and −61°, and only the first one is close to that anticipated from X-ray studies of CO hemoglobins. Similar behavior is found for other proteins, so it is clear that varying ν_0, a, and b is only capable of producing a small number of acceptable solutions.

We also investigated the effect of a small deviation in dipole moment (μ) orientation using a set of charges computed via an ab initio method, where for the two tautomers of methyl imidazole (a histidine model) we found orientations of 24.9 ($H^{\epsilon 2}$) and 15.6 ($H^{\delta 1}$) (rotated toward the methyl group). Using the Brookhaven Protein Data Bank coordinates for MbCO (File No. 1 mb5), we determined a best fit having an rms error of 0.95 cm^{-1}, with $\nu_0 = 1949$ cm^{-1}, $a = -16.7$ cm^{-1}, and $b = 7.2°$. This compares quite well with the values of $\nu_0 = 1953.4$ cm^{-1}, $a = -19.8$ cm^{-1}, and $b = -12.7°$ shown in Table I. Given the uncertainties in the Fe–C–O orientation, and the simplicity of the model, we believe this represents very good agreement, and confirms our basic assignments.

This very simple model appears to explain, or at least offer an alternative way of thinking about, many observations of "conformational substates", and related phenomena seen in heme proteins, which we now enumerate: (1) The model predicts *four* substates in HbCO, corresponding to states I, II, III, and IV, providing an explanation of the over 100 vibrational frequencies reported by Potter et al.[2] in a wide range of CO–hemoglobins. (2) The model provides an explanation of the four major substates, A_0–A_3, observed by Frauenfelder et al.[1] in sperm whale MbCO, giving them a molecular basis. The $\bar{\nu}_0$, a, and b values found for sperm whale MbCO are also given in Table I, and comparison with experiment is shown graphically in Figure 3b. (3) The model suggests a molecular basis for the "four, and only four" conformers (I–IV) found in bovine heart MbCO by Caughey et al.,[8] and his

Table I. Experimental and Calculated C–O Infrared Vibrational Stretch Frequencies for the Major Conformational Substates of Heme Proteins

		$\nu(A_0, IV)$		$\nu(A_1, III)$		$\nu(A_2, II)$		$\nu(A_3, I)$		fitting parameters		
protein	temp (°C), pH	exptl (cm^{-1})	calcd (cm^{-1})	exptl (cm^{-1})	calcd (cm^{-1})	exptl (cm^{-1})	calcd (cm^{-1})	exptl (cm^{-1})	calcd (cm^{-1})	ν_0 (cm^{-1})	a (cm^{-1})	b (deg)
human HbA[a]	28, 7.4	1969.5	1969.4	1951.1	1951.2	1943	1943.1	1932	1931.9	1954	−22.53	−10.83
human HbA[a]	30, 5.0	1968.5	1969	1951.5	1950.9	1943	1942.1	1930	1930.9	1953.5	−23.07	−11.63
human HbA[a]	30, 9.5	1969	1969.3	1951	1950.7	1942.5	1942	1930	1930.5	1953.4	−23.39	−11.27
horse Hb[a]	30, 7	1968	1967.8	1949.7	1949.9	1942	1942.3	1931.5	1931.2	1952.7	−21.9	−10.49
horse Hb[a]	9, 7	1969	1968.8	1949.3	1949.4	1941.5	1941.7	1930	1929.8	1952.7	−23.28	−10.02
cow Hb[a]	36, 7	1968	1967.5	1950	1949.5	1941	1940.7	1932	1932.5	1950.8	−18.87	14.23
cow Hb[a]	7, 7	1968	1968.3	1949.6	1949.2	1940.5	1940.3	1931.5	1931.9	1950.5	−19.15	14.22
cotton tail rabbit Hb[a]	30, 7	1968	1968.1	1950.6	1950.4	1943.5	1943.4	1935	1935.2	1952.2	−17.4	12.27
cotton tail rabbit Hb[a]	10, 7	1970	1969.8	1950.3	1950.6	1944	1944.2	1935	1934.7	1953	−18.62	10.42
white rabbit I Hb[a]	29, 7	1970	1971.5	1951.8	1950.4	1946.8	1944.5	1929.1	1931.5	1954.6	−23.33	−7.63
white rabbit I Hb[a]	29, 5	1968	1968.9	1951.2	1950.2	1945	1943.4	1930.4	1931.9	1953.5	−21.91	−9.36
white rabbit I Hb[a]	5, 7	1971	1971.7	1951.2	1950.5	1944	1942.9	1928.7	1929.8	1954.3	−24.78	−9.31
guinea pig Hb 30[a]	30, 7	1965.6	1965.9	1949.2	1948.9	1941.5	1941	1930	1930.5	1951.4	−21.33	−11.21
guinea pig Hb 30[a]	8, 7	1965.6	1965.8	1948.8	1948.5	1941.5	1941.1	1930	1930.4	1951.3	−21.22	−10.56
rat Hb[a]	31, 7	1966.8	1966.8	1950.3	1950.3	1943	1943.1	1933	1932.9	1952.9	−20.31	−10.85
rat Hb[a]	4, 7	1966.8	1966.7	1949.6	1949.7	1941	1941.2	1931	1930.8	1952.1	−21.76	−11.87
mouse Hb[a]	28, 7	1967.5	1967.4	1950.8	1950.9	1943	1943.1	1933	1932.9	1953.3	−20.82	−11.29
mouse Hb[a]	5, 7	1967.5	1967.5	1950.3	1950.3	1941.5	1941.5	1931	1931	1952.6	−22.17	−12.02
sperm whale Mb[a]	20, 7.8	1966	1966.4	1952	1951.6	1944	1943.3	1933.4	1934.1	1953.4	−19.77	−12.7
rabbit HbI α[a]	NR[b]	1970	1971.3	1950.9	1949.5	1946.8	1944.6	1929.1	1931.2	1954.2	−23.14	−6.41
rabbit HbI β[a]	NR[b]	1970	1971.6	1952.4	1950.9	1946.8	1944.4	1929.1	1931.6	1954.8	−23.46	−8.43
human Hb α[c]	4, 8.2	1969	1968.5	1950.4	1950.8	1943.5	1944.2	1934	1933.3	1953.9	−20.92	−9.58
human Hb α Zürich[d]	30, 7.2	1969.1	1968.6	1950	1950.6	1943	1943.9	1933.5	1932.7	1953.7	−21.29	−9.50
human Hb β Zürich[d]	30, 7.2	1969.1	1969.7	1958.2	1957.6	1943	1942	1933.5	1934.5	1956.6	−23.6	−20.31
human Hb α/B Sydney[e]	NR[b]	1967.7	1967.1	1951.7	1952.3	1944.1	1945.1	1937	1936	1954.4	−18.8	−11.63
bovine Mb[f]	35, 7.8	1965	1964.6	1953.5	1953.9	1943.5	1944.1	1938	1937.4	1954.1	−17.49	−17.09
bovine Mb[f]	8, 7.8	1965	1964.6	1953.6	1954	1943.4	1944	1938	1937.4	1954.2	−17.56	−17.38
bovine Mb[f]	20, 5.2	1965	1964.8	1953	1953.2	1945.5	1945.8	1939	1938.7	1954.4	−16.17	−13.87
bovine Mb[f]	20, 7.8	1965	1964.8	1954.2	1954.4	1947.6	1944	1938	1937.6	1954.4	−17.67	−18.12
HRP (C isoenzyme)[g]	15, 7.4	1934	1934.4	1932	1931.6	1906	1905.4	1903	1903.6	1925.1	−25.53	−32.71
HRP (C isoenzyme)[h]	~20, 5–9	1933	1933.8	1929	1928.2	1905	1904.7	1900	1901.3	1923.3	−25.22	−29.32
HRP (A_2 isoenzyme)[h]	~20, 5–9	1938	1936.1	1925	1924.9	1906	1908.5	1903	1901.5	1923.5	−23.56	−21.53

[a] Reference 2. [b] NR, not reported. [c] Potter, W. T.; Hazzard, J. H.; Kawanishi, S.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 719. [d] Potter, W. T.; Houtchens, R. A.; Caughey, W. S. *J. Am. Chem. Soc.* **1985**, *107*, 3350. [e] Caughey, W. S.; Houtchens, R. A.; Lanir, A.; Maxwell, J. C.; Charache, S. In *Biochemical and Clinical Aspects of Hemoglobin Abnormalities*; Caughey, W. S., Ed.; Academic Press: New York, 1978; p 29. [f] Reference 8; also Shimada H.; Caughey, W. S. *J. Biol. Chem.* **1982**, *257*, 11893. [g] Reference 15. [h] Reference 15.

data points are shown on our cosine curve in Figure 3c. (4) The presence of an isosbestic point[1] between A_1 (1946 cm^{-1}, $\theta = -82°$) and A_0 (1966 cm^{-1}, $\theta = 134°$) on changing temperature (or pressure) in sperm whale MbCO can be explained as being due to a 180° ring flip, probably of the $H^{\epsilon 2}$ tautomer. (5) The observation of an interconversion between the CI and CIII conformers of human adult HbCOA (1950 and 1968 cm^{-1}) as a function of temperature or pH[9] can similarly be explained as a ring-flip isomerization. (6) In the case of extremely acid pH effects on MbCO, the observation of a shift from ~1947 to ~1967 cm^{-1}, previously attributed to a protonation of the distal His,[10] with concomitant removal from the heme pocket, may have an alternative explanation. The more conservative ring flip can similarly result in a 1947 → 1967 cm^{-1} conversion, and would be in line with the temperature and pressure results already mentioned, as would the increase of a 1968-cm^{-1} conformer in HbCO on alkaline transition.[9] (7) The observation by Brown et al.[11] that dehydration of MbCO shifts ν_{CO} to 1967 cm^{-1} could likewise be explained as due to the same ring-flip process. The observation by Brown et al.[11] that the early stages of denaturation of HbCO are characterized by the appearance of a broad band ($\Delta\nu_{1/2} \approx 13$–$22$ cm^{-1}) at 1968–1970 cm^{-1}, as opposed to the more normal $\Delta\nu_{1/2} = 8$ cm^{-1} form, are consistent with one of the postulates of Hong et al.[12] that unfolding (of MbCO) may occur through the process $A_1 \rightarrow A_0 \rightarrow A_0' \rightarrow$ unfolded. Hong et al. dislike this postulate because A_0' and A_0 must have the same ν_{CO} but be different thermodynamically. In our work, we believe that ~1968–1970-cm^{-1} forms having different structures do exist. In the presence of a distal His, one conformer absorbs at ~1968 cm^{-1} (Figure 1), due to the *E* field influence. However, we also note below that, e.g., a His E7 → Gly mutant also absorbs at this same frequency—and there is no distal histidine, so clearly the 1968-cm^{-1} form does not require His. Given the preponderance of the evidence, we believe that the native ~1968-cm^{-1} ($\Delta\nu_{1/2} = 8$ cm^{-1}) conformer is a ring-flip isomer, while with the denatured species, the distal His is removed away from the CO and the electrical interactions are more akin to those found in say, the His E7 → Gly or Val MbCO mutants, or e.g., in a free porphyrin. (8) The observation of bands at 1933, 1944, and 1967 cm^{-1} in crystalline MbCO,[13] with intensities varying as a function of the amount of met or deoxy protein present in the MbCO sample, can similarly be rationalized as due to the presence of the two ring-flip isomers mentioned above, together with one form of the other distal His tautomer. (9) The observation by Hong et al.[12] that transitions between the A substates do not occur if the solvent is a glass or a solid, and that even when photolysed, transitions between the A substates cannot be induced below a glass temperature, appears quite consistent with the idea that a ring flip is involved. (10) The model is not restricted to substates of HbCO and MbCO. For example, in horseradish peroxidase (HRP), Uno et al.[14] have observed four vibrational frequencies for HRPC, at 1903, 1906, 1932, and 1934 cm^{-1}, and these and previous researchers have noted that these states interconvert as a function of pH.[12] We obtain excellent agreement with the experimental observations by using $\bar{\nu}_0 = 1925$ cm^{-1}, $a = -25.5$ cm^{-1}, and $b = -33°$, as shown in Figure 3d,e and Table I. (11) With the A_2 isoenzyme of HRP, we again obtain quite good agreement with observation (Figure 3f), although it is difficult to resolve the two low-frequency vibrations in the

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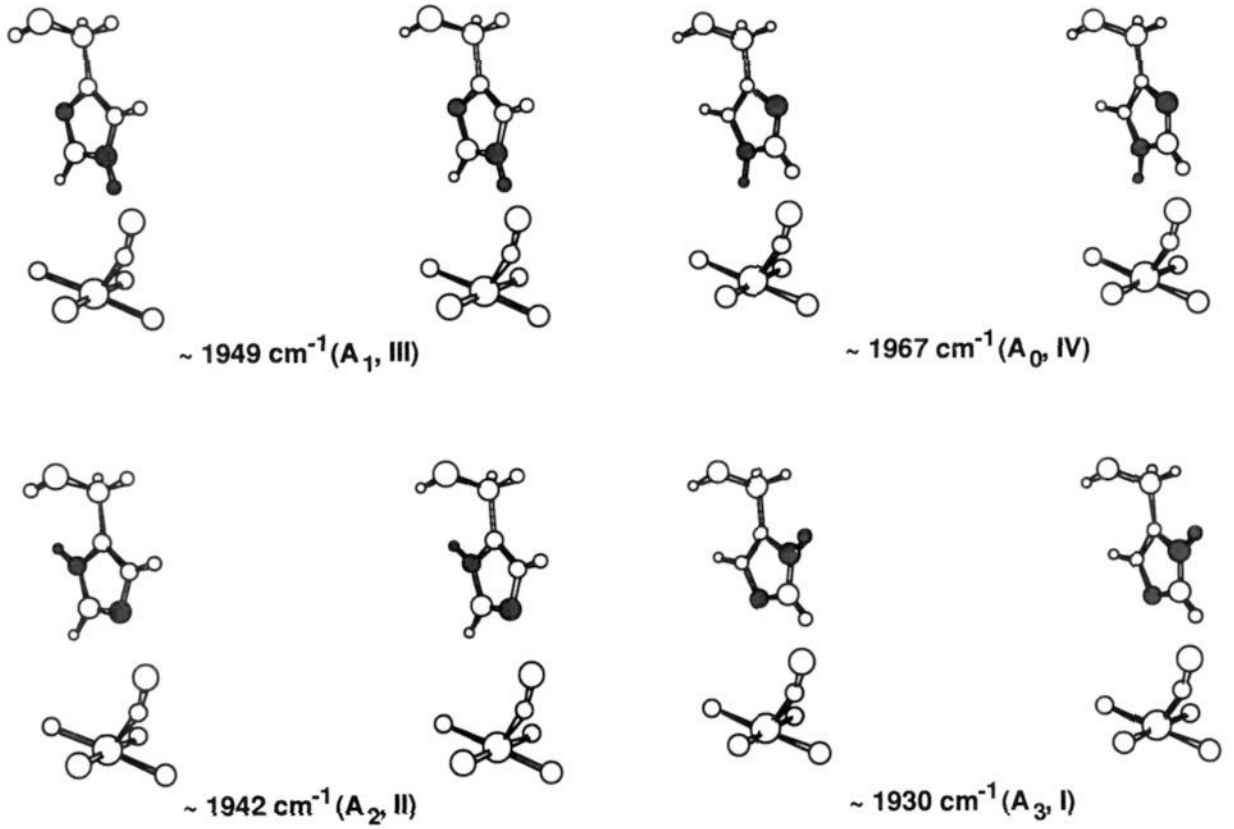

Figure 4. Suggested structure of the four conformational substates found in CO heme proteins (coordinates based on the neutron structure of MbCO, ref 27).

experimental spectrum.[15] (12) In cytochrome *c* peroxidase, which is thought to have many structural similarities with HRP,[16] the vibrations at 1948 and 1922 cm^{-1} [17] can be explained by using $\bar{\nu}_0$ = 1929 cm^{-1}, a = −24 cm^{-1}, and b = −36° (Table I). Although it is clearly premature to make an attribution of these two forms to the two tautomers (of either or both ring-flip isomers), the coefficients used to fit the data are very close to those used in HRPC, where four substates were previously noted by Uno et al.[14] (13) In horseradish peroxidase isoenzymes A_2 and C, and in chloroperoxidase, we previously found a 7-ppm ^{17}O NMR chemical shift change,[18] on acid–base transition, which using the empirical relation between ^{17}O chemical shift and ν_{CO} presented previously,[4] corresponds to a ~25-cm^{-1} change in ν_{CO}, upon acid–base transition (basically ~a in eq 1). The pK values for the transition were found to be 6.1 (HRPA), 10.7 (HRPC), and 4.7 (CPO). The very large differences in pK, but similarities in $\Delta\delta_i(^{17}O)$ and $\Delta\nu_{CO}$, may be readily explained in the present model as being due to changes in the tautomer population induced by slight changes in protein structure caused by the titration of neighboring residues in each of the peroxidases (basically as seen in HbCO and MbCO, but with tautomerism dominating). (14) The rates of exchange between the HRP states (1.05×10^3, 1.88×10^2 s^{-1}; ref 18) in this model can be attributed to tautomerism. (15) A similar rate of exchange in HRP–CN has been observed by Lecomte and LaMar et al.,[19] and could have the same origin. In this case, the effect was generated by $^1H \rightarrow {}^2H$ exchange, with an "anomalously large" isotope effect on the heme 5-Me resonance being observed. This observation could be an electric field effect due to modulation of the population of the tautomers, due to extensive proton exchange (with ^2H) in the protein. (16) Our model should be applicable to other proteins and ligands. For example, in leghemoglobin, ν_{CO} = 1947.7 cm^{-1} (an average, ref 20). We would attribute this major conformer to the A_1/III substate, although further work will be necessary in order to support this postulate. Interestingly, Stetzkowski et al.[21] have observed conformational substates in LbCO, at 1935, 1947, and 1963 cm^{-1}, consistent with our proposed model. (17) It also seems likely that the two forms of nitrosyl hemoglobin found[21] with, or without, inositol hexaphosphate ($\Delta\nu$ = 50 cm^{-1}) may originate from ring-flip isomers. (18) The model predicts only a single conformational substate in His E7 → Gly, Val, or Phe mutants,[23] since Gly and Val generate no appreciable electric field, and Phe effects would be degenerate with respect to a 180° flip. The model is silent on the observation that $\bar{\nu}$ for these species is around 1970 cm^{-1}, a point that will require a more comprehensive theory of electric field effects in proteins, for resolution. The model predicts the possibility of multiple conformational substates in His E7 → Gln species, which can produce dipole fields oriented in several directions. (19) With respect to the question of Fe–CO tilt, our model is very simple, and basically ignores dihedral angle effects. When these are fully taken into account, then an off-axis tilt of the CO, due to the electric field interaction, may become apparent as the E field rotates in the His E7 plane. (20) In the general context of consideration of our model with previous approaches

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based on back-bonding—there is no conflict with the back-bonding picture. Rather, we are suggesting that changes in back-bonding are a direct or indirect consequence of mutual electrical interaction. A charge field changes by a small amount the equilibrium internuclear separation in CO, via its interaction with the dipole moment (mostly) and polarizability tensors, this changes ν_{CO}, and as expected, ν_{FeC} responds to the change in charge density. Chemical shift changes are brought about via the E field interaction with the shielding polarizability tensor, $A = \partial\sigma/\partial E_x$, as discussed elsewhere.[5] (21) We note that, for a number of different proteins, the a terms in eq 1 are quite similar, ranging from ~ -18 to ~ -25 cm^{-1}. This maximal value of the electric field effect may reflect primarily the fairly small variation in the electric field caused by changes in distance from CO to the imidazole ring in the various proteins. The variation in ν_0 between the globins and peroxidases may arise from proximal side differences.

We now consider some more speculative topics. For example, consider the following: (22) In the A_0, A_1, and A_3 substates of MbCO, there are significant differences in CO rebinding kinetics after MbCO photolysis.[24] A_0 rebinds fastest, A_1 next, followed by A_3 (A_2 was not observed). We believe that the electric field produced by the distal His could contribute to the rebinding kinetics, since in our model A_0, A_1, and A_3 fields contribute as cos 134° = −0.69, cos −82 = 0.14, and cos −10 = 0.98, that is, the A_0, A_1, and A_3 fields will be roughly aligned parallel, orthogonal, or antiparallel to the CO dipole moment vector. (23) Other electric field effects may be worth considering in the framework of the present model. For example, when reviewing a (limited) range of k_{off} rates in heme proteins, Uno et al.[14] noted inverse relationships between log k_{off} and ν_{FeC}, as well as between $(\nu_{CO})^2$ and $(\nu_{FeC})^2$, which means $\nu_{CO}\alpha$ log k_{off}. It may thus be possible to relate electric field effects (on ν_{CO}) to kinetic off-rate parameters, and thus via (22) above, to thermodynamic parameters. (24) An interesting correlation between the oxidation–reduction potential, E_{m7}, and ν_{CO}, in a range of proteins, has been reported.[15] Electric field effects from the protein may contribute to this phenomenon, and could be of interest in the context of electron-transfer rates. (25) The A_0 and A_1 substates of MbCO are very different with respect to pressure. For example, A_1 shows neither an elastic nor a conformational shift with pressure,[24] while A_0 displays both. It is possible that A_1 (III, θ = −82°, $H^{\epsilon 2}(+)$) is the "native" form of HbCO/MbCO (i.e., the $H^{\epsilon 2}(+)$ MbO_2 form, ref 25), and is best tailored to ligand binding, including stabilization of the Fe=O^+—O^- species in HbO_2/MbO_2, via an electric field interaction, while the ring-flip isomer is more distorted, and thus needs to modify its structure more than the native form, as P increases. A related observation might be that of Potter et al.[2] who note "how tenaciously the environment that gives rise to band III remains intact even after prolonged periods at an elevated temperature." Whether ring flips have any functional role in O_2 binding remains, of course, to be seen.

Overall, we believe that our results provide a plausible explanation of a considerable body of data. Additional detailed calculations of electrical potentials and mutual polarization in proteins will need to be performed in order to test our ideas. However, at present there is no generally accepted alternative view, and it would indeed be surprising, given the huge T, P, and pH perturbations heme proteins have been subjected to, if ring flips and tautomerism did *not* occur. The ubiquitous presence of "four, and only four" conformational substates in HbCO and MbCO, and in HRPC, is a direct prediction of the model, and is consonant with experiment. Suggested structures for each of the conformational substates are shown in Figure 4.

Finally, we note that the implications of this work extend considerably beyond the question of conformational substates in heme proteins. Our results support the electric field model[5] of chemical shifts in proteins, and via mediation of the shielding polarizability and hyperpolarizability, should give a full explanation of the chemical shift nonequivalences of amino acids observed in proteins due to folding into their native conformations, first observed almost 20 years ago,[25] and currently of considerable interest in the context of higher dimensional NMR studies of protein (and possibly nucleic acid) structure. It also appears likely that IR and NMR results may help to test the veracity of the various electrostatic models of protein structure. Further work is in progress.

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