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Alternative Carbon Monoxide Binding Modes for Horseradish Peroxidase Studied by Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman (RR) spectroscopy and infrared spectroscopy have been used to characterize the three vibrational modes, CO and FeC stretching and FeCO bending, for carbon monoxide bound to reduced horseradish peroxidase, with the aid of ^{13}CO and $C^{18}O$ isotope shifts. At high pH, one species, I, is observed, with ν_{FeC} = 490 cm^{-1} and ν_{CO} = 1932 cm^{-1}. The absence of a band attributable to δ_{FeCO} suggests a linear FeCO unit normal to the heme plane. The data were consistent with I having a strongly H-bonded proximal histidine, as shown by a comparison with imidazole and imidazolate adducts of Fe^{II}PPDME(CO) (PPDME = protoporphyrin IX dimethyl ester), with ν_{FeC} = 497 and 492 cm^{-1} and ν_{CO} = 1960 and 1942 cm^{-1}. At low pH an additional species, II, is observed, with ν_{FeC} = 537 cm^{-1}, ν_{CO} = 1904 cm^{-1}, and δ_{FeCO} = 587 cm^{-1}; it is attributed to FeCO that is H bonded to a protonated distal histidine, the H bond strongly lowering ν_{CO} and raising ν_{FeC}. The appearance of δ_{FeCO} in the RR spectrum suggests that the FeCO unit in II is tilted with respect to the heme plane. At low pH, the population of I and II depends on the CO concentration. I dominates at low CO/protein levels but is replaced by II as the amount of CO is increased. This behavior is suggested to arise from secondary binding of CO, which induces a conformation change involving the distal residues of the heme pocket.

The peroxidases are of much interest in connection with ideas about how O_2 is activated toward O–O bond scission. They contain the same prosthetic group as hemoglobin (Hb) and myoglobin (Mb), noncovalently bound heme coordinated to a histidine residue. Peroxidases do not form stable O_2 adducts, however. Instead, they catalyze the oxidation of organic substrates by peroxide via the agency of highly oxidized intermediates containing the Fe^{IV}=O unit (Roberts et al., 1981; LaMar et al., 1983; Terner et al., 1985). The crystal structure of cytochrome *c* peroxidase (CCP) (Poulos & Kraut, 1980; Poulos & Finzel, 1984; Finzel et al., 1984) has focused attention on differences among heme proteins, particularly with

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regard to H bonding in the heme pocket. The proximal histidine is involved in a strong H bond to an anionic residue (aspartate) in CCP but only a weak H bond to a backbone carbonyl in Mb and Hb (Lander et al., 1977). For the related enzyme horseradish peroxidase (HRP), striking differences relative to Mb are seen in the Fe–imidazole stretching Raman frequency (Teraoka & Kitagawa, 1980; Stein et al., 1980) and in the imidazole NH proton NMR shift (LaMar & DeRopp, 1982), which are attributable to strong H bonding. On the distal side, both CCP and Mb have a histidine residue available for interaction with bound ligands, but in CCP this imidazole is H bonded to a side-chain carbonyl, while in Mb it is exposed to solvent (Poulos & Finzel, 1984). In addition, CCP contains an arginine residue on the distal side, which is close enough to the Fe atom to interact with ligands (Edwards et al., 1984).

The importance of distal H bonding in peroxidases is emphasized by infrared spectroscopic studies of the CO adducts. The IR spectrum of HRP shows CO stretching IR bands at 1932 and 1904 cm^{-1} (Barlow et al., 1976). The latter shifts down by 2.5 cm^{-1} when the protein is dissolved in D_2O (Smith et al., 1983), and it disappears at high pH (Barlow et al., 1976). The CCP adduct has a band at 1920 cm^{-1}, which shifts 2.0 cm^{-1} in D_2O (Satterlee & Erman, 1984). This evidence supports the CO adducts being strongly H bonded by a distal residue, probably histidine.

Resonance Raman (RR) spectroscopy can be used to determine the FeC stretching frequency of heme-bound CO (Tsubaki et al., 1982; Armstrong et al., 1982) and, in favorable cases, the FeCO bending frequency as well (Tsubaki et al., 1982). These Raman bands can readily be identified via characteristic ^{13}CO and $C^{18}O$ isotopic shifts, as demonstrated by Yu and co-workers for heme proteins (Tsubaki et al., 1982) and model compounds (Yu et al., 1983). The appearance of the FeCO bending mode in the Raman spectrum has been suggested to be associated with FeCO bonds that are off-axis relative to the heme normal, a situation demonstrated crystallographically for MbCO (Norvell et al., 1975) and HbCO (Baldwin, 1980). In the present study, we find that the 1904-cm^{-1} IR band of HRP–CO is associated with an unprecedentedly high 537-cm^{-1} FeC stretching Raman band and also a 587-cm^{-1} FeCO bending mode, whose appearance in the Raman spectrum suggests a tilted geometry for the H-bonded CO. The 1932-cm^{-1} IR band is associated with a 490-cm^{-1} FeC stretch; these frequencies are shown via model compounds to be consistent with a strong H bond for the proximal imidazole ligand. The absence of an FeCO bending Raman band suggests that the CO is normal to the heme plane in this form. The population shifts from the 537-cm^{-1} species to the 490-cm^{-1} species as the pH is raised, and also, unexpectedly, as the CO exposure is decreased. This CO concentration effect is suggested to be due to the perturbation of a conformational equilibrium involving the distal residues via binding of a second CO molecule in the vicinity of the heme group.

Experimental Procedures

Horseradish peroxidase (Sigma, type VI), mainly the C isozyme, was dissolved in 0.1 M phosphate buffer at the desired pH, and a slight excess of solid sodium dithionite (Aldrich) was added to prepare reduced protein. The CO adduct was prepared by gently flowing CO (Matheson) over the surface of the Fe^{II} solution for 45 min (high CO form) or by introducing carefully regulated smaller amounts of CO from a 0.25-L glass flask (Cambridge Isotope Labs) to an evacuated NMR tube (low- and medium-CO forms). The effect of reducing the amount of CO is mimicked by increasing the protein concentration at a given CO exposure. To obtain the high-pH forms of the CO adduct, HRP was dissolved in phosphate buffer, pH 11, to which a minimal amount of a dithionite solution was added (no significant pH change), or to which a slight excess of solid $Na_2S_2O_4$ was introduced (pH 7.5). Under identical experimental conditions, no significant difference was observed when the CO adducts of Sigma, type VI and electrophoretically pure isozyme C_2 (Aibara, 1982) were compared. In the case of ^{13}CO (99%) and $C^{18}O$ (98%) (Cambridge Isotope Labs), only the low- and medium-pressure forms could be obtained, because of the small volumes of gas introduced from a 0.25- or 0.1-L glass flask. The model compound Fe^{II}PPDME(ImH)(CO)[1] and its deprotonated analogue [Fe^{II}PPDME(Im)(CO)]$^-$ were prepared via literature procedures (Mincey & Traylor, 1983). The Fe^{II} porphyrin bis(imidazole) adduct was reduced under a CO atmosphere with a slight excess of a methanolic solution of 18-crown-6/$Na_2S_2O_4$ complex. Addition of deprotonated dimethyl sulfoxide anion, prepared from Me_2SO and sodium hydride, yielded the imidazolate–heme–CO complex for Raman characterization. For IR spectroscopy, the imidazolate complex was prepared by addition of solid NaH to the imidazole–heme–CO complex and subsequent filtration of undissolved NaH. UV/visible spectra were obtained before and after spectroscopic characterization to check sample integrity.

Raman spectra were obtained with excitation from the 413.1-nm line of a Kr^+ laser (Spectra Physics 171) via backscattering from a slowly rotating NMR tube. The scattered light was collected and focused into a computer-controlled double monochromator (SPEX 1401) equipped with a cooled photomultiplier (RCA) and photon-counting electronics. The spectra were obtained at room temperature (25 °C) at low laser powers (less than 5 mW). Under these conditions photolysis was negligible, as could be determined from the frequency of the ν_4 porphyrin skeletal mode.

Infrared spectra were recorded at 25 °C with a Digilab FDS-20C FTIR spectrophotometer. The samples were transferred by a syringe flushed with CO to a CaF_2 IR cell (0.1-mm path length) that had been flushed with N_2. Buffer solution was used as a reference. The CaF_2 windows permitted UV/vis absorption spectra to be recorded immediately before and after IR spectroscopy.

Results

Figure 1 shows Raman spectra in the 500-cm^{-1} region under conditions that we label high, medium, and low CO. high CO simply refers to extended flushing (45 min; see Experimental Procedures) with CO from a tank with 15 psi exit pressure. For the medium- and low-CO samples, smaller amounts of CO were carefully introduced to reduced HRP under N_2. The extent of CO binding was monitored with UV/vis spectra, as well as high-frequency RR spectra, in which HRP and HRP–CO are clearly distinguished by the position of the C–N breathing mode, ν_4, at 1355 and 1369 cm^{-1} (Choi & Spiro, 1983). In all cases the HRP was fully ligated. Nevertheless, the CO dose has a marked effect on the spectrum. As the exposure to CO increases, a band at 537 cm^{-1} grows in, at the expense of the 490-cm^{-1} band, which appears to shift to 495 cm^{-1}, while the 587-cm^{-1} band increases in intensity and the 515-cm^{-1} band remains essentially unchanged. These changes were reversed by raising the protein concentration for a given

[1] PPDME, protoporphyrin IX dimethyl ester; ImH, imidazole; Im^-, imidazolate; FTIR, Fourier transform infrared; EXAFS, extended X-ray absorption fine structure.

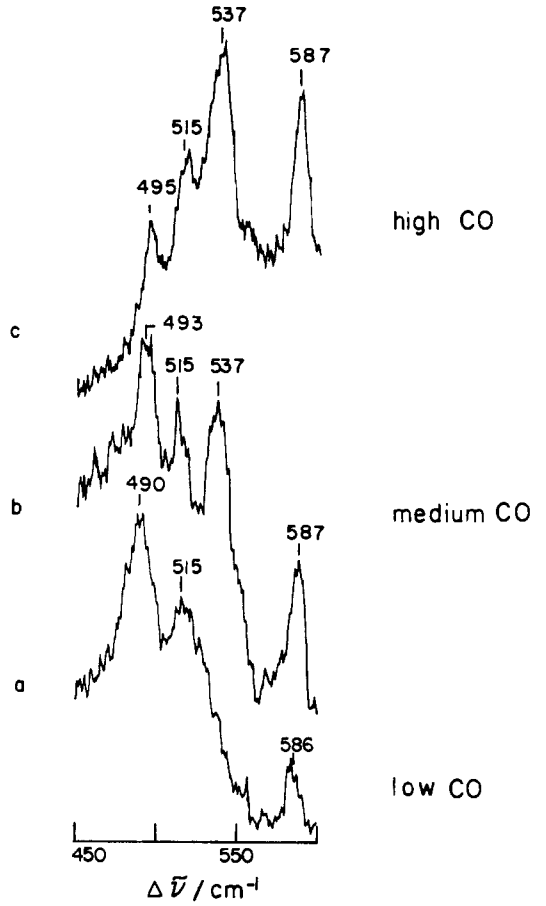

FIGURE 1: Resonance Raman spectra with 413.1-nm laser excitation of HRP–CO (1.5×10^{-3} M) in phosphate buffer (0.1 M), pH 6 at (a) low, (b) medium, and (c) high CO concentrations (see text). Experimental conditions: laser power, ~5 mW at the sample; spectral slit width, 5 cm^{-1}; accumulation time, (a) 15, (b) 12, and (c) 30 s/0.5 cm^{-1}.

CO exposure; presumably the higher uptake of CO by the concentrated protein leaves a lower CO concentration in solution.

These spectra contain a total of six bands. Three of them are porphyrin modes, at 495, 515, and 587 cm^{-1}, which are seen in myoglobin and protoheme complexes, and have been assigned (Choi & Spiro, 1983) to out-of-plane pyrrole folding (495, 515 cm^{-1}) and an in-plane infrared-type (E_u, ν_{48}) vinyl-coupled skeletal mode (587 cm^{-1}). The other three bands, at 490, 537, and 587 cm^{-1}, are associated with the bound CO, as demonstrated by the isotopic spectrum shown in Figure 2. Under low-CO conditions, the 490-cm^{-1} band is seen to shift down by 5 cm^{-1} for the ^{13}CO adduct and by 10 cm^{-1} for the $C^{18}O$ adduct. The shifts are typical for Fe–C stretching vibrations (Tsubaki et al., 1982). [The $C^{18}O$ spectrum shows a band at 550 cm^{-1}, which is assigned to another E_u type mode (ν_{49}), seen also in myoglobin (Choi & Spiro, 1983); its apparent gain in intensity is due to the additional downshift of a remnant of the 537-cm^{-1} FeC band.] The medium-CO spectra (obtained with the same aliquoting procedure as the low-CO spectra, but with half the protein concentration, 1.75 mM instead of 3.5 mM) show downshifts of the 537-cm^{-1} band, by 4 cm^{-1} for ^{13}CO and by 8 cm^{-1} for $C^{18}O$, again as expected for an FeC mode. In addition the ^{13}CO spectrum shows a new band at 567 cm^{-1} and a significant loss in intensity at 587 cm^{-1}. Thus the 567-cm^{-1} band has shifted out from under the 587-cm^{-1} band; this selective ^{13}CO downshift is consistent with assignment of the mode to the FeCO bend (Tsubaki et al., 1982; Yu et al., 1983). Only the C atom moves significantly

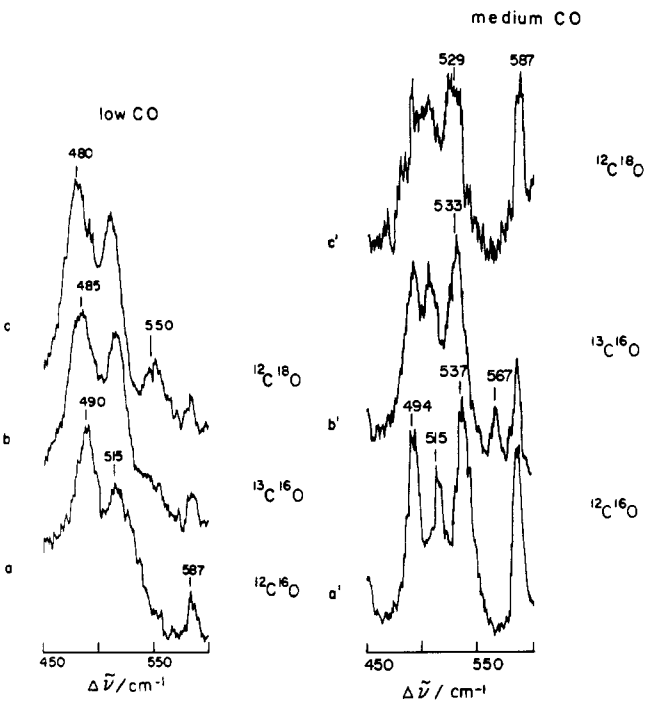

FIGURE 2: Resonance Raman spectra with 413.1-nm Kr^+ laser excitation of HRP–CO (1.5×10^{-3} M) and its ^{13}CO and $C^{18}O$ analogues at low and medium CO concentrations. Experimental conditions as in Figure 1, except accumulation time: (a and b) 15, (c) 24, (a′ and c′) 12, and (b′) 27 s/0.5 cm^{-1}.

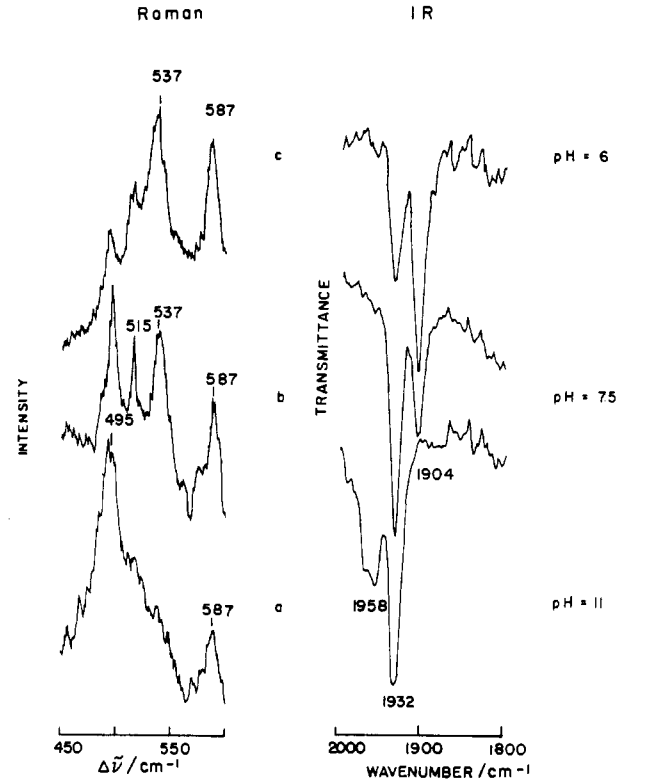

FIGURE 3: Resonance Raman and IR spectra of HRP–CO at high CO concentrations. Experimental conditions for RR as in Figure 1, except accumulation time: (a and b) 30 and (c) 9 s/0.5 cm^{-1}. Accumulation time for IR, (a, b, and c) 128 transients; spectral resolution, 4 cm^{-1}.

in this mode, and consequently the $C^{18}O$ frequency is unshifted and coincident with the underlying ν_{48} mode at 587 cm^{-1}.

Figure 3 shows the effect on the high-CO sample of raising the pH from 6 to 11.5. There is a loss of intensity at both 537

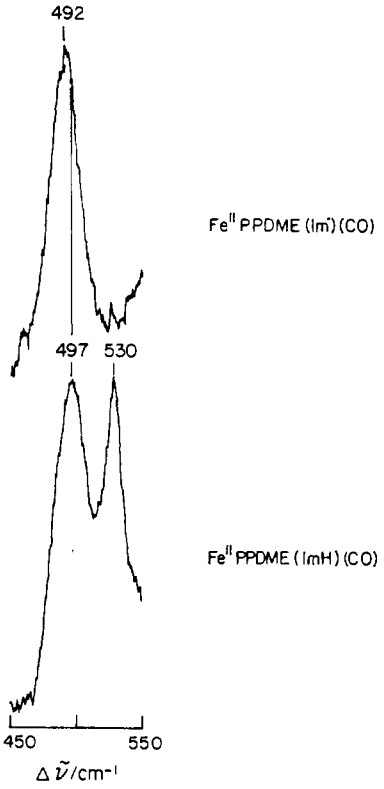

FIGURE 4: Resonance Raman spectra with 413.1-nm Kr^+ laser excitation of Fe^{II}PPDME(ImH)(CO) and $[Fe^{II}PPDME(Im)(CO)]^-$ in Me_2SO (2×10^{-3} M). Experimental conditions: laser power, ~15 mW on the sample; spectral slit width, 5 cm^{-1}; accumulation time, 9 s/0.5 cm^{-1}. The 530-cm^{-1} band in the bottom spectrum is due to some ImH-free CO adduct present in this solution.

and 587 cm^{-1}, accompanied by an increase in 490-cm^{-1} intensity. In parallel with this trend, the two CO stretching infrared bands, previously identified by Paul and co-workers (Barlow et al., 1976), change their relative intensities, the 1932-cm^{-1} band growing in at the higher pH, at the expense of the 1904-cm^{-1} band. At pH 11, both the 537-cm^{-1} Raman band and the 1904-cm^{-1} IR band have disappeared. [The 1958-cm^{-1} IR band is due to heme dissociated from the protein, a small amount of which is always seen at high pH (Barlow et al., 1976).]

Figure 4 shows the 500-cm^{-1} region of the Raman spectra for model compounds prepared by adding imidazole and CO to Fe^{II}PPDME in dimethyl sulfoxide solution and then deprotonating the bound imidazole. The effect of deprotonation is to shift the FeC stretching frequency from 497 to 492 cm^{-1}. [The 530-cm^{-1} band in the lower spectrum is due to some imidazole-free 5-coordinate CO–heme (Yu et al., 1983) that was present in this sample.] The CO stretching frequencies of these two imidazole adducts were determined by IR spectroscopy to be 1960 and 1942 cm^{-1} in methylene chloride solution; in this solvent the Fe–C stretching frequencies could not be obtained in Me_2SO because of interfering solvent bands.

The frequencies determined in this work are listed in Table I, where they are compared with data from other proteins and model compounds.

Discussion

The existence of multiple forms of bound CO for HRP was first demonstrated by Alben and Bare (1973), who found two IR CO stretching bands, at 1933 and 1905 cm^{-1}. Paul and co-workers (Barlow et al., 1976) demonstrated that the lower frequency band disappears at high pH, while the intensity of the higher frequency band increases, the titration curves giving pK_a's of 6.7 and 8.8 for isozymes A2 and C, respectively. They assigned the 1905-cm^{-1} band to bound CO that is H bonded to a protonated distal histidine residue. H bonding was subsequently confirmed directly via a 2.5-cm^{-1} downshift of the 1905-cm^{-1} band when the protein was prepared in D_2O (Smith et al., 1983). The 1933-cm^{-1} band (whose 1-cm^{-1} shift in D_2O was considered to be within experimental error) was assigned to non-H-bonded bound CO, which is partially, but not entirely replaced by the H-bonded form at low pH. The 1933-cm^{-1} band actually contained two components, separated by 13 and 4 cm^{-1} for isozymes A2 and C, respectively, suggesting two slightly different conformations of the non-H-bonded CO.

Table I: Bound CO Frequencies (cm^{-1})

CO adduct	ν_{FeC}	δ_{FeCO}	ν_{CO}
HRP I	490[a]		1932[a,b]
HRP II	537[a]	587[a]	1904[a,b]
HB	507[c]	578[c]	1951[c,d]
Mb	512[c]	577[c]	1944[c,e]
cyt a_3	520[f]	578[f]	1964[g]
Fe^{II}PPDME(ImH)[h]	495[a]		1960[a]
Fe^{II}PPDME(Im^-)[h]	490[a]		1942[a]

[a] This work. [b] Barlow et al., 1976. [c] Tsubaki et al., 1982. [d] Alben et al., 1968. [e] Makinen et al., 1979. [f] Argade et al., 1984. [g] Yoshikawa et al., 1977. [h] In methylene chloride.

Our HRP samples (predominantly the C isozyme) showed a single 1932-cm^{-1} IR band at high pH (Figure 3), which is replaced partially by a band at 1904 cm^{-1} as the pH is lowered. These bands are associated with the 537- and 490-cm^{-1} FeC stretching bands in the RR spectra. We label the FeCO units I and II with ν_{FeC} at 490 and 537 cm^{-1}, respectively, and ν_{CO} at 1932 and 1904 cm^{-1}, respectively. Form II has a δ_{FeCO} bending mode at 587 cm^{-1}, while no bending mode can be identified in the Raman spectrum for form I.

The appearance of the δ_{FeCO} Raman mode has been associated by Yu and co-workers (Tsubaki et al., 1982; Yu et al., 1983) with tilting of the FeCO unit relative to the heme plane. The mode is not observed for ordinary 6-coordinate CO–heme adducts, but it does show up for adducts with "strapped" hemes, in which an organic chain spans one side of the heme, offering steric hindrance to the binding of CO in its normal perpendicular fashion (Yu et al., 1983). Off-axis CO has been observed in crystal structures of MbCO (Norvell et al., 1975) and HbCO (Baldwin, 1980) whose RR spectra do show the δ_{FeCO} mode (Tsubaki et al., 1982). There is some question as to which angle, NFeC or FeCO, is distorted in the structures; a bent FeCO linkage, with an FeCO angle equal to 135°, has been reported for MbCO on the basis of neutron diffraction data (Norvell et al., 1975) and also EXAFS analysis of a frozen sample at 4 K (Powers et al., 1984), whereas the HbCO X-ray crystal structure (Baldwin, 1980) shows tilted, but linear, FeCO units. The vibrational frequencies and isotope shifts favor a linear, but tilted, structure in solution for both proteins (Yu et al., 1983). Since no δ_{FeCO} mode is observable for form I of HRP–CO, we tentatively conclude that the FeCO bonds are normal to the heme plane. In contrast, form II shows a strong δ_{FeCO} band and seems clearly to have a tilted or bent FeCO unit.

No δ_{FeCO} mode is observed in the Raman spectra of the complexes (ImH)FePPDME(CO) and (Im^-)FePPDME(CO), as expected. These complexes were examined in order to model the effect of strong H bonding from the proximal imidazole to a nearby acceptor group, which is seen in the CCP crystal structure (Poulos & Kraut, 1980; Poulos & Finzel, 1984) and is also evident in the high Fe–imidazole stretching

frequency (Teraoka & Kitagawa, 1980; Stein et al., 1980) and in the NMR spectra (LaMar & DeRopp, 1982) of reduced HRP. Imidazolate is a stronger donor ligand than imidazole, and the decrease in ν_{CO}, from 1960 to 1942 cm^{-1} for the FePPDME complexes, is attributable to the expected increase in back-donation to the CO π^* orbitals from the more electron-rich Fe. Less predictable is the observed 5-cm^{-1} downshift of ν_{FeC} from 495 to 490 cm^{-1} in the imidazolate complex (Table I). π-bonding arguments alone would suggest a stronger Fe–C bond associated with the increased back-bonding. However, the σ-bonding interactions of the imidazole and CO ligands compete for the same Fe d_{z^2} orbital, so that stronger σ-bonding from imidazolate may weaken the σ-FeC bond; this effect apparently predominates, although the net frequency shift is relatively small. The observed ν_{Fe-C} for HRP–CO form I, 490 cm^{-1}, is the same as that of the imidazolate model compound in CH_2Cl_2, consistent with the strong proximal imidazole H bonding mentioned above. The ν_{CO} frequency is even lower in the protein, by 10 cm^{-1}, than in the imidazolate model, a difference that is likely due to differences in polarity between the heme pocket and the methylene chloride solution.

The very low frequency of ν_{CO} for form II, 1904 cm^{-1}, is consistent with strong H bonding from the distal histidine (Barlow et al., 1976). Interaction of Lewis acids with the O atom of bound CO depresses ν_{CO} substantially (Shriver, 1984). The ν_{FeC} frequency of form II, 537 cm^{-1}, is unprecedentedly high. A comparison with other heme proteins (Table I) shows the cytochrome a_3 unit of cytochrome oxidase as giving the highest previous ν_{FeC} frequency, 520 cm^{-1} (Argade et al., 1984); a similar frequency is also seen for a minor component of the cytochrome *o*–CO complex of the terminal oxidase from *Escherichia coli* (Uno et al., 1985). In these cases, however, the ν_{CO} frequency is also high, 1963 cm^{-1} (Yoshikawa, 1977; Choc et al., 1982), while the Fe–imidazole frequency (Ogura et al., 1983) of reduced cytochrome a_3, 215 cm^{-1}, is quite low, suggesting a relatively weak Fe–imidazole bond. An increase in ν_{FeC} is known, on the basis of model compounds (Yu et al., 1983), to accompany the weakening of the transaxial bond. If the sixth ligand is missing, ν_{FeC} is ~530 cm^{-1} (Yu et al., 1983), and one possible interpretation of the high ν_{FeC} shown by HRP–CO form II is that the Fe–proximal imidazole bond is broken. In view of the strength of this bond in the CO-free protein, reflected in the high Fe–imidazole frequency, 244 cm^{-1} (Teraoka & Kitagawa, 1980), this explanation is unlikely. We attribute the elevated ν_{FeC} frequency instead to the effect of the distal H bonding, which draws electrons from Fe into the CO π^* orbitals, thereby raising the FeC bond order while lowering that of the CO. In contrast to the influence of proximal H bonding, which increases the imidazole donor strength and decreases ν_{FeC} (due to σ competition between the transaxial ligands) as well as ν_{CO}, distal H bonding is expected to increase ν_{FeC} while decreasing ν_{CO}.

Figure 5 is a sketch of the proposed binding modes associated with forms I and II of HRP–CO. This scheme differs from that given earlier by Paul and co-workers (Barlow et al., 1976) in two respects: (1) form I involves a normal CO linkage, perpendicular to the heme plane, while form II is tilted, and (2) deprotonation of the distal imidazole eliminates form II. Paul and co-workers had suggested, on the basis of the multiple components they observed for the 1933-cm^{-1} ν_{CO}IR band, that deprotonation leaves a tilted, but non-H-bonded, CO. Were this to happen, we would expect to see associated ν_{FeC} and δ_{FeCO} RR bands at ~510 cm^{-1} and ~570 cm^{-1}, as in MbCO and HbCO (see Table I). These are not observed for HRP, under any conditions, but we have found

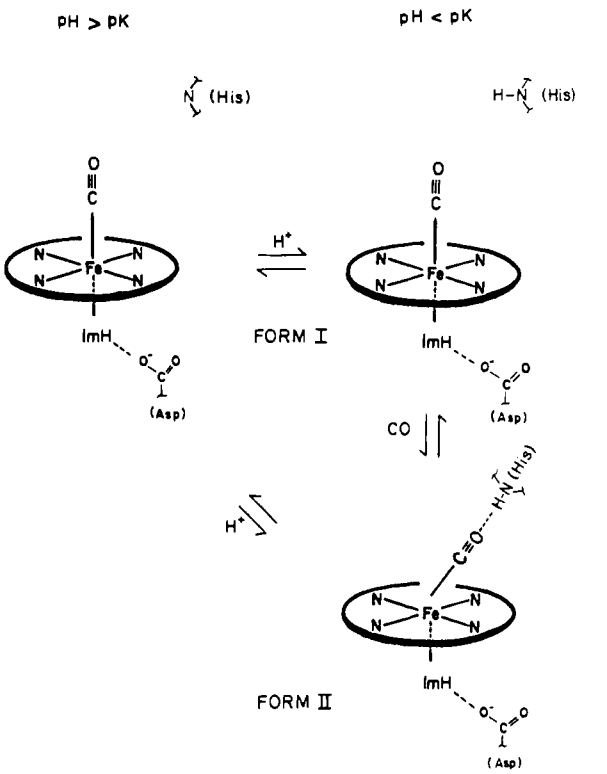

FIGURE 5: Schematic diagram of the two proposed CO binding modes for horseradish peroxidase at acidic and alkaline pHs. Strong H bonding from the proximal imidazole, consistent with the spectroscopic data, is suggested to involve an aspartate residue, as in CCP.

this pair of signals for the CO adduct of CCP at high pH (Smulevich et al., 1986). The multiple 1933-cm^{-1} IR components, not readily seen for isozyme C of HRP (see Figure 3), must represent conformational variants of the form I unit.

An unexpected finding is the CO concentration dependence of the equilibrium, at low pH, between forms I and II. This effect was discovered in the course of obtaining RR spectra with ^{13}CO and $C^{18}O$, for which the usual method of preparing the CO adduct with a flowing stream of the gas was not feasible. The spectra became interpretable when introduction of similar small amounts of natural abundance CO to reduced HRP was found to favor form I at the expense of form II. While the CO concentration dependence could not be quantitated, it was observed to be at least partially reversible; thus when a sample prepared under high-CO conditions was left to stand at ambient pressure, the Raman spectrum relaxed back to a point similar to the medium-CO spectrum shown in Figure 1. The effect is not ascribable to the hydrostatic pressure, per se, since exposure of the sample to N_2 at increasing pressures had no detectable effect on the RR spectra. The CO-specific spectral shift must be due to a perturbation of the distal interactions favoring form I relative to form II. We speculate that the heme pocket has an additional weak-binding CO site (not coordinated to the Fe) whose occupancy changes the conformation of the distal residues in a manner that favors the formation of an H bond from the protonated distal histidine to the Fe-bound CO. This site may be similar to the Xe-binding cavities known for Mb (Kretsinger et al., 1968), although in the present case occupancy of the site shifts a conformational equilibrium. An attempt was made to gauge the temperature dependence of the equilibrium by recording spectra of samples cooled to 4 °C, but no marked changes were observed.

Whether this secondary CO binding has any biological relevance is uncertain, since peroxidase–CO adducts are

presumably not involved in enzyme function. However, the conformational equilibrium itself may well be important since distal H bonding is probably involved in peroxidase activation and heterolysis (Poulos & Finzel, 1984). We note that a comparison of the crystal structure of the CCP fluoride adduct with that of the native protein (Edwards et al., 1984) shows the distal arginine to have swung into position to interact with the bound fluoride. The secondary binding of CO in the distal region might induce a similar conformation change, which could force the Fe-bound CO off-axis into an orientation favorable for interaction with the distal imidazole. The CO binding characteristics of CCP itself (Smulevich et al., 1986) also favor this hypothesis.

Registry No. HRP, 9003-99-0; Fe^{II}PPDME(ImH)(CO), 69971-14-8; $[Fe^{II}PPDME(Im)(CO)]^-$, 69971-13-7; CO, 630-08-0; L-His, 71-00-1.

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