Infrared Spectra of Carbonyl Horseradish Peroxidase and Its Substrate Complexes: Characterization of pH-Dependent Conformers

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Abstract: The FTIR spectra of carbonyl horseradish peroxidase (HRP) were re-investigated over an extended pH range between pH 3 and 11.5. Two ν(CO) bands were observed at 1934 and 1905 cm^{-1} and the relative absorbance intensity of these bands varied with pH. The absorbance of the 1905-cm^{-1} band increased in intensity on ionization of a group with a pK_{a1} = 4.0 ± 0.1, and decreased in intensity on ionization of a second group with a pK_{a2} = 8.7 ± 0.1. Since the vibrational spectrum of HRP−CO was not recorded below pH 5 previously, pK_{a1} was not observed and pK_{a2} was assigned to deprotonation of the distal His42 (Barlow, C. H.; Ohlsson, P. I.; Paul, K. G. *Biochemistry* **1976**, *15*, 2225). pK_{a2} is re-assigned here to a residue involved in the H-bonding network between the distal and proximal heme cavities, and pK_{a1} to deprotonation of the distal His42, since a pK_a <4 has been assigned to this residue in ferric HRP. Parallel pH-dependent changes were observed in the amide I′ region of HRP−CO, suggesting that shifts in the population of the CO conformers are accompanied by conformational changes in the helices surrounding the heme. Formation of substrate−HRP−CO ternary complexes with substrates of the type Ph−CO−NH−X (X = H, OH, NH_2, CH_3) resulted in shifting of the FeCO conformation equilibrium to a single form at pH 7.0 with ν(CO) values (cm^{-1}) of 1904 (X = H), 1911 (X = OH), 1916 (X = NH_2), and 1900 (X = CH_3). Examination of the pH dependence of the FTIR spectrum of the benzhydroxamic acid (BHA; X = OH) ternary complex revealed that the single ν(CO) band at 1911 cm^{-1} persists between pH 3 and 11, indicating that BHA binding inhibits the pH-dependent conformation equilibria of the FeCO unit. The combined FTIR results on the binary and ternary complexes are consistent with the involvement of Arg38, and *not* His42, in H-bonding to the CO ligand in HRP.

Introduction

The nature of the active-site residues in heme proteins such as myoglobin (Mb), catalases, cytochromes P_{450}, and peroxidases determines the function carried out by the heme.[1] From a comparison of their crystal structures, the increased polarity of the distal heme cavity in peroxidases compared to Mb is immediately apparent, and this is believed to promote heterolysis of H_2O_2 by peroxidases.[2] Particularly noteworthy in the fungal peroxidases is the presence of a distal arginine residue (Figure 1) at a position corresponding approximately to Phe43 in Mb. From crystal structures of cytochrome *c* peroxidase (CCP) adducts (F^-, CN^-, NO, and CO) it is apparent that the distal Arg48 has the conformational flexibility to adapt to different heme ligands.[3]

While many heme proteins possess distal and proximal histidines, the H-bonding and acid−base properties of these residues differ significantly.[1,2] For example, in the fungal peroxidases of known structure such as CCP,[4] lignin peroxidase (LIP),[5] *Arthromyces ramosus* (ARP),[6] and manganese[7] peroxidases, the proximal histidine is strongly H-bonded to a carboxylate side chain (Figure 1), but weakly H-bonded to a backbone carbonyl in Mb.[1,2] Furthermore, in the fungal peroxidases the imidazole ND1 atom of the distal histidine is H-bonded to the carboxamide of an asparagine residue, but partially exposed to solvent in Mb.[1,2] This ensures that NE2 of the neutral imidazole ring is a H-bond acceptor from Fe-

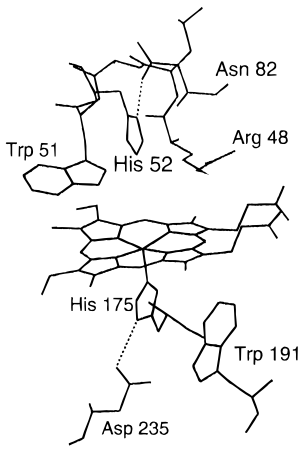

Figure 1. The heme and active-site residues in the X-ray structure of cytochrome *c* peroxidase (CCP).[4] The residues equivalent to the catalytic Arg48, His52, and His175 in HRP are Arg38, His42, and His170. Key H-bonds in the crystal structure of the ferric enzyme are shown as dashed lines. The δ-meso heme edge, facing the viewer, lies at the bottom of the substrate access channel that leads into the active site of heme peroxidases, and Phe142 and Phe143, which line the hydrophobic substrate binding pocket in HRP,[30] are adjacent to this edge.

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bound ligands in the peroxidases, but can act as either a H-bond acceptor or donor in Mb.[1,2] The pK_a of the distal histidine in ferric heme peroxidases is depressed to values between 4 and 5, which is attributed to the proximity of the δ-guanidinium group of the distal arginine (Figure 1).[8−10]

The prominence of H-bonding in the distal and proximal heme cavities of peroxidases has been highlighted by numerous vibrational studies of their CO adducts. For example, extensive resonance Raman (RR) studies on CCP−CO and its mutants[8,11−14] revealed two conformers at neutral pH, designated forms I and II. Form I is linear with a non-H-bonded FeCO unit, and the proximal His175 ligand possesses substantial imidazolate character due to strong H-bonding to Asp235.[8,11−14] The FeCO unit is tilted and H-bonded in form II, and the proximal Fe^{II}−His175 bond is weakened. Consistent with distal H-bonding in CCP−CO, the ν(CO) band is shifted by ~2 cm^{-1} in D_2O.[15]

The existence of multiple conformers of the FeCO unit in HRP−CO was first observed in the ν(CO) region of its IR spectrum.[16] Support for strong H-bonding between the CO ligand and a distal residue came from shifts (2−3 cm^{-1}) of ν(CO) in D_2O.[17] RR results confirmed the presence of pH-dependent FeCO conformational equilibria;[18,19] at neutral pH two Fe−CO stretching modes and one Fe−C−O bending mode were observed indicating linear and tilted FeCO conformers.[18,19] As in CCP, the CO ligand appears to be non-H-bonded in the linear conformer (form I), but strongly H-bonded in the tilted conformer (form II). Form I alone is observed at high pH and the conversion of the absorbance intensity to this conformer is coupled to an acid−alkaline transition with a pK_a of 8.8.[20] This is close to the pK_a of 8.5 observed by RR for H-bonding to the oxene (Fe^{IV}=O) ligand in HRP compound II,[21a] and identical to the pK_a of 8.8 which controls exchange of the oxene ligand with H_2O.[21b] It is noteworthy that the alkaline form of compound II is unreactive with aromatic substrates such as *p*-cresol.[22]

An acid−alkaline transition with an apparent pK_a of 7.5 has been reported for CCP−CO, where ν(CO) shifts from 1922 cm^{-1} in both acid forms I and II to 1948 cm^{-1} in the alkaline form I′.[23] This transition, which was originally assigned to deprotonation of the distal His52,[23] is also observed in the CO adduct of the CCP(H52L) mutant.[8,13] Disruption of the H-bonding chain that connects the proximal and distal sides of the heme in CCP, and involves the active-site residues Trp51 and Arg48 as well as a heme propionate and His181,[2,14,16] is now believed to convert CCP−CO to its alkaline form. RR[24] and NMR[25] studies on the active-site R38K mutant of HRP suggest that a similar H-bonding network exists in the wild-type enzyme. Thus, disruption of this network may also give rise to the reported acid−alkaline transition in HRP−CO, rather than deprotonation of the distal His42, as has been proposed.[18−20]

The structurally-related aromatic substrates, Ph−CO−NH−X [X = OH, benzhydroxamic acid (BHA); X = NH_2, benzhydrazide (BZH); X = H, benzamide (BZA); X = CH_3, *N*-methylbenzamide (NMBZA)] are believed to bind in a similar manner to HRP, with the aromatic ring occupying a hydrophobic binding site close to the heme edge such that the side chain extends into the distal heme cavity.[26−31] The Ph−CO−NH−X substrates are structurally related to aromatic peracids (Ph−CO−O−OH),[26,32] which react with ferric HRP to form compound I at diffusion-controlled rates.[33] In the BHA−HRP−CO ternary complex only a single ν(CO) band at 1911 cm^{-1} was reported at neutral pH.[18] This provides strong evidence that the hydroxamic acid side chain reaches into the distal heme cavity and reduces the number of conformations available to the FeCO unit. The ν(CO) frequency in BHA−HRP−CO shifts by ~2 cm^{-1} in D_2O,[18] revealing a H-bonded CO ligand, and the RR spectrum[18] exhibits ν(Fe−C) and δ(Fe−C−O) modes similar to those assigned to form II of HRP−CO. Thus, the single FeCO conformer in the ternary BHA complex resembles form II of the binary HRP−CO complex in that the FeCO unit is tilted and H-bonded.

The Ph−CO−NH−X substrates can partake in polyfunctional H-bonding and possess side chains of varying sizes and pK_a values. BHA and BZH can potentially form the same number of H-bonds but have pK_as of 8.8 and ~12, respectively, whereas the side chains of NMBZA and BZA exhibit pK_as ≥15 and possess non-H-bonding X groups. Hence, the substrate−HRP−CO ternary complexes were investigated to probe the effects on the ν(CO) frequencies of altering the H-bonding capability of the aromatic substrate, in addition to reducing the space available to the heme-bound CO ligand in the distal cavity. The FTIR spectra of the ternary complexes were also examined in D_2O to probe distal H-bonding to the CO ligand.

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The combined FTIR results, examined over a more extensive pH range (3−11.5) than previously,[18−20] provide compelling evidence that the distal His42 is *not* a H-bond donor to the CO ligand in HRP as previously suggested.[18−20] In light of a recent study on CO adducts of active-site mutants of Mb,[34] we interpret the low ν(CO) frequencies observed for form II of HRP−CO in terms of H-bond donation by the distal Arg38 to the CO ligand. The distal Arg38 probably also H-bonds to the oxene ligand in the acid, catalytically active form of HRP compound II.

Experimental Section

Materials. Horseradish peroxidase (E.C. 1.11.1.7) grade I was obtained from Boehringer Mannheim. Benzhydroxamic acid (99%), benzhydrazide (98%), benzamide (99%), *N*-methylbenzamide (99%), and D_2O (99.9%) were purchased from Aldrich and used without further purification. CO gas (99.3%) was obtained from Union Carbide or Air Products. All other reagents were of the highest quality available and were used without further purification. IR spectra were recorded on a Nicolet 205 FTIR spectrometer equipped with a deuterated triglycerine sulfate (DTGS) detector and purged with dry air. A dismountable IR cell (Model 116) with 13- × 2-mm CaF_2 windows and a 50-μm Teflon spacer were obtained from Wilmad, New Jersey.

FTIR Spectroscopy. Solutions of ∼2−4 mM ferric HRP were prepared spectrophotometrically (ϵ_{403} = 102.2 mM^{-1} cm^{-1})[26] in the following buffers: 0.1 M sodium formate pH 3−4; 0.1 M sodium acetate pH 4−6; 0.1 M sodium phosphate pH 6−8 and 11−11.5; 0.1 M sodium borate pH 8−10. The pD values for buffers prepared in D_2O were calculated from pD = pH (measured) + 0.4.[35] CO gas was passed over 10 μL of 2−4 mM ferric HRP in a sealed Eppendorf tube for ∼10 min, and ∼0.5 μL of buffered saturated dithionite solution was added using a gas-tight syringe. The solution was then rapidly pipeted into the IR cell and the absorbance of the HRP−CO complex was measured at 422 (ϵ_{422} ∼ 161 mM^{-1} cm^{-1})[26] on a diode array spectrophotometer to ensure complete formation of the CO adduct. For the ternary HRP−CO−substrate complexes, stock solutions of the substrates (150−275 mM) were prepared by weight, and ∼2 μL of stock substrate solution was added to 10 μL of protein solution to give final concentrations of 26 mM BHA, 46 mM BZA, 37 mM BZH or 42 mM NMBZA, and ∼2 mM HRP−CO in the IR cell. At these substrate concentrations the ternary complexes were fully formed since further increasing the substrate concentration caused no additional changes in the ν(CO) bands. Both substrates and HRP were equilibrated overnight in D_2O to ensure complete H/D exchange prior to sample preparation. The FTIR spectra reported here are an average of 256 or 512 scans recorded at a speed of 73 scans/min and a resolution of 4 cm^{-1}. To ensure no structural changes in the protein at low and high pH, the second set of 256 scans recorded were compared to the first set.

Results

pH Dependence of the ν(CO) Frequencies of HRP−CO. Figure 2 shows the FTIR spectra of HRP−CO in the ν(CO) region at pHs between 3 and 10. A single strong ν(CO) band is observed at low and high pH, with a frequency (1934 cm^{-1}) corresponding to that previously assigned to the linear, non-H-bonded form I of the FeCO unit.[18,19] A second conformer with ν(CO) at 1905 cm^{-1} grows in intensity between pH 3 and 6, and starts to decrease above pH 7. The frequency of this band is the same as that assigned to form II, the tilted H-bonded conformer of FeCO.[18,19] Barlow et al. also carried out a pH titration of the free HRP−CO complex but starting at pH 5,[20] and reported that below pH 5 HRP−CO exhibited profound structural changes leading to a broad ν(CO) band at 1970 cm^{-1}. However, we observed only a weak band in the spectra of HRP−CO at ∼1965 cm^{-1} at low pH, and no spectral changes occurred over the time course of our measurements.

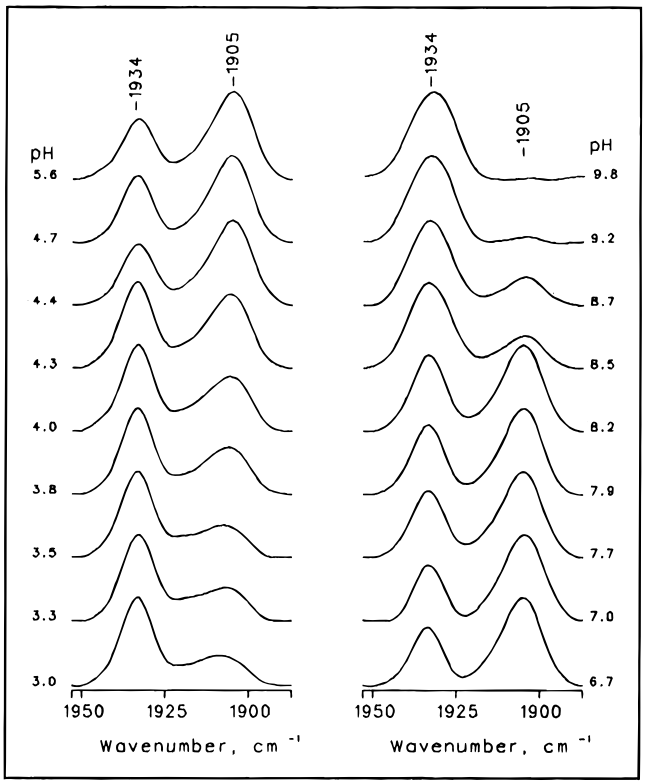

Figure 2. FTIR spectra of the ν(CO) region of HRP−CO vs pH. Experimental conditions: ∼2−4 mM HRP in CO-saturated 0.1 M buffer; 4-cm^{-1} resolution; 50-μm path length.

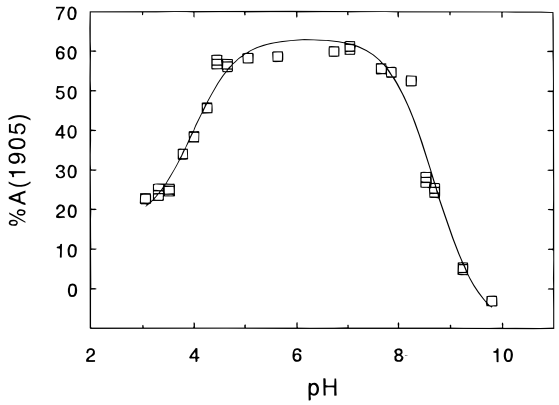

Figure 3. Relative integrated absorbance intensity of the ν(CO) band centered at 1905 cm^{-1} of HRP−CO [%A(1905)] vs pH. The squares represent the integrated intensity between 1887 and 1921 cm^{-1} as a percentage of the integrated intensity between 1887 and 1953 cm^{-1}. The curve shows the fit of eq 2 to the 34 data points, which yields pK_{a1} = 4.0 ± 0.1 and pK_{a2} = 8.7 ± 0.1.

The relative integrated absorbance intensity [%A(1905)] of the band centered at 1905 cm^{-1} between pH 3 and 10 is plotted in Figure 3. The growth of the 1905-cm^{-1} band can be associated with deprotonation of an amino acid residue at low pH and its disappearance at high pH is associated with deprotonation of a second residue. Thus, %A(1905) is a weighted sum of the contributions from three different forms of the enzyme, EH_2, EH, and E:

$$\%A(1905) = A_1 f_1 + A_2 f_2 + A_3 f_3 \qquad (1)$$

where f_1, f_2, and f_3 are the fractions at a given pH of forms EH_2, EH, and E, respectively, and A_1, A_2, and A_3 are the corresponding integrated intensities for 100% of each form. With acid dissociation constant K_{a1} relating forms EH_2 and EH, and K_{a2} relating forms EH and E, then:

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$$\%A(1905) = \frac{(A_1[H^+]^2 + A_2K_{a1}[H^+] + A_3K_{a1}K_{a2})}{([H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2})} \quad (2)$$

Nonlinear fitting of eq 2 to the 34 data points in Figure 3 gives pK_{a1} = 4.0 ± 0.1 and pK_{a2} = 8.7 ± 0.1. Since the two pK_as are well separated, both can be determined separately, and the values obtained are indistinguishable from those given.

As discussed below, the distal His42 in *ferric* HRP and other heme peroxidases has been assigned a pK_a ~4; hence, the pK_{a1} at 4.0 is attributed to deprotonation of this residue in HRP−CO. Consequently, the absorbance intensity shift to form II of the FeCO unit is linked to changes in the distal cavity that accompany *deprotonation* of the distal His42 at low pH. Above pH 9, form II disappears and only the linear non-H-bonded form I of FeCO is observed (Figure 2). The loss of form II intensity at high pH is linked to a second acid−alkaline transition in HRP−CO with a pK_{a2} of 8.7, which corresponds to the single pK_a of 8.8 reported by Barlow et al. from their IR studies,[20] but higher than the pK_a of 8.25 obtained from visible absorption measurements.[36] Contrary to our interpretation, the CO ligand in form II was assumed to be H-bonded to the distal His42, and its disappearance at high pH was attributed to deprotonation of His42 with a pK_a above 8.[18−20,36]

pD Dependence of the Amide I′ Frequencies of HRP−CO. The secondary structure of HRP−CO was also examined by FTIR to probe pH-induced global changes in protein conformation. Figure 4 shows the amide I′ region of free HRP−CO, which can be deconvolved into bands representing different secondary structural components.[37] The secondary structure of HRP is dominated by α-helices,[38] which give rise to bands between 1650 and 1660 cm^{-1}, random structure, which gives rise to the band at 1644 cm^{-1}, and turns or loops giving bands at 1671 and 1682 cm^{-1}.[37] Figure 4 reveals that the amide I′ bands of HRP−CO are essentially identical at pD 3 and 11, conditions where a dominant broad ν(CO) band is observed at 1934 cm^{-1} in H_2O (Figure 2). At intermediate pDs, corresponding to the pHs where the low-frequency ν(CO) band at 1905 cm^{-1} is more dominant in H_2O, the α-helical bands, particularly the higher frequency component at 1659 cm^{-1}, gain in intensity at the expense of the bands arising from random loop structure. Thus, it appears that the changes in population of the FeCO conformers may be associated with subtle pH-dependent conformational changes in the secondary structure of HRP−CO.

pH Dependence of the ν(CO) Frequencies of the Substrate−HRP−CO Ternary Complexes. Figure 5 shows the effects of substrate binding on the ν(CO) vibrations at pH 7.0, and Table 1 summarizes the observed ν(CO) frequencies and $\Delta\nu_{1/2}$ values for the binary HRP−CO and ternary substrate−HRP−CO complexes at two pHs and pDs. As can be seen from Figure 5, substrate binding dramatically alters the ν(CO) spectrum of HRP−CO at pH 7.0. In the four ternary complexes the spectra are dominated by a single narrow band that falls between 1916 and 1900 cm^{-1}. Such alteration in the FTIR spectra can be attributed to a reduction in the space available in the distal heme pocket on substrate binding, which reduces the conformational flexibility of the bound ligand, as was reported for certain distal heme-cavity mutants of Mb.[34] Essentially no absorbance intensity is observed around 1934 cm^{-1}, the frequency assigned to the linear non-H-bonded form I of free HRP−CO, indicating that substrate binding shifts the FeCO conformational equilibria toward the tilted H-bonded form II. Evidence for H-bonding in both free HRP−CO and BHA−HRP−CO was obtained previously by recording the IR spectra in D_2O, where 2−3-cm^{-1} shifts were observed in ν(CO) for form II of HRP−CO at 1905 cm^{-1} and for the single band of BHA−HRP−CO at 1911 cm^{-1}, indicating strong H-bonding to the terminal oxygen of CO.[17,18] Similar shifts were observed here in D_2O for the low-frequency ν(CO) bands (Table 1), supporting the assignment of ν(CO) in the ternary complexes to the H-bonded, tilted form II of FeCO. It is of interest that the Ph−CO−NH−X substrates

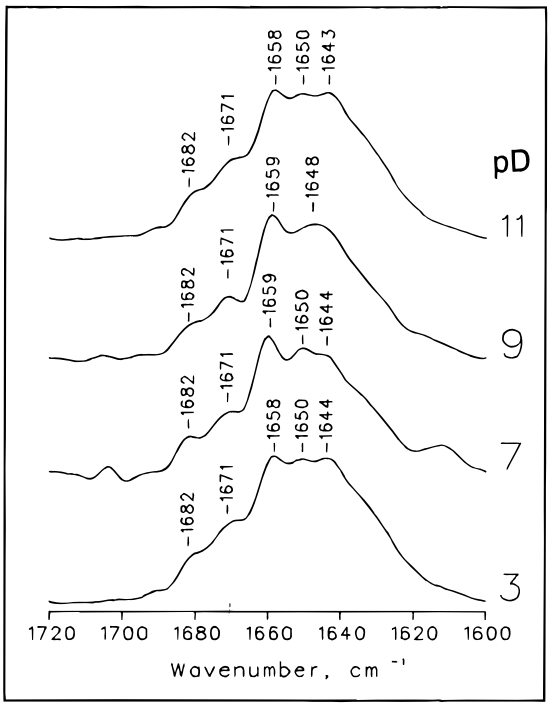

Figure 4. Deconvolved spectra of the amide I′ region of HRP−CO at pD 3.0 to 11.0. Experimental conditions: ~2 mM HRP in CO-saturated 0.1 M buffer prepared in D_2O; 4-cm^{-1} resolution; 50-μm pathlength.

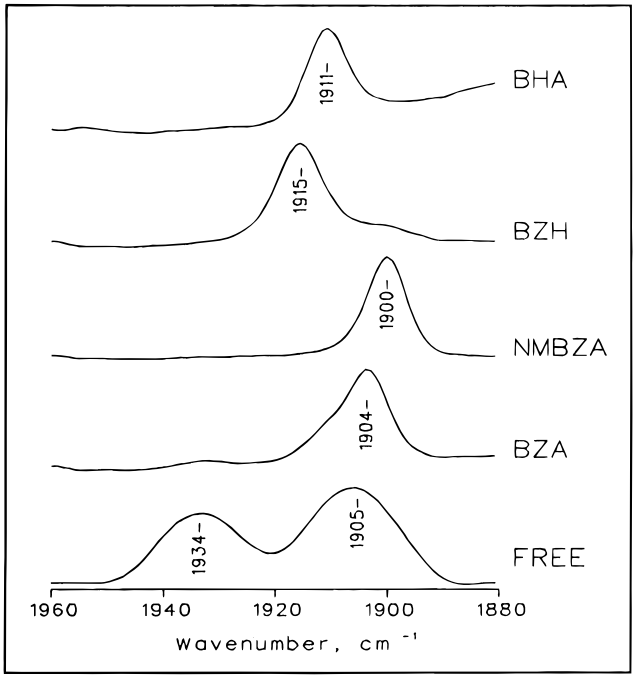

Figure 5. FTIR spectra of the ν(CO) region of substrate−HRP−CO ternary complexes at pH 7.0. Experimental conditions: ~2 mM HRP in CO-saturated 0.1 M phosphate buffer, 26 mM BHA, 46 mM BZA, 37 mM BZH, 42 mM NMBZA; resolution 4 cm^{-1}, 50-μm pathlength.

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Table 1. ν(CO) Frequencies (cm^{-1}) in HRP−CO and Substrate−HRP−CO Ternary Complexes[a]

substrate Ph−CO−NH−X	pH 7.0 ($\Delta\nu_{1/2}$)[b]	pD 7.0	pH 9.1	pD 9.9
none	1933.7 (11.2)	1932.6	1932.2	1933.4
	1905.0 (15.3)	1903.3		
BHA (X = OH)	1910.7 (8.5)	1909.7	1910.9	1909.9
BZH (X = NH_2)	1915.5 (10.6)	1913.2	1914.8	1913.4
BZA (X = H)	1903.8 (10.4)	1901.2	1903.3	1899.6
NMBZA (X = CH_3)	1900.1 (8.3)	1898.2	1899.8	1898.3

[a] Experimental conditions: 0.1 M CO-saturated buffers (pH 7.0 and pD 7.0, sodium phosphate; pH 9.1 and pD 9.9, sodium borate) containing dithionite-reduced HRP (∼2 mM) and substrate where indicated. Substrate concentrations (≥10K_d; mM): BHA, 26; BZH, 37; BZA, 46; NMBZA, 42; FTIR cell path length, 50 μm; 512 scans at 4-cm^{-1} resolution. [b] ν(CO) bandwidth at half maximum intensity.

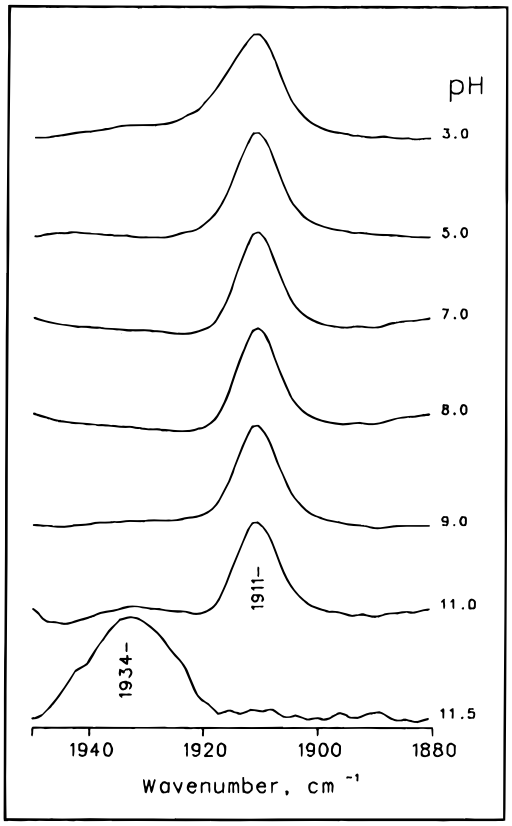

Figure 6. FTIR spectra of the ν(CO) region of BHA−HRP−CO ternary complex vs pH. The bandwidth at half maximum intensity ($\Delta\nu_{1/2}$) of the band centered at 1911 cm^{-1} is 10.9, 9.4, 8.5, 8.5, 9.1, and 8.6 at pH 3, 5, 7, 8, 9, and 11, respectively. Experimental conditions: ∼2 mM HRP and 26 mM BHA in CO-saturated 0.1 M buffer; 4-cm^{-1} resolution; 50-μm path length.

with H-bonding capacity in X (OH, NH_2) shift the ν(CO) frequency of form II to higher wavenumbers (1911 cm^{-1} for BHA−HRP−CO; 1915 cm^{-1} for BZH−HRP−CO) compared to the free HRP−CO value, while substrates with non-H-bonding X (CH_3, H) cause a shift to lower frequencies (1900 cm^{-1} for NMBZA−HRP−CO; 1904 cm^{-1} for BZA−HRP−CO).

The ν(CO) spectra of the substrate−HRP−CO complexes do not change between pH 7.0 and 9.1, nor between pD 7.0 and 9.9 (Table 1). Thus, substrate binding inhibits the high pH conversion of form II to form I with a pK_a of 8.7 in free HRP−CO (Figures 2 and 3). To further investigate the effects of substrate binding on ν(CO), the FTIR spectrum of BHA−HRP−CO was recorded between pH 3.0 and 11.5 (Figure 6). A single ν(CO) band is observed at 1911 cm^{-1} up to pH 11, indicating that the FeCO unit of BHA−HRP−CO adopts the tilted H-bonded conformer II, irrespective of pH. This is in marked contrast to free HRP−CO, where the population of form II is largely restricted to the pH region between the two acid−alkaline transitions (Figures 2 and 3). Ionization of bound BHA above pH 11 results in dissociation of the substrate from the ternary complex, and the FTIR spectrum resembles that of free HRP−CO at high pH (Figures 2 and 6). Free BHA has a pK_a of 8.5,[39] so its ionization is suppressed by ∼3 pH units on binding to HRP−CO, as was reported for BHA bound to ferric HRP.[26] Protonation of free BHA occurs below pH 3, thus the neutral form of BHA is bound to HRP−CO between pH 3 and 11.

The narrow ν(CO) bandwidth ($\Delta\nu_{1/2}$, Table 1 and caption to Figure 6) in the ternary complexes reflects little inhomogeneous broadening, indicating that substrate binding reduces the conformational flexibility of the FeCO unit in HRP.

Discussion

HRP belongs to the plant peroxidase superfamily, which consists of evolutionarily-related heme peroxidases from bacteria, fungi, and plants.[40] There are nine invariant residues in the plant peroxidase superfamily, and five of these are at the active site (Figure 1), including both the distal histidine and arginine residues. In the ferric enzymes, the distal histidine is deprotonated at low pH (pK_a ∼ 4),[1,2] which allows it to act as a general acid−base catalyst in heterolytic cleavage of H_2O_2 by accepting a proton from the O_α atom and transferring it to the departing O_β atom.[41] Consistent with this key role for the distal histidine, the H52L mutant of CCP reacts with H_2O_2 10^5-fold more slowly than the wild-type enzyme.[9] It has been postulated that the low pK_a of the distal histidine in the peroxidases is due to the presence of arginine in the heme pocket,[8−10] and also the distal His64 in Mb−CO is reported to have a pK_a of ∼4 due to the close proximity of Arg45 at the surface of the protein.[42] Despite the low pK_a of the distal His42 in ferric HRP, heme-linked ionizations with pK_as between 8 and 11 in other oxidation and ligation states of the enzyme have been attributed to deprotonation of the distal His42. For example, the acid−alkaline transitions in HRP compound II[21a,22] and ferric HRP−CN,[43] which have reported pK_as of 8.5 and 10.6, respectively, were attributed to deprotonation of the distal His42. The CO adducts of both CCP(H52L)[8,16] and the wild-type enzyme[23] exhibit a cooperative two-proton ionization with an apparent pK_a of ∼7.5. This transition is believed to involve disruption of the Trp51−Arg48−heme propionate−His181 H-bonding network that connects the proximal and distal sides of the heme. The involvement of His181 rather than the distal His52 has been confirmed since CCP(H181G) exhibits only a single-proton ionization.[8,13] Furthermore, the acid form II with a H-bonded CO ligand is observed for CCP(H52L)[8] but *not* for CCP(R48L),[14] indicating that Arg48 and *not* His52 is required for H-bonding to the CO ligand in the wild-type enzyme. The presence of a H-bonding network similar to that in wild-type CCP has been proposed for HRP from the results of RR[24] and NMR[25] studies on ferric HRP(R38K). Thus, disruption of this H-bonding chain may give rise to the acid−alkaline transition of HRP−CO with a $pK_{a2} = 8.7$.

The FTIR spectra, shown in Figures 2, 5, and 6 for HRP−CO and the substrate−HRP−CO ternary complexes, can also be interpreted in terms of the involvement of the distal Arg38 in H-bonding to the CO ligand in form II of the FeCO unit.

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Figures 2 and 3 reveal that population of form II of HRP−CO increases on *deprotonation* of the distal His42 with a $pK_{a1} = 4.0 \pm 0.1$. Although the neutral form of the distal His42 could also H-bond to the CO ligand, as may occur in Mb,[34,44] strong H-bonding of the ND1 atom to a carboxamide group in the peroxidases (Figure 1) favors the ND1-H tautomer over the NE2-H tautomer, which could H-bond donate to heme ligands. In the substrate−HRP−CO complexes, only form II is observed at both pH 7.0 (Figure 5) and 9.1 (Table 1). If the CO ligand in form II were H-bonded to the distal His42 with a pK_a of 8.2−8.8 as previously proposed,[18−20,36] a second ν(CO) band should appear in the FTIR spectra of the ternary complexes at high pH, but this is not observed. Furthermore, the extended pH titration of the ν(CO) frequency centered at 1911 cm^{-1} in BHA−HRP−CO (Figure 6) reveals no change in this band between pH 3 and 11, except for slight broadening at low pH. Hence, it is unlikely that the CO ligand in the BHA complex interacts with a distal residue that titrates between pH 3 and 11, excluding His42 as a possible H-bond donor. The strongly basic δ-guanidinium group of arginine side chains usually has a pK_a of ~12 in proteins, and can act as a H-bond donor over the pH range examined here. Also, interaction with the positive charge on Arg38 would give rise to the red-shifted ν(CO) frequencies observed for form II, which are at the low end of the reported range for heme proteins (1970−1900 cm^{-1}).[34]

It is proposed that the distal arginine in peroxidases stabilizes the developing negative charge on the peroxide O_β atom during heterolysis of the O−O bond.[1,2,45] The 10^3-fold decrease in peroxide activation by the R38K mutant of HRP[46] clearly indicates the key catalytic role of Arg38 in HRP. A second role of the distal arginine is to depress the pK_a of distal histidine, permitting the neutral imidazole side chain to accept a proton from H_2O_2 at the active site. Since Arg38 is not expected to titrate below pH 12, the pK_{a2} of 8.7 in HRP−CO must arise from deprotonation of another residue, such as the proximal His170, which may affect the H-bonding network around the heme.

The amide I′ spectra shown in Figure 4 reveal that HRP−CO retains secondary structure at the pD extremes (3 and 11) examined. Nonetheless, small but significant changes occur in the amide I′ region between the two heme-linked acid−alkaline transitions observed here for free HRP−CO. A pD-induced conformational change in HRP−CO in the vicinity of the heme would be consistent with the changes observed in the α-helical components in the FTIR spectra, since the heme is sandwiched between helix B in the distal pocket and helix F in the proximal pocket.[1,2] In CCP, the distal Arg48 and His52 extend from helix B, and the proximal His175 in the F helix is coordinated to the iron.[1,2] Despite large differences in physiological reducing substrates,[1,2] the structural homology between members of the plant peroxidase superfamily[40] is remarkable. Hence, a similar arrangement of the distal Arg38 and His42 and proximal His170 is expected in HRP and CCP, and NMR data to date support this assumption.[47] Since the growth of H-bonded form II of HRP−CO overlaps with increased intensity in the 1659-cm^{-1} amide I′ component (Figure 4), the population of form II may be controlled by the conformation of the B and F helices. This in turn may control the integrity of the H-bonding network that connects the proximal and distal heme pocket.[24,25] Parallel changes in the amide I′ bands and the ν(CO) frequencies were observed in a study on the thermal denaturation of HRP−CO.[38b]

A pK_a of 10.6 has been assigned to the distal His42 in ferric HRP−CN,[43] which is considerably higher than the present assignment of pK_{a1} at 4.0 to this residue in HRP−CO. It is generally accepted that HCN binds to HRP and other peroxidases to form the CN^- adduct and a proton simultaneously binds to the distal His42 to form the imidazolium side chain which H-bonds strongly to the CN^- ligand.[43] The X-ray structure of CCP(W191F)−O_2 indicates that the O_β atom is in a position to strongly H-bond to the distal His52,[45] but in CCP compound I Arg48 swings into position to H-bond to the oxene ligand.[45,48] Thus, the CO and CN^- peroxidase adducts may provide models of distal H-bonding in compounds I/II and the ES (or peroxy anion, Fe^{III}−OOH) complex of HRP, respectively. The latter unstable complex should be structurally similar to the stable Fe^{II}−O_2 adduct of CCP(W191F).[45] However, it should be noted that a recent RR study of ferric HRP−CN revealed the presence of two FeCN conformers which had not been resolved by NMR,[43] and H-bonding of CN^- to both the distal Arg38 and His42 is proposed.[49] To acertain whether H-bonding to the CN^- ligand in HRP is indeed different from that to the CO ligand, pH titration of ν(CN), observed at 2131 cm^{-1} at pH 8.0 for ferric HRP,[50] is required.

There is mounting evidence that the side chain of BHA may also H-bond to the distal Arg38 in HRP. Titration of the ferric HRP−CN complex with BHA and three methyl-substituted BHA derivatives resulted in perturbation of the Arg38 resonances in the ^1H NMR spectra.[30] The His42 CE1H proton resonance is also significantly perturbed,[30] and NOE connectivities between this proton and protons of the BHA nucleus[28,30] implicate His42 in the BHA binding site. A recent computer model of the BHA−HRP complex shows, however, the side chain of BHA H-bonding exclusively with Arg38,[29] and in addition HRP(R38K) does not bind BHA.[24] We speculate that in BHA−HRP−CO and BZH−HRP−CO, the substrates H-bond to Arg38 via their X groups (Table 1), since these ternary complexes exhibit ν(CO) frequencies 6−15 cm^{-1} higher than the BZA and NMBZA complexes. The higher values could arise from competition between CO and the substrate X group for H-bonding with Arg38.

Finally, it is of interest to compare the present results with those published for *Pseudomonas putida* cytochrome P-450_{cam}.[51] The camphor-free CO adduct of this enzyme gives rise to two ν(CO) vibrations at 1963 and 1942 cm^{-1}, while the camphor-bound ternary complex exhibits a single, narrow CO band at 1940 cm^{-1}.[51] A recent X-ray structure of the ternary complex reveals that the bound camphor provides steric contraint to the heme-bound CO in the distal pocket of P-450_{cam}.[52]

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