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Oxygen-17 Nuclear Magnetic Resonance Spectroscopic Studies of Carbonmonoxyperoxidases*

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We have obtained oxygen-17 (^{17}O) nuclear magnetic resonance (NMR) spectra of $C^{17}O$ ligands bound to ferrous horseradish peroxidase isozyme A, isozyme C, and ferrous chloroperoxidase, as a function of pH. Our results show that the peroxidases exist in two distinct states, the acidic and alkaline forms, which undergo reversible acid-base-induced transitions characterized by a single p*K* value. The two forms are characterized spectroscopically in much the same way in all three proteins, suggesting a similar structural origin for the transition process. In particular, the ^{17}O NMR signal of the acidic form is approximately 7 ppm more shielded than that of the alkaline form, and the CO ligand in the acidic form appears to have a smaller ^{17}O nuclear quadrupole coupling constant than that of the alkaline form. We have also obtained the p*K* values and exchange rates for all three peroxidases. The results indicate that a similar structural change may be involved in the transition process in all three peroxidases.

External ligands occupy the 6th coordination position of the heme iron in many hemoproteins, including peroxidases. Carbon monoxide is one such external ligand and has been extensively used in many spectroscopic studies, including infrared (1–4) and ^{13}C nuclear magnetic resonance (NMR) studies of peroxidases (5). Most recently, ^{17}O NMR of $C^{17}O$-ligated hemoproteins has been shown to be an informative probe for characterizing the active site structure of the O_2 transport hemoproteins, myoglobin and hemoglobin (6). Analysis of both relaxation and chemical shift data for these proteins has provided information on both the structural and dynamic properties of the heme group. In this paper, we describe our recent ^{17}O NMR studies of the CO complexes of the ferrous peroxidases: horseradish peroxidase isozyme A, horseradish peroxidase isozyme C, and chloroperoxidase.

Horseradish peroxidase (EC 1.11.1.7) is a hemoprotein which catalyzes the oxidation of various substrates by hydrogen peroxide. Like myoglobin and hemoglobin, horseradish peroxidase contains iron-protoporphyrin IX with an axially coordinated histidine and can bind a variety of exogeneous ligands, in both the ferrous and ferric states. There are, however, significant differences between horseradish peroxidase and the oxygen-carrying hemoproteins, which may be related to the very different reactivity of horseradish peroxidase. One of these is the stronger hydrogen-bonding (7, 8) ability in peroxidase of a heme-linked ionizable group with the protein or with external ligands, an effect that is weaker or absent in myoglobin (9). Thus, the role of ionizable groups, such as the proximal His (10), the distal His (11), and Asp (12) have been particularly stressed for the catalytic activity of horseradish peroxidase.

Chloroperoxidase (EC 1.11.1.10) is a unique hemoprotein whose biological function involves catalysis of the chlorination reaction involved in the biosynthesis of caldariomycin (13). Extensive studies of chloroperoxidase have revealed a close correspondence between its spectral properties and those of cytochrome P-450. Thus, it has been suggested (14–19) that cysteinyl sulfur is coordinated to the 5th position of the heme iron of chloroperoxidase. Despite this unusual heme iron coordination structure, chloroperoxidase still catalyzes the peroxidative oxidation of classical peroxidase substrates, in addition to its primary function in the halogenation reaction (20). Equilibrium binding studies have also revealed (21) the typical peroxidase-like ligand binding properties of chloroperoxidase with many exogeneous ligands, suggesting a similar heme active-site environment of chloroperoxidase and the other peroxidases. However, the structural details of the active site of chloroperoxidase are not yet known precisely, and the relationship between the active site structure and the catalytic activity of chloroperoxidase remains unclear. One of the goals of the present study is to further characterize the active site environments of chloroperoxidase and horseradish peroxidase, which must be responsible for their unique catalytic activities.

In order to obtain further information about the active site structures and heme environments of peroxidases, we have studied in detail the pH-dependent behavior of the CO-peroxidases, over a wide pH range, using ^{17}O NMR spectroscopy. Our present results demonstrate that CO-peroxidases can exist in two different forms, namely the acidic and alkaline forms, and that transitions between the two forms may occur due to the ionization of what appears to be a single group. Our results provide the p*K* values and rates for the transition process, the possible nature of the ionizable group involved, as well as the structural origin for the different transition rates between the various peroxidases. The results are also of purely spectroscopic interest, since they demonstrate that ^{17}O NMR of $C^{17}O$-labeled proteins is a viable new method for probing protein structure. In particular, the ^{17}O line widths observed are all quite narrow, so that adequate NMR sensitivity is not a major problem.

EXPERIMENTAL PROCEDURES

Horseradish peroxidase, type II, was purchased as a lyophilized, salt-free powder from Sigma. Isozymes A and C were purified by the methods of Shannon *et al.* (22). The samples had RZ (Reinheitszahl) values of at least 3.3, with a maximum value of 3.55 obtained for very pure samples. Chloroperoxidase was purified from *Caldariomyces*

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fumago grown on fructose (23) as described by Morris and Hager (13). The samples had RZ values of 1.4 or higher. All chemicals were of reagent grade, and purchased from J. T. Baker Chemical Co., and Sigma.

For the preparation of the CO complex of the peroxidase, native ferric peroxidase in a 50 mM phosphate buffer was reduced anaerobically with sodium dithionite and then carbonylated. $C^{17}O$ (36% ^{17}O) was obtained from ICON Services, Inc. (Summit, NJ). A buffer solution having the desired pH was made by titrating a 50 mM monobasic phosphate solution with either 50 mM dibasic, or tribasic, phosphate solutions. The pH of all samples were measured before and after the NMR experiments using a Corning (Park Ridge, IL) pH/ion meter 150, and Fisher buffer standards.

^{17}O NMR spectra were obtained at 11.7 T (corresponding to an ^{17}O resonance frequency of 67.8 MHz) on a "homebuilt" spectrometer, which consists of an 11.7-T 52-mm bore superconducting solenoid (Oxford Instruments, Osney Mead, United Kingdom), a Nicolet (Madison, WI) Model 1280 data acquisition system, an Amplifier Research (Souderton, PA) Model 200L amplifier for radio frequency pulse generation, and a 10-mm multinuclear probe (Cryomagnet Systems, Indianapolis, IN). All the spectra were recorded from static samples, using a spin-echo pulse sequence (90°-τ-180°-τ-acquire). The 90° pulse widths were between 35 and 40 μs, and typical recycle times of 200–300 ms were employed. Chemical shifts were referenced to $H_2{}^{17}O$ within the protein samples, at 0 ppm, using the convention that high frequency, low field, paramagnetic, deshielded values are positive (IUPAC δ-scale). All experiments were performed at ambient probe temperature (~26 °C).

RESULTS

The ^{17}O NMR spectra of CO-horseradish peroxidase isozyme A at pH values between 4.5 and 9.5 are shown in Fig. 1. This enzyme yields a single, pH-dependent signal, in the chemical shift range 355.5–362.6 ppm. The pH dependence of the chemical shifts of all species investigated is listed in Table I. The narrow signal at 353.2 ppm, which is also observed from CO-horseradish peroxidase isozyme C and CO-chloroperoxidase, can be assigned to free CO in solution. Fig. 2 shows a plot of $C^{17}O$ chemical shift *versus* pH, for CO-horseradish peroxidase isozyme A. The chemical shift follows a simple Henderson-Hasselbach dissociation curve having an apparent $pK = 6.1$, suggesting that CO-horseradish peroxidase isozyme A undergoes a transition between two different forms, namely the acidic (A) and alkaline (B) forms, with protonation and deprotonation of a single ionizable group being responsible for the chemical shift change of the bound CO ligand. Thus, the signals at 355.5 and 362.6 ppm represent the purely acidic (A) and purely alkaline (B) forms of the enzyme, respectively, while the signals in the region between them, which are significantly broadened by chemical exchange, represent mixtures of the two forms. The exchange rate between the acidic and alkaline forms, τ^{-1}, can be obtained from the pH 6.1 spectrum, in which the signal spreads over the entire (acid-base) chemical shift range. For chemical exchange between two equally populated sites this requires (24) that

$$\tau^{-1} = \pi \cdot \Delta\nu/\sqrt{2} \qquad (1)$$

where $\Delta\nu$ is the separation (in hertz) between the two signals in the absence of chemical exchange. The exchange rate estimated at pH = 6.1 is thus ~1.05×10^3 s^{-1} for CO-horseradish peroxidase isozyme A.

Table I also lists the spin-lattice relaxation times (T_1), and the apparent line widths, of the signals from the acidic and alkaline forms of CO-horseradish peroxidase isozyme A, at pH 4.5 and 9.5. The spin-lattice relaxation times were obtained by using the inversion-recovery method, and Fig. 3 shows representative partially relaxed spectra, at pH 4.5. It can be seen that T_1 values are an order of magnitude longer than the T_2 values deduced from the apparent line widths,

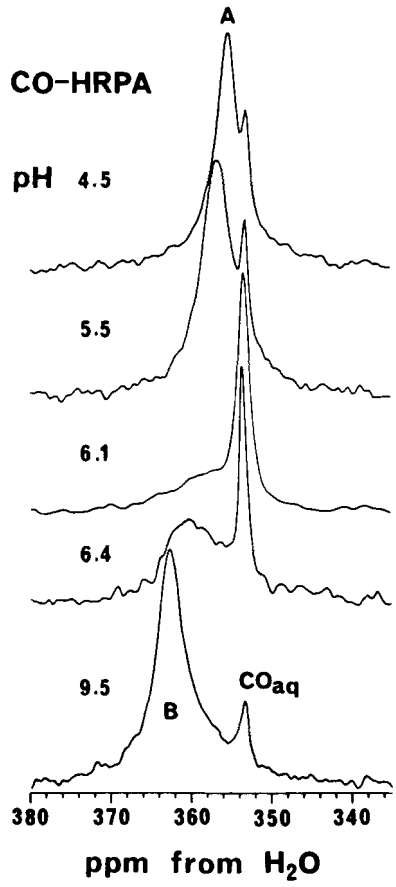

FIG. 1. **Oxygen-17 NMR spectra of CO-horseradish peroxidase isozyme A (*CO-HRPA*) (1 mM in 50 mM phosphate buffer) at 11.7 T, between pH 4.5 and 9.5.** 100,000–400,000 scans/spectrum, 150–250 ms recycle times, 38 μs 90° pulse widths, and 50 Hz line broadening. *A*, *B*, and CO_{aq} denote the acidic form of CO-horseradish peroxidase isozyme A, the alkaline form of CO-horseradish peroxidase isozyme A, and free CO in solution, respectively.

indicating that the rotational correlation time of the CO ligand is in the slow motion limit ($\omega_O\tau_c > 1$), where ω_O is the Larmor frequency, and τ_c the correlation time of the ^{17}O nucleus. It can also be seen that T_1 for the acidic form is considerably longer than that for the alkaline form and that the $C^{17}O$ line width of the acidic form is appreciably less than that of the alkaline form.

It has been shown (6) that the *predominant* relaxation mechanism for the ^{17}O NMR of $C^{17}O$, bound to hemoproteins, is quadrupolar. In such cases, the decay of the transverse magnetization is not a simple exponential but a weighted sum of three exponentials, in which the line width of the narrow component *decreases* as τ_c increases, in the slow motion limit ($\omega_O\tau_c > 1$), while the line widths of the intermediate and broad components increase (25, 26). The apparent line width, which is dominated by the narrow component of the $C^{17}O$ resonance, might thus be expected to be smaller at a longer τ_c. On the other hand, the decay of the longitudinal magnetization can be approximated by a single exponential for all correlation times, such that T_1 increases as τ_c increases in the slow motion limit. Thus, the differences between the acidic and alkaline forms could in principle be due to either a longer rotational correlation time, or a smaller ^{17}O quadrupole coupling constant (QCC),[1] of the CO ligand in the acidic form. However, it has also been shown (6) that the ^{17}O chemical shift aniso-

[1] The abbreviation used is QCC, quadrupole coupling constant.

TABLE I

Oxygen-17 NMR chemical shifts and relaxation data for CO-peroxidases

Sample	pH	δ_i[a]	T_1[b]	Line width[c]
		ppm	*ms*	*Hz*
Horseradish peroxidase *isozyme A* (EC 1.11.1.7)	4.5	355.5	52	195
	5.5	356.7		
	6.1	358.6		
	6.4	360		
	7	361.9		
	9.5	362.6	32	256
Horseradish peroxidase *isozyme C* (EC 1.11.1.7)	4.5	358.4		227
	7	358.3		221
	10.5	358.2		280
		+365		373
Chloroperoxidase (EC 1.11.1.10)	3.5	362.5		
	4.6	365.3		
	5.1	367.4		
	5.7	368.2		
	6	368.6		

[a] Chemical shift in parts/million from H_2O.
[b] Obtained by using the inversion-recovery method.
[c] Obtained by computer simulation of the spectra, using the Nicolet program NMRCAP. An exponential line broadening of 50–100 Hz was applied to the spectrum, and the corrected line widths are given in this column.

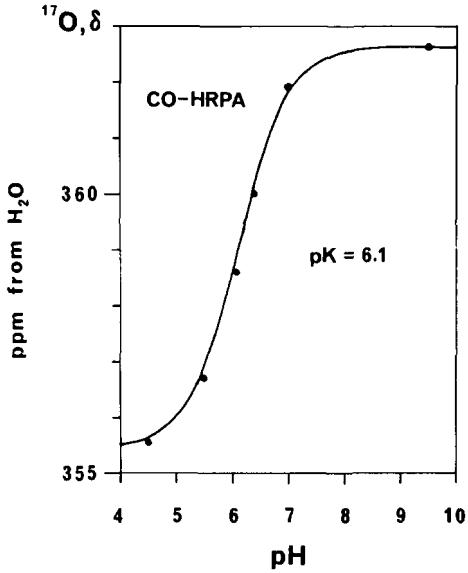

FIG. 2. **Plot of ^{17}O NMR chemical shift *versus* pH for CO-horseradish peroxidase isozyme A (*CO-HRPA*) (1 mM in 50 mM phosphate buffer).** *Circles* denote experimental data, the *line* is a theoretical (Henderson-Hasselbalch) dissociation curve having a pK = 6.1.

tropy of C^{17}O bound to hemoproteins is very large (~800 ppm) and that the C^{17}O line width is not very sensitive to τ_c in proteins, due to simultaneous contributions from both quadrupolar and chemical shift anisotropy interactions, while the chemical shift anisotropy contribution to T_1 is negligible. Thus, the apparent line width of MbC^{17}O is not much different from that of HbC^{17}O, even though Hb is almost four times larger than Mb. In this respect, the line-width decrease of some 60 Hz from the alkaline form to the acidic form of CO-horseradish peroxidase isozyme A appears to be significant, and we conclude that a smaller ^{17}O QCC of the C^{17}O is more likely responsible for the longer T_1 and narrower line width for the acidic form of CO-horseradish peroxidase isozyme A, than is a change in τ_c.

The pH dependence of the ^{17}O NMR spectrum of CO-horseradish peroxidase isozyme C is illustrated in Fig. 4. Whereas a single hemoprotein C^{17}O signal is observed (at 358.3 ppm) below pH 10, two separate signals are observed at pH 10.5. The new signal, assigned to the alkaline form of CO-horseradish peroxidase isozyme C, is broader and approximately 7 ppm shifted downfield from the signal of the acidic form. A similar pH-dependent change in the chemical shift

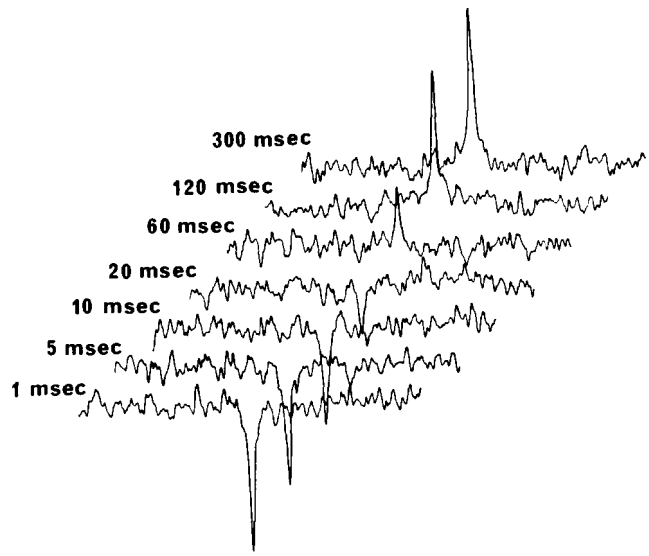

FIG. 3. **11.7 Tesla oxygen-17 inversion-recovery (180°-τ-90°) partially relaxed Fourier transform NMR data set for CO-horseradish peroxidase A (1 mM in 50 mM phosphate buffer), at pH = 4.5.** 20,000 scans, 250 ms recycle time, 80 μs 180° and 38 μs 90° pulse widths, and 200 Hz line broadening. The τ values used are given on the figure.

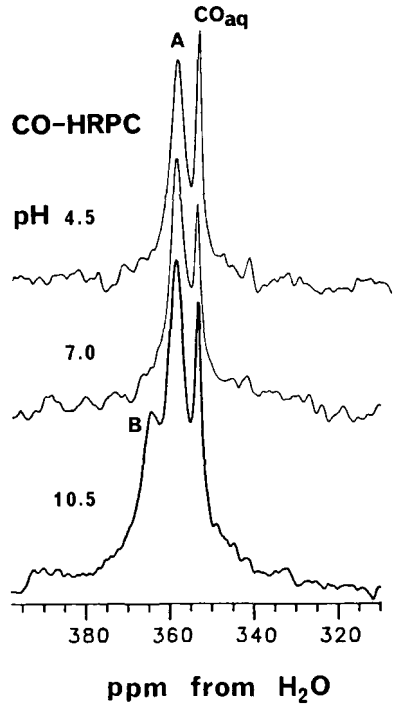

FIG. 4. **Oxygen-17 NMR spectra of CO-horseradish peroxidase isozyme C (*CO-HRPC*) (1 mM in 50 mM phosphate buffer) at 11.7 T, between pH 4.5 and 10.5.** 50,000–250,000 scans, 100–150 ms recycle times, 38 μs 90° pulse widths, and 100 Hz line broadening. *A*, *B*, and *CO_{aq}* denote the acidic form of CO-horseradish peroxidase isozyme C, the alkaline form of CO-horseradish peroxidase isozyme C and free CO in solution, respectively.

and line width was observed in the A ↔ B transition for CO-horseradish peroxidase isozyme A, suggesting the same structural origins in both CO-horseradish peroxidase isozyme A and CO-horseradish peroxidase isozyme C. In addition, the new signal decreases in intensity upon lowering the pH, while remaining at the same position. Thus, at pH 10.0 only a trace of the new signal is observed (data not shown). We therefore conclude that CO-horseradish peroxidase isozyme C undergoes a transition between the acidic and alkaline forms in much the same way as does CO-horseradish peroxidase isozyme A, but here the transition is *slow* on the NMR time scale ($\tau > 10^{-3}$ s). The pK can be estimated at ~10.7 from comparison of the relative areas of the two signals at pH 10.5, obtained by computer simulation of the spectrum shown in Fig. 5. The relative area of the signal from the alkaline form at pH 10.5 is approximately 40% of the total (protein) signal intensity.

The results of a computer simulation of the pH 10.5 CO-horseradish peroxidase isozyme C spectrum (Fig. 5) also indicate that both signals are broadened by the acid-base transition. In particular, the line width of the signal from the acidic form at pH 10.5 is about 60 Hz greater than that of the pH 4.5 or 7 signals (simulations not shown), which represent the purely acidic form of the enzyme. When two signals broaden but do not overlap strongly (slow exchange limit), the rate is related to the line broadening by (24)

$$\tau^{-1} = \pi \cdot \Delta\nu_{1/2} \qquad (2)$$

where $\Delta\nu_{1/2}$ is the difference in line width in the presence and absence of chemical exchange. Using Equation 2, the exchange rate between the acidic and alkaline forms of CO-horseradish peroxidase isozyme C is thus estimated to be ~1.88×10^2 s^{-1}.

The ^{17}O NMR spectra of CO-chloroperoxidase between pH 3.5 and 5.7 are illustrated in Fig. 6. A single, pH-dependent signal is observed, and its chemical shifts are listed in Table I. A plot of chemical shift *versus* pH is shown in Fig. 7, in which the chemical shift once again follows simple Henderson-Hasselbalch behavior, having a pK = 4.7. Although the pH titration of CO-chloroperoxidase was not extended to extreme pH values, due to protein instability, the chemical shifts of the purely acidic, and purely alkaline forms can nonetheless be determined to be 362 ± 0.5 and 369 ± 0.5 ppm, respectively, from the dissociation curve shown in Fig. 7. In the intermediate range of pH values, the signal is located at

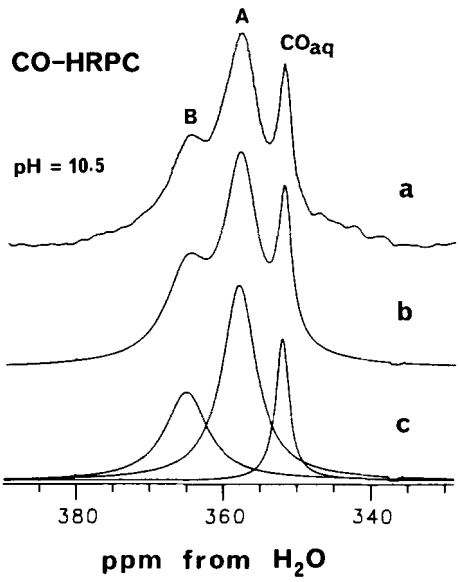

FIG. 5. **Simulation of the ^{17}O NMR spectrum of CO-horseradish peroxidase isozyme C (*CO-HRPC*), at pH = 10.5.** *a*, experimental spectrum, from Fig. 1. *b*, simulated spectrum. *c*, component peaks of *b*. The chemical shifts, line widths, and relative areas used were as follows: *A* (358.3 ppm, 380 Hz, 53%), *B* (365 ppm, 473 Hz, 32%), and CO_{aq} (353.2 ppm, 150 Hz, 15%). *A*, *B* and CO_{aq} denote the acidic form of CO-horseradish peroxidase isozyme C, the alkaline form of CO-horseradish peroxidase isozyme C, and free CO in solution, respectively.

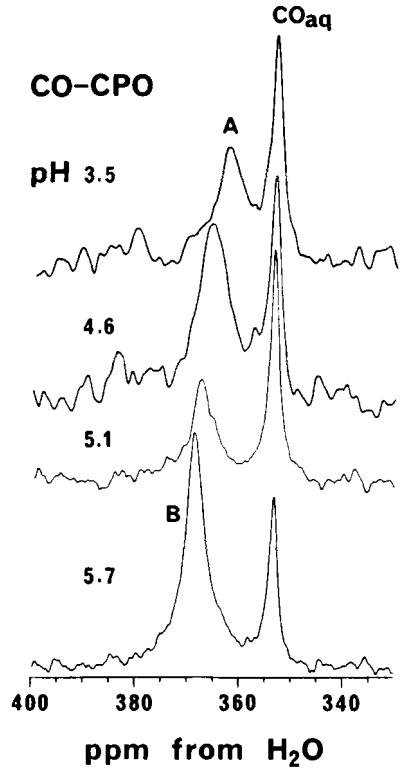

FIG. 6. **Oxygen-17 NMR spectra of CO-chloroperoxidase (*CO-CPO*) (2 mM in 50 mM phosphate buffer) at 11.7 T, between pH 3.5 and 5.7.** 300,000–600,000 scans/spectrum, 100–150 ms recycle times, 38 μs 90° pulse widths, and 100 Hz line broadening. *A*, *B* and CO_{aq} denote the acidic form of CO-chloroperoxidase, the alkaline form of CO-chloroperoxidase, and free CO in solution, respectively.

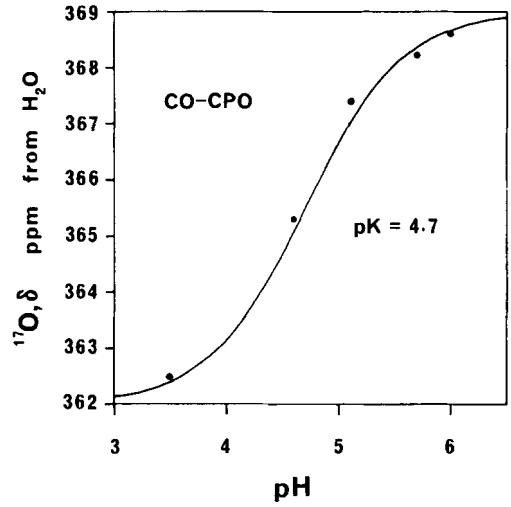

FIG. 7. **Plot of ^{17}O NMR chemical shift *versus* pH for CO-chloroperoxidase (*CO-CPO*) (2 mM in 50 mM phosphate buffer).** *Circles* denote experimental data, the *line* is a theoretical (Henderson-Hasselbalch) dissociation curve having pK = 4.7.

the weighted average position of the two forms, indicating that the transition is *faster* than the ^{17}O NMR time scale ($\tau \leq 10^{-3}$ s). An attempt to estimate the transition rate from the line widths was not made, since it is subject to large errors due to relatively poor signal to noise ratios of the ^{17}O NMR spectra at the acidic pH values.

DISCUSSION

Our results clearly demonstrate that horseradish peroxidase isozyme A, horseradish peroxidase isozyme C, and chloroperoxidase exist in two distinct forms: acidic and alkaline. All three proteins undergo reversible acid-base-induced transitions characteristic of a single ionizable group. Although the pK values and rates for the transition vary considerably between the three, as shown in Table II, the two forms of each may be characterized in much the same way in all three peroxidases. *First,* the ^{17}O NMR signal of the acidic form is approximately 7 ppm more shielded than that of the alkaline form. *Second,* the CO ligand in the acidic form appears to have a smaller ^{17}O nuclear QCC than that of the alkaline form. This pattern of change in both the chemical shift and ^{17}O QCC may be explained if one assumes that the CO ligand in the acidic form is hydrogen-bonded with a protonated, distal amino acid residue, since a similar change in ^{17}O chemical shift (27) and ^{17}O QCC (28) has been observed for the carbonyl oxygen of a variety of simple organic compounds upon hydrogen bond formation.

Another possibility is that the ^{17}O NMR chemical shift may be closely related to steric hindrance in the heme pocket, where the CO ligand in the more hindered heme pocket yields a more upfield shifted signal, as we have noted previously for several other heme proteins (6). Thus, the acidic form of the peroxidase could have a more hindered Fe-CO unit than the alkaline form. At present, we are unable to adequately assess the relative importance of such steric effects, and more work on determining ^{17}O chemical shifts and QCC changes due to hydrogen bonding/protonation of model organometallic compounds seems worthwhile. However, since in the previous study (6) we found *no* pH dependence for the ^{17}O chemical shifts of oxygen-carrying hemoproteins, while in this study both δ_i and QCC change with pH, and similar pH-induced changes have been seen in model systems, the "protonation" mechanism is clearly simpler, although we cannot completely rule out additional steric effects.

The present study also provides information on the exchange rate between the acidic and alkaline forms of the peroxidases. As shown in Table II, the time scale of the chemical exchange between the two forms is in the millisecond range, and the exchange rate is CO-chloroperoxidase > CO-horseradish peroxidase isozyme A > CO-horseradish peroxidase isozyme C. The rate appears to be much too slow to be attributed to a simple protonation-deprotonation reaction of a distal residue, as the proton dissociation rate of the imidazole coordinated to ferric myoglobin has been reported (29) to be much faster than 10^4 s^{-1}. Presumably, the exchange for the peroxidases involves a significant conformational change of the protein polypeptide chain itself.

TABLE II

Ionization constants for ferrous peroxidases (pK_r) and CO-peroxidases (pK_{CO}), and NMR-determined exchange rates for CO-peroxidases

	pK_r	pK_{CO}	pK_{CO} (NMR)[a]	τ_{CO}^{-1} [b]
				s^{-1}
Horseradish peroxidase				
Isozyme A	5.8[c]	6.7[d]	6.1	1.05×10^3
Isozyme C	7.25[e]	8.25,[e] 8.8[d]	10.7	1.88×10^2
Chloroperoxidase	4.7,[f] 5.5[g]		4.7	$>1.05 \times 10^3$

[a] This work. See Figs. 1, 5, and 7.
[b] The exchange rates were estimated from line widths by using Equations 1 and 2 in the text.
[c] Ref. 10.
[d] Ref. 1.
[e] Ref. 33.
[f] Ref. 34.
[g] Ref. 30.

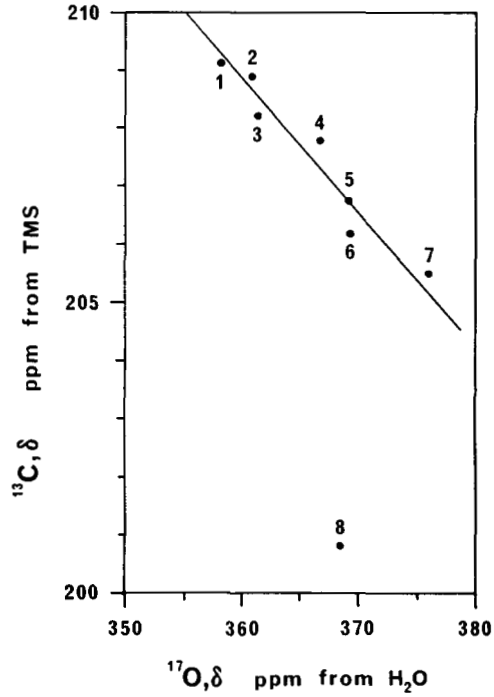

FIG. 8. **Plot of ^{17}O NMR chemical shifts *versus* ^{13}C NMR chemical shifts of CO ligands in various hemoproteins.** *1,* CO-horseradish peroxidase isozyme C, pH = 6.4, from Ref. 5. *2,* CO-horseradish peroxidase isozyme A, pH = 6.8, from Ref. 5. *3,* rabbit Hb α chain, from Refs. 6 and 48. *4,* sperm whale Mb, from Refs. 6 and 49. *5,* human Hb α chain, from Refs. 6 and 48. *6,* human Hb β chain and rabbit Hb β chain, from Refs. 6 and 48. *7,* CO-picket fence porphyrin, from Ref. 6. *8,* CO-chloroperoxidase, pH = 5.8, from Ref. 5. *TMS,* tetramethylsilane.

The presence of a heme-linked ionizable group in the ferrous peroxidases, and their carbon monoxide complexes, has been suggested by many spectroscopic (1, 30–33) and potentiometric (10, 34) studies. The pK values determined in these studies are compared with our results in Table II. These ionizations have been interpreted as protonations and deprotonations of distal residues and are attributed to the ionization of the imidazole ring of a histidine residue. In addition, it has also been suggested (10, 31, 32) that the distal residue is hydrogen-bonded to the ligand at the 6th coordination position, and the differences in pK values are caused by differences in the strength of this hydrogen bonding. Thus, the characteristic differences between horseradish peroxidase isozyme A and horseradish peroxidase isozyme C have been attributed to differences in hydrogen bond strength (31, 32), and the increase in pK from ferrous horseradish peroxidase to CO-horseradish peroxidase has been explained by the presence of hydrogen bonding in CO-horseradish peroxidase, which is absent in ferrous horseradish peroxidase (1, 33). Such explanations would require that the hydrogen bonding is much stronger in CO-horseradish peroxidase isozyme C than in CO-horseradish peroxidase isozyme A, since the shift of pK value in horseradish peroxidase isozyme C is much greater than that in horseradish peroxidase isozyme A. Furthermore, the

hydrogen bonding in CO-chloroperoxidase should be very weak, since the pK value of CO-chloroperoxidase is even smaller than that of ferrous chloroperoxidase. However, our results clearly show that whatever the nature of the interaction between the CO ligand and the distal residue, its effect on the CO ligand is the same for all three peroxidases, being characterized by the ~7 ppm upfield shift of the ^{17}O NMR signal of the acidic peroxidase. These data argue against the hypothesis that differences in hydrogen bond strength are responsible for the observed pK values.

The pK of 6.1 for CO-horseradish peroxidase isozyme A is compatible with the value of 6.7 determined (1) by infrared spectroscopic studies. A pK of 6.1 could suggest that the ionizable group in CO-horseradish peroxidase isozyme A is a histidine imidazole group. This pK value is quite close to that of 5.8 determined (10) for ferrous horseradish peroxidase isozyme A, suggesting that the same ionizable group may be involved in the acid-base transition of both ferrous horseradish peroxidase isozyme A and CO-horseradish peroxidase isozyme A.

For CO-horseradish peroxidase isozyme C, the pK of 10.7 does not agree with the values of 8.25 and 8.8 determined by optical (33) and infrared (1) spectroscopy, respectively, but is consistent with the observations by Kertesz *et al.* (35) that in the reaction of ferrous horseradish peroxidase isozyme C with CO the equilibrium constant is almost independent of pH between 7 and 9.18. The pK of 10.7 for CO-horseradish peroxidase isozyme C appears to be quite high for a dissociation constant for a histidyl residue, but rather close to the pK of a tyrosyl or lysyl residue.

Native horseradish peroxidase isozyme C in the ferric state also undergoes a transition with pK = 10.8, accompanied by a high spin to low spin transition (36, 37). This transition has generally been attributed (10, 38–40) to proton dissociation of an iron-bound water molecule, and the slow transition rate has been explained by assuming that hydroxide ion is replacing the water molecule (39) or that the dissociable proton of the water molecule of the 6th coordination position is strongly hydrogen-bonded to the protein (40). On the other hand, Epstein and Schejter (41) have proposed that a conformational change of the protein is involved in the transition process of ferric horseradish peroxidase isozyme C and is responsible for the slow transition rate. Our results show that the slow exchange rate is not a unique property of ferric horseradish peroxidase isozyme C but is also applicable to the transition of CO-horseradish peroxidase isozyme C. Furthermore, the exchange rate of 1.88×10^2 s^{-1} for CO-horseradish peroxidase isozyme C is remarkably similar to that of ~1.6×10^2 s^{-1} for ferric horseradish peroxidase isozyme C determined (41) by the pH jump method, suggesting that the slow rate is caused by the protein itself, rather than the nature of the ligand. Thus, our results support the suggestions that the conformational change of the protein is involved in the acid-base transition process of ferric horseradish peroxidase isozyme C.

The pK of 10.7 for CO-horseradish peroxidase isozyme C is about the same as that of 10.8 for ferric horseradish peroxidase isozyme C and also is very close to the values of 10.6 for CN-horseradish peroxidase isozyme C and 10.8 for NO-horseradish peroxidase isozyme C, determined by proton NMR (42) and flash photolysis (43), respectively. Thus, it seems likely that horseradish peroxidase isozyme C undergoes the same acid-base transition *regardless* of the oxidation state of the heme iron and the nature of the ligands. This invariance in pK may indicate a strong hydrogen-bonding network (4) of the distal residues in horseradish peroxidase isozyme C, such that the geometry of the distal residues is not greatly affected by reduction or carbonylation. On the other hand, the pK of 6.1 for CO-horseradish peroxidase isozyme A is much smaller than the value of 9.2 for ferric horseradish peroxidase isozyme A (31, 44), and the transition rate of CO-horseradish peroxidase isozyme A is markedly increased compared to that of the ferric horseradish peroxidase isozyme A (40). This change of pK and transition rate could be due to partial breakage of the hydrogen-bonding network in horseradish peroxidase isozyme A by reduction or carbonylation, which makes the protein less resistant to the pH-dependent conformational change of the protein and results in a relatively faster transition rate, having a pK characteristic of free histidine. We therefore propose that one characteristic difference between horseradish peroxidase isozyme A and horseradish peroxidase isozyme C lies in the increased strength of the hydrogen-bonding network of the distal residues in horseradish peroxidase isozyme C.

For CO-chloroperoxidase, the pK value of 4.7 is compatible with the pK values of 4.7 and 5.5 determined for *ferrous* chloroperoxidase by potentiometric (34) and kinetic studies (29), respectively, suggesting the involvement of the same ionizable group in the transition process in both CO-chloroperoxidase and ferrous chloroperoxidase. The pK of 4.7 is in the range of the pK of a carboxylic acid group; however, the possibility of a histidine residue with a low pK value cannot be excluded. In any case, our results show that the exchange rate between the acidic and alkaline forms of CO-chloroperoxidase is fast, compared with those of CO-horseradish peroxidase isozyme A and CO-horseradish peroxidase isozyme C, suggesting that CO-chloroperoxidase is more flexible and less resistant to the conformational change than CO-horseradish peroxidase isozyme A and CO-horseradish peroxidase isozyme C, which might be due in part to the unusual coordination structure of the heme iron at chloroperoxidase.

The unusual behavior of CO-chloroperoxidase is demonstrated by the results of Fig. 8, in which ^{17}O chemical shifts of the heme bound CO ligands are plotted against the corresponding ^{13}C chemical shifts in various hemoproteins. The ^{17}O chemical shifts for most of the hemoproteins (including myoglobin, hemoglobin, and horseradish peroxidase) are inversely correlated with the ^{13}C chemical shifts, as has been observed previously for the transition metal carbonyls (45–47). The chemical shifts of CO-chloroperoxidase, however, deviate markedly from this correlation. Since it is generally accepted that the CO ligand coordinates to the heme iron via the carbon atom, we believe that the unusual behavior of CO-chloroperoxidase is due to differences in the proximal side of the heme plane.

REFERENCES

1. Barlow, C. H., Ohlsson, P.-I., and Paul, K. G. (1976) *Biochemistry* **15,** 2225–2229
2. Smith, M. L., Paul, J., Ohlsson, P. I., and Paul, K. G. (1984) *Biochemistry* **23,** 6776–6785
3. Satterlee, J. D., and Erman, J. E. (1984) *J. Am. Chem. Soc.* **106,** 1139–1140
4. Iizuka, T., Makino, R., Ishimura, Y., and Yonetani, T. (1985) *J. Biol. Chem.* **260,** 1407–1412
5. Behere, D. V., Gonzalez-Vergara, E., and Goff, H. M. (1985) *Biochem. Biophys. Res. Commun.* **131,** 607–613
6. Lee, H. C., and Oldfield, E. (1988) *J. Am. Chem. Soc.*, in press
7. Teraoka, J., and Kitagawa, T. (1981) *J. Biol. Chem.* **256,** 3969–3977
8. Smith, M. L., Ohlsson, P. I., and Paul, K. G. (1983) *FEBS Lett.* **163,** 303–305
9. Norvell, J. C., Nunes, A. C., and Schoenborn, B. P. (1975) *Science* **190,** 568–570
10. Yamada, H., and Yamazaki, I. (1974) *Arch. Biochem. Biophys.* **165,** 728–738

11. Mincey, T., and Traylor, T. G. (1979) *J. Am. Chem. Soc.* **101,** 765–766
12. Dunford, H. B., and Araiso, T. (1979) *Biochem. Biophys. Res. Commun.* **89,** 764–768
13. Morris, D. R., and Hager, L. P. (1966) *J. Biol. Chem.* **241,** 1763–1768
14. Hollenberg, P. F., and Hager, L. P. (1973) *J. Biol. Chem.* **248,** 2630–2633
15. Champion, P. M., Chiang, R., Münck, E., Debrunner, P., and Hager, L. P. (1975) *Biochemistry* **14,** 4159–4166
16. Cramer, S. P., Dawson, J. H., Hodgson, K. O., and Hager, L. P. (1978) *J. Am. Chem. Soc.* **100,** 7282–7290
17. Hollenberg, P. F., Hager, L. P., Blumberg, W. E., and Peisach, J. (1980) *J. Biol. Chem.* **255,** 4801–4807
18. Sono, M., Dawson, J. H., and Hager, L. P. (1984) *J. Biol. Chem.* **259,** 13209–13216
19. Bangcharoenpaurpong, O., Champion, P. M., Hall, K. S., and Hager, L. P. (1986) *Biochemistry* **25,** 2374–2378
20. Thomas, J. A., Morris, D. R., and Hager, L. P. (1970) *J. Biol. Chem.* **245,** 3129–3134
21. Sono, M., Dawson, J. H., Hall, K., and Hager, L. P. (1986) *Biochemistry* **25,** 347–356
22. Shannon, L. M., Kay, E., and Lew, J. Y. (1966) *J. Biol. Chem.* **241,** 2166–2172
23. Pichard, M. A. (1980) *Can. J. Microbiol.* **27,** 1298–1305
24. Johnson, C. S., Jr. (1965) *Adv. Magn. Reson.* **1,** 33–102
25. Hubbard, P. S. (1970) *J. Chem. Phys.* **53,** 985–987
26. Bull, T. E., Forsén, S., and Turner, D. L. (1979) *J. Chem. Phys.* **70,** 3106–3111
27. Reuben, J. (1969) *J. Am. Chem. Soc.* **91,** 5725–5729
28. Butler, L. G., and Brown, T. L. (1981) *J. Am. Chem. Soc.* **103,** 6541–6549
29. Iizuka, T., and Morishima, I. (1975) *Biochim. Biophys. Acta* **400,** 143–153
30. Campbell, B. N., Jr., Araiso, T., Reinisch, L., Yue, K. T., and Hager, L. P. (1982) *Biochemistry* **21,** 4343–4349
31. Yamada, H., Makino, R., and Yamazaki, I. (1975) *Arch. Biochem. Biophys.* **169,** 344–353
32. Yamada, H., and Yamazaki, I. (1975) *Arch. Biochem. Biophys.* **171,** 737–744
33. Hayashi, Y., Yamada, H., and Yamazaki, I. (1976) *Biochim. Biophys. Acta* **427,** 608–616
34. Makino, R., Chiang, R., and Hager, L. P. (1976) *Biochemistry* **15,** 4748–4754
35. Kertesz, D., Antonini, E., Brunori, M., Wyman, J., and Zito, R. (1965) *Biochemistry* **4,** 2672–2676
36. Theorell, H., and Ehrenberg, A. (1951) *Acta Chem. Scand.* **5,** 823–848
37. Harbury, H. A. (1957) *J. Biol. Chem.* **225,** 1009–1024
38. Ellis, W. D., and Dunford, H. B. (1969) *Arch. Biochem. Biophys.* **133,** 313–317
39. Job, D., Richard, J., and Dunford, H. B. (1977) *Arch. Biochem. Biophys.* **179,** 95–99
40. Arasio, T., and Yamazaki, I. (1978) *Biochemistry* **17,** 942–946
41. Epstein, N., and Schejter, A. (1972) *FEBS Lett.* **25,** 46–48
42. de Ropp, J. S., La Mar, G. N., Smith, K. M., and Langry, K. C. (1984) *J. Am. Chem. Soc.* **106,** 4438–4444
43. Kobayashi, K., Tamura, M., and Hayashi, K. (1982) *Biochemistry* **21,** 729–732
44. Ohlsson, P. I., and Paul, K. G. (1973) *Biochim. Biophys. Acta* **315,** 293–305
45. Cozak, D., Butler, I. S., Hickey, J. P., and Todd, L. J. (1979) *J. Magn. Reson.* **33,** 149–157
46. Hickey, J. P., Wilkinson, J. R., and Todd, L. J. (1979) *J. Organomet. Chem.* **179,** 159–168
47. Buchner, W., and Schenk, W. A. (1982) *J. Magn. Reson.* **48,** 148–151
48. Moon, R. B., and Richards, J. H. (1974) *Biochemistry* **13,** 3437–3443