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Infrared Spectroscopic Studies of Carbonyl Horseradish Peroxidases†

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ABSTRACT: Infrared difference spectra, $Fe^{II}CO$ vs. Fe^{III}, of horseradish peroxidase isoenzymes A2 and C were recorded from 2000 to 1800 cm^{-1}. Under alkaline conditions, pH 9, both isoenzymes exhibit two CO stretching bands, at 1938 and 1925 cm^{-1} for A2 and at 1933 and 1929 cm^{-1} for C. As the pH is lowered the low-frequency band for each isoenzyme decreases in intensity with a concommitant appearance and increase in intensity of a band at 1906 and 1905 cm^{-1} for the A2 and C isoenzymes, respectively. These changes conform to pK values of 6.7 for the A2 and 8.8 for the C isoenzymes of horseradish peroxidase. The interpretation of the infrared results was simplified by the observation that a linear relationship exists between the redox potential, E_{m7}, for the Fe^{III}/Fe^{II} system vs. the infrared CO stretching frequency, ν_{CO}, for cytchrome a_3, hemoglobin, myoglobin, and cytochrome P-450 cam with substrate. This relationship suggests that the primary force altering ν_{CO} in these heme proteins is a variation in electron density at the heme iron and not direct protein interactions with the CO ligand. The horseradish peroxidase infrared bands in the 1930-cm^{-1} region correlate well with this relationship. The large deviation of the 1905-cm^{-1} band from the linear relationship and its dependence upon hydrogen ion concentration are consistent with horseradish peroxidase having a single CO binding site which can hold in two geometries, one of which contains an amino acid moiety capable of forming a hydrogen bond to the carbonyl oxygen.

The infrared spectra of most carbonyl heme proteins exhibit a single band due to the C-O stretching mode of CO bound to the heme iron, and only in a few instances have more than one band been detected. Blood from some animal species shows more than one band because of the presence of hemoglobin variants with deviant binding sites (Barlow et al., 1973). Homogeneous preparations of myoglobin from various species yielded spectra with a main band at 1944 cm^{-1} and a shoulder at 1935 cm^{-1}, which led to the assumption of a site allowing two geometries for CO binding (McCoy and Caughey, 1971). Recent reports of multiple CO stretching bands for the CO compounds of horseradish peroxidase (1933 and 1905 cm^{-1}) (Alben and Bare, 1973) suggest a site capable of binding CO in more than one manner. To explore this possibility we have compared the infrared spectra of the CO compounds of two horseradish isoperoxidases and studied in detail the effects of variations in pH.

Materials and Methods

The acid type HRP[1]A2, pH 3.9, was isolated as described by Paul and Stigbrand (1970) and Marklund et al. (1974). It gave A_{403}/A_{280} = 4.08 and was homogeneous in polyacryl-

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[1] Abbreviations used are: HRP, horseradish peroxidase; Hb, hemoglobin; Mb, myoglobin; ir, infrared; E_{m7}, midpoint potential at pH 7; ν_{CO}, wavenumber in cm^{-1} for the CO stretching absorption band; $\Delta\nu_{CO}$, the half-bandwidth of this band.

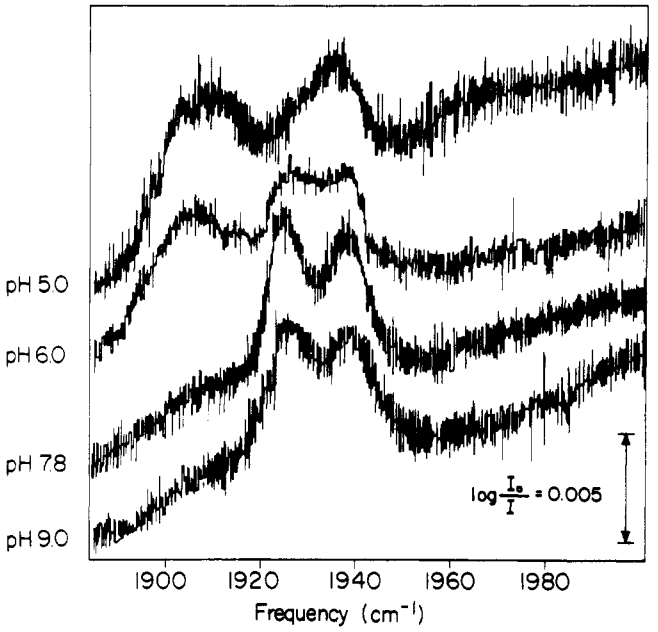

FIGURE 1: Infrared difference spectra of CO Fe^{II}HRP A2 vs. Fe^{III}HRP A2. HRP 0.75 mM in both cuvettes. The spectra are shifted arbitrarily vertically.

amide electrophoresis and in isoelectric focusing. HRP of the slightly alkaline type was purchased from Sigma Chemical Co. (lot no. 44C-9570, A_{403}/A_{275} = 2.68). In polyacrylamide gel electrophoresis it gave a main band corresponding to HRP type C, pH 8.8, with a minor component, approximately 10%, corresponding to type B (Paul, 1958). At pH 6 the CO complex of this material gave an ir spectrum identical with that of authentic HRP C2. The peroxidases were dialyzed against distilled water overnight, concentrated in collodion bags, and diluted to the given HRP concentrations in 100 mM buffer. The buffers used were: sodium citrate (pH 4 and 5), sodium phosphate (pH 6, 7, and 11), sodium carbonate (pH 10), and Tris-HCl (pH 8 and 9). To convert HRP to its ferrous carbonyl compound CO was passed over the surface of HRP in the buffer for 10 min, and the solution was then transferred, by means of a 1-ml plastic syringe filled with CO and containing some solid dithionite, to the ir cell which had been pregassed with CO. With proper handling, the pH changed less than 0.15 unit from the buffer value due to HRP and dithionite, as found in separate tests.

The reference cell contained Fe^{III}HRP of the same concentration as the sample cell although sometimes in a different buffer. In the ir region of interest water is the principal absorber, with neither the protein nor the buffers employed exhibiting any sharp or intense broad bands except for that of heme-bound CO (Maxwell et al., 1974). Thus, either the pathlength could be adjusted or the protein concentration varied to balance the absorption due to water.

The ir spectra were recorded with a Perkin-Elmer Model 225 ir spectrophotometer at a resolution of 2.6 cm^{-1} and a scanning time of 22 min for the range 2000–1870 cm^{-1}. The cells were equipped with CaF_2 windows with 0.05-mm Teflon spacers and water-jacketed holders to maintain sample temperatures between 20 and 25 °C. The CaF_2 windows allowed the registration of the visible and Soret region spectra on a Cary 14 instrument immediately following the recording of the ir spectrum.

The apparent integrated absorption intensity, B, was determined from the relationship

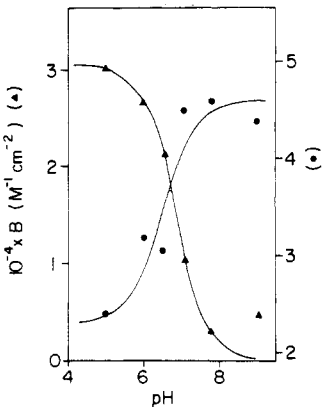

FIGURE 2: Effect of pH on the intensities of the 1906- (▲) and 1938- + 1925- (●) cm^{-1} bands of CO Fe^{II}HRP A2 with n = 1 theoretical dissociation curves for pK = 6.85 and 6.55, respectively.

$$B = \frac{1}{cl}\int \log \frac{I_0}{I}\,d\nu$$

where c is the molar concentration, l is the pathlength in cm, log (I_0/I) is the absorbance, and ν is the frequency in cm^{-1} (Ramsey, 1952). The areas of the bands were determined by cutting and weighing, the baseline being drawn from the nearly linear segment on one side of a band to the corresponding segment on the other side. The bands in the 1925–1937-cm^{-1} region could not be quantitated separately, but were cut out and weighed together. The weights were converted to the proper units M^{-1} cm^{-2} by means of separate determinations of the weight per cm^2 of the paper.

Results

The ir spectrum of CO HRP A2 exhibits no less than four bands (1970, 1938, 1925, and 1906 cm^{-1}) attributable to C–O stretching, but appearing under different conditions. At neutral and slightly alkaline conditions, two bands of about equal intensit appear at 1925 and 1938 cm^{-1} with the half-band widths of 10 and 11 cm^{-1} (Figure 1). At a lower pH the 1925-cm^{-1} band vanishes whereas the 1938-cm^{-1} band remains essentially unchanged, and simultaneously a band appears at 1906 cm^{-1}, half-band width 19 cm^{-1}. The decrease in the sum of the 1925- and 1938-cm^{-1} bands as well as the increase in the 1906-cm^{-1} band follows simple dissociation curves with pK = 6.55 and 6.85, respectively (Figure 2). The difference is not significant, and the changes are assigned a common pK of 6.70. Figure 2 also shows that half of the (1925 + 1938) area persists as the protonation is complete, confirming the impression of a persisting 1938-cm^{-1} band in Figure 1. At pH < 5 the 1905- and 1938-cm^{-1} bands slowly decrease with time and simultaneously a broad band with a half-bandwidth of 20 cm^{-1} appears at 1970 cm^{-1} (not shown in the figures). In those experiments where the 1970-cm^{-1} band had appeared, the contents of the cell had gelatinized irreversibly.

The spectrum of CO HRP C shows under neutral conditions bands at 1905 and 1933 cm^{-1} with half-bandwidths of 17 and 12 cm^{-1} (Figure 3). As the pH is raised the area and bandwidth of the latter band increase somewhat (16 cm^{-1}), with the formation of a shoulder at 1929 cm^{-1} and the disappearance of the 1905-cm^{-1} band. As the pH is lowered, the 1933-cm^{-1} band decreases without disappearing fully while the 1905-cm^{-1} band increases in intensity. These changes in intensity conform to a simple dissociation curve with pK = 8.80 (Figure 4). HRP C, like A2, shows time-dependent changes below pH 5.

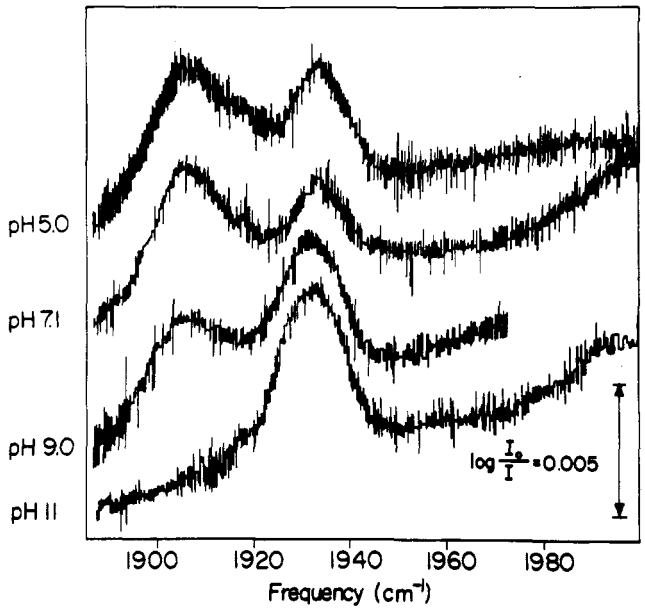

FIGURE 3: Infrared difference spectra of CO Fe^{II}HRP C vs. Fe^{III}HRP C. HRP = 0.55 mM in both cuvettes. The spectra are shifted arbitrarily vertically.

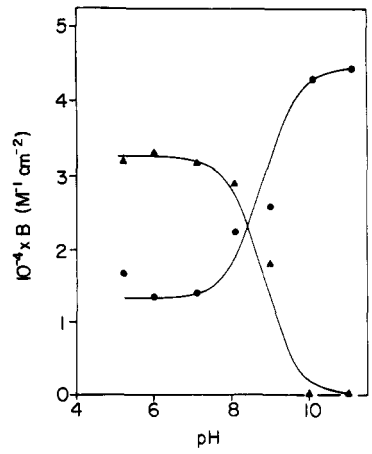

FIGURE 4: The effect of pH on the intensities of the 1905- and 1933- + 1929-cm^{-1} bands of CO Fe^{II}HRP C with $n = 1$ theoretical dissociation curves for $pK = 8.80$.

The presence of 3.2 mM hydroquinone did not alter the C-O stretching of CO HRP C at pH 6. At ~100 mM hydroquinone or 1 M 3-amino-1,2,4-triazole the 1933-cm^{-1} band shifted 3 cm^{-1} toward lower frequencies and a broad band grew slowly between 1964 and 1970 cm^{-1}. The lack of pronounced spectral changes under conditions when half of the peroxidase in the ferric form would be bound to the hydrogen donor hydroquinone (K_{diss} = 3.2 mM)[2] is in agreement with the absence of large effects of benzhydrazide or benzhydroxamic acid on the visible spectrum of CO HRP C (Schonbaum, 1973).

The infrared spectra of CO HRP A2 and CO HRP C are thus very similar, and they respond to changes in pH in much the same way. Under neutral or slightly alkaline conditions a pair of bands (shoulder in HRP C) appear, symmetrically spaced around 1931 cm^{-1}, the bands being 13 and 4 cm^{-1} apart in HRP A2 and C. In both peroxidases the band at higher frequency is little affected by changes in pH, whereas the lower frequency band is depressed and concomitantly replaced by a broader and slightly more intense band at 1905 cm^{-1} as the pH is lowered. The changes follow dissociation curves with n = 1, $pK_{HRP\,A2}$ = 6.7, and $pK_{HRP\,C}$ = 8.8. At pH < 5 both peroxidases show signs of profound structural changes, slowly yielding a broad band at ~1970 cm^{-1}.

Discussion

E_m and ν_{CO}. Three parameters describe the ir absorption due to CO stretching in CO ferrous heme proteins: the position of the absorption band ν_{CO}, its half-band width $\Delta\nu_{CO}$, and its intensity B. In addition, the number of bands may provide further information.

Factors that affect ν_{CO} in carbonyl hemes and heme proteins may be categorized as those modifying the electron density at the heme iron and those interacting with the carbonyl ligand directly. The strength of the iron-carbonyl bond depends on the extent of the $d\pi$-$p\pi$ back-bonding from the heme iron to the carbonyl ligand which in turn depends on cis (equatorial) and trans (axial) ligand interactions with heme iron (Caughey et al., 1973). Changing the basicity of these ligands in model heme complexes results in predictable changes in ν_{CO} (Alben and Caughey, 1968), so that increasing ligand basicity lowers ν_{CO}. The effects of ligand interactions have been less clear in heme proteins where charge-transfer interactions, electron withdrawal or donation by variable orientations of porphyrin peripheral substituents, and dipolar interactions between the ligands and amino acid residues may considerably modify the strength of π bonding between the heme iron and the carbonyl ligand.

On the other hand, differences in ν_{CO} between heme proteins also depend on direct effects of the protein matrix on the carbonyl ligand. Dipole-dipole interactions between the carbonyl ligand and the immediate solvent environment decrease ν_{CO} in carbonyl hemes by 15-20 cm^{-1} as the dipole moment of the solvent increases from 0-4 D.[3] In an ir study of cobalt carbonyl complexes ν_{CO} migrated about 25 cm^{-1} toward lower frequency as cyclohexane was replaced by chloroform as solvent. Mixtures of the two solvents produced two ν_{CO} maxima, demonstrating the immediate solvent environment, and not the bulk properties, to be decisive (Beck and Lottes, 1964). Steric (Caughey, 1970), charge-transfer (Caughey, 1970), and hydrogen-bonding (Yonetani et al., 1974) interactions of the distal histidine of hemoglobin and myoglobin with bound CO or O_2 have been proposed and should, when operable, profoundly modify ν_{CO}. Unfortunately, model systems for these effects either do not exist or are not well defined.

The interpretation of the physical parameters which govern the position of ν_{CO} in carbonyl heme proteins would be simplified if data were available to rate the heme iron charge-mediated shifts in ν_{CO} vs. the shifts in ν_{CO} caused by direct interactions of the protein or solvent environment with bound CO. The redox potential expresses the difference in ligand exchange energies between two oxidation states and can, for heme proteins, be considered as the result of two effects, the formal charge on the iron atom and the interactions with the protein. A plot of the literature values in Table I for the redox potentials E_{m7} and ν_{CO} for several heme proteins reveals a linear relationship between these two parameters (Figure 5). Our data for HRP show that the higher energy bands also obey this correlation. This linear relationship assigns a dominating role to the electron density on the heme iron in these heme proteins, whereas deviations indicate that the other factors

[2] Ohlsson, P. I., and Paul, K.-G., manuscript in preparation.

[3] Maxwell, J. C., Barlow, C. H., and Caughey, W. S., manuscript in preparation.

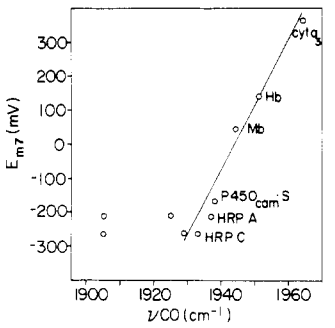

FIGURE 5: Relationship of CO stretching frequencies (ν_{CO}) to half-oxidation potentials (E_m) of the Fe^{III}/Fe^{II} system in heme proteins at pH 7. Slight variations in pH, ionic strength, or temperature may exist between the two values of a pair.

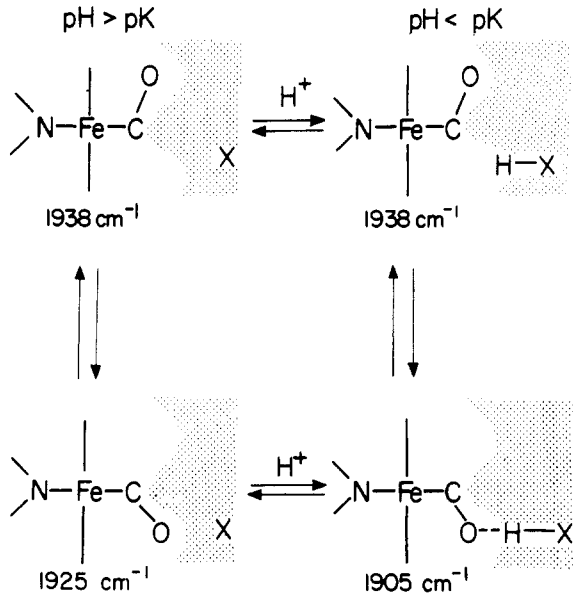

FIGURE 6: Proposed structures of CO Fe^{II}HRP A and C, giving the three ir stretching frequencies ν_{CO} 1906–1905 cm^{-1}, 1925/1929 cm^{-1}, and 1938/1933 cm^{-1}.

mentioned above are also operating. Thus, the infrared bands in the 1931-cm^{-1} region for the two peroxidases may be considered the normal result of CO binding to an electron-rich heme iron and the shift to 1905–1906 cm^{-1} the result of a force operating directly on the bound CO.

The magnitude of $\Delta\nu_{CO}$ depends upon steric factors and upon interactions between solvent and the CO dipole and hence to the extent of polarization of CO, i.e., ultimately the allocation of electrons to the metal atom. An inverse linear relation between ν_{CO} and $\Delta\nu_{CO}$ has been demonstrated for cyclopentadienyliron carbonyl derivatives (Darensbourgh, 1972). Such a relationship also exists for several carbonyl heme proteins (Table I). Thus, discussions concerning the randomness of the CO binding site based on bandwidth measurements must also allow for changes in $\Delta\nu_{CO}$ due to changes in the dipolar character of the carbonyl ligand.

The intensity B of carbonyl bands has been shown to be highly dependent on the extent of π bonding between the metal ion and CO in several transition metal complexes (Darensbourgh, 1972). The intensity data in Table I correlate with trends in E_{m7} and ν_{CO}, suggesting that a similar relationship holds for B vs. the extent of π bonding in heme proteins.

Acid CO Heme Proteins. HRP, Hb, and Mb are protohematin proteins, probably with histidine as the fifth ligand to iron. Upon acidification to pH <2.5 their ferric forms become spectrally identical (Maehly, 1958), which suggests that the surroundings of the chromophoric heme have become identical, i.e., unspecific. In HRP this change is reversible. The CO complexes of the ferrous forms of these heme proteins likewise yield nearly identical ir spectra upon acidification to pH <5 with the formation of a broad, intense band at ~1970 cm^{-1}. The unspecificity of the corresponding structure is in agreement with the width of the band ($\Delta\nu_{CO} \sim 20$ cm^{-1}) (O'Toole, 1972). Thus, at sufficient acidity the heme protein structure seems to be loosened at either state of oxidation of the iron, and ir spectra will yield little information on the structures of acid CO heme proteins. Moreover, some caution should be exercised about the conditions under which acid CO heme protein ir spectra have been registered. We found for both peroxidases a time factor in the appearance of the ~1970 cm^{-1} band, and the CO HRP A2 became irreversibly altered. Dithionite is decomposed at pH <5, and abnormal reactions at this acidity in other studies of HRP have been attributed to decomposition products of dithionite (Yamada and Yamazaki, 1974).

TABLE I: Redox Potentials for Fe^{III}/Fe^{II} and Parameters for the Infrared Spectra of CO Heme Proteins at pH 7.

Heme Protein	E_{m7} (mV)	ν_{CO} (cm^{-1})	$\Delta\nu_{CO}$ (cm^{-1})	$10^{-4}B$ (M^{-1} cm^{-2})
Cytochrome a_3	+365[a]	1963.5[b,c]	5.5[c]	3.0[c]
Hemoglobin	+139[d]	1951[e]	8[e]	2.2[c]
Myoglobin	+46[f]	1944[g]	8[g]	2.5[c]
Cytochrome P-450 cam + substrate	−150[h]	1938[i]	14[i]	
Horseradish peroxidase	−212[j,l]	1938	11	4.6
		+1925		
A2		1906	19	6.0
Horseradish peroxidase	−265[j,k]	1933	~12	4.5
		+1929		
C[m]		1905	17	4.5

[a] Dutton et al. (1970). [b] Caughey et al. (1970). [c] Volpe et al. (1975). [d] Taylor and Hastings (1939). [e] Alben and Caughey (1968). [f] Taylor and Morgan (1942). [g] McCoy and Caughey (1971). [h] Gunsalus and Lipscomb (1972). [i] Volpe, J. A., Griffin, B., Peterson, J. A., and Caughey, W. S., unpublished results. [j] Makino and Yamazaki (1975). [k] Harbury (1957). [l] Ohlsson, P. I., and Paul, K. G., found $E_{m7} = -212$ mV for HRP A2 (unpublished). [m] Infrared values for HRP A2 and C are taken from the present study.

Mode of CO Binding in HRP. While the midpoints, 1931 cm^{-1}, of the double bands 1925/1938 and 1929/1933 cm^{-1} for CO HRP A2 and C fit well into the linear relation between E_{m7} and ν_{CO} (Figure 5) the two actual values for ν_{CO} in the doublets correspond to two probability maxima for the position of CO, separated by a potential energy barrier. Transition metal carbonyl complexes commonly have a nearly linear M–C–O linkage, but this is not necessarily the case for carbonyl heme proteins. X-ray analysis of carbonyl erythrocruorin revealed an Fe–C–O angle of 145 ± 15°, the result of a possible steric interaction between the carbonyl ligand and an isoleucine (Huber and Formanek, 1970). In HRP a similar interaction could allow CO to bind in two geometries (Figure 6).

Whatever the nature of the barrier may be, the two probability maxima must have a structural correspondence in two niches for CO of somewhat different character. The difference in energy between CO bound in the two niches is well within the previously mentioned range of ν_{CO} for variations in polarity

of solvent environment. The two values for the bands centered at 1931 cm^{-1} are split further in HRP A (12 cm^{-1}) than in HRP C (4 cm^{-1}). This may reflect greater differences in polarity for the two sites in HRP A than in HRP C. In each of the HRP's the niche giving the higher frequency stretching is insensitive to pH and may be less polar in character. The other position of CO produces two communicating stretching frequencies, the intrinsic acidity being decisive in the choice between them. The protonation of a sterically suitable group "X" in the protein would permit the establishment of a hydrogen bond as shown in Figure 6. The energy of the CO stretching frequency is lowered from 1925 and 1929 cm^{-1} for HRP A2 and C, respectively, to 1905 and 1906 cm^{-1}, which is consistent with hydrogen-bond formation. The existence of the hydrogen bond C-O-H-X is substantiated by the dependence of the formation of the 1905- and 1906-cm^{-1} bands upon a single dissociation constant. Moreover, the large deviation from the linear E_{m7} vs. ν_{CO} relationship in Figure 5 corresponds to a major change in CO bonding not transmitted through the heme iron and thus a result of a direct action on the bound CO. If the effect of increasing hydrogen ion activity were not to form a CO- -H-X bond, but rather to protonate the backside ligand or some amino acid residue interacting with the porphyrin, the shift in ν_{CO} would be toward higher frequencies. The hydrogen bond is suggested as being formed with the carbonyl oxygen rather than with the carbon for two reasons. The oxygen of metal-bound carbonyls is more electronegative than the carbon, and hydrogen-bond formation with the carbon would reduce the frequencies of both bands in the doublet.

The pH_{red} values determined by potentiometric titrations of HRP A and C are 5.8 and 7.3, respectively (Makino and Yamazaki, 1975). These can probably be identified with the protonation constants $pK_A = 6.7$ and $pK_C = 8.8$ for "X" in the CO Fe^{II}HRP's, for the following reason. The ir spectra of CO-Hb and Co-Mb are insensitive to changes in pH within the rane pH 4-10 (McCoy and Caughey, 1971; O'Toole, 1972) and no protonations can be detected potentiometrically within the range of pH 5-9 in Fe^{2+} Hb (Taylor and Hastings, 1939) and pH 5.9-7.4 in Fe^{2+} Mb (Taylor and Morgan, 1942). In HRP there exist dissociation constants, operable by both ir spectroscopy and potentiometry. The sixth position at the iron(II) atom is probably empty in HRP, and the potentiometrically operable $pK_{red} = 5.8$ and 7.3 in HRP A and C, respectively, are only slightly sensitive to 2,4 substitutions on the porphyrin ring (Makino and Yamazaki, 1975). In Fe^{III} HRP water occupies the sixth position, giving a bridging mechanism, and the optically and potentiometrically operable $pK_{ox} = 9.2$ and 11.1 in HRP A and C are sensitive to 2,4 substitutions (Makino and Yamazaki, 1975; Ohlsson and Paul, 1973). A similar mechanism may operate in the CO Fe^{II}HRP's. The presence of a hydrogen bond between CO and protein will increase the apparent pK for the protonation of "X" and thereby account for the discrepancy between the two sets of dissociation constants in Fe^{II}HRP ($pK_{CO\text{-}Fe^{II}} - pK_{Fe^{II}} = 0.9$ for HRP A and 1.5 for HRP C, respectively.)

The experimental data would also be accounted for if each of the isolated HRP isoenzymes were a mixture of two components in roughly equal amounts, with only one component capable of forming a hydrogen bond with the carbonyl ligand. This would require that both HRP's have been modified to the same extent and in the same way. This possibility is rejected as unlikly, and it has no support in the homogeniety tests of the material.

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