Ligand and Halide Binding Properties of Chloroperoxidase: Peroxidase-Type Active Site Heme Environment with Cytochrome P-450 Type Endogenous Axial Ligand and Spectroscopic Properties†

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ABSTRACT: Equilibrium binding studies of exogenous ligands and halides to the active site heme iron of chloroperoxidase have been carried out from pH 2 to 7. Over twenty ligands have been studied including C, N, O, P, and S donors and the four halides. As judged from changes in the optical absorption spectra, direct binding of the ligands to the heme iron of ferric or ferrous chloroperoxidase occurs in all cases; this has been ascertained for the ferric enzyme in several cases through competition experiments with cyanide. All of the ligands except for the halides, nitrate, and acetate form exclusively low-spin complexes in analogy to results obtained with the spectroscopically related protein, cytochrome P-450-CAM [Sono, M., & Dawson, J. H. (1982) *J. Biol. Chem. 257*, 5496–5502]. The titration results show that, for the ferric enzyme, (i) weakly acidic ligands ($pK_a > 3$) bind to the enzyme in their neutral (protonated) form, followed by deprotonation upon ligation to the heme iron. In contrast, (ii) strongly acidic ligands ($pK_a < 0$) including SCN^-, NO_3^-, and the halides except for F^- likely bind in their anionic (deprotonated) form to the acid form of the enzyme: a single ionizable group on the protein with a $pK_a < 2$ is involved in this binding. For the ferrous enzyme, (iii) a single ionizable group with the pK_a value of 5.5 affects ligand binding. These results reveal that chloroperoxidase, in spite of the previously established close spectroscopic and heme iron coordination structure similarities to the P-450 enzymes, clearly belongs to the hydroperoxidases in terms of its ligand binding properties and active site heme environment. Magnetic circular dichroism studies indicate that the alkaline form (pH 9.5) of ferric chloroperoxidase has an RS–ferric heme–N donor ligand coordination structure with the N donor likely derived from histidine imidazole.

Chloroperoxidase is a unique protein with which to test the relationships between structure and function in the heme iron class of enzymes. Extensive studies of chloroperoxidase (vide infra) have revealed close correspondence between its spectral properties and those of cytochrome P-450.[1] Such similarities are generally presumed to result from essentially identical heme iron coordination structures. And yet, in terms of catalytic activity, chloroperoxidase belongs to the hydroperoxidase category of enzymes while P-450 is a monooxygenase. The spectral parallels that have been observed for chloroperoxidase and P-450 have included studies with UV–visible absorption (Hollenberg & Hager, 1973), Mössbauer (Champion et al., 1973, 1975), magnetic circular dichroism (MCD) (Dawson et al., 1976; Sono et al., 1984), EPR (Hollenberg et al., 1980), resonance Raman (Champion et al., 1976; Hall et al., 1985), and extended X-ray absorption fine structure (Cramer et al., 1978) spectroscopy. In particular, both enzymes exhibit hyperporphyrin (split Soret) spectra with unusually red-shifted Soret peaks around 450 nm upon binding of CO to the ferrous enzyme (Hollenberg & Hager, 1973), thiolate ligands to the ferric enzyme (Dawson et al., 1983; Sono et al., 1984), and phosphine ligands to either the ferric or ferrous enzyme (Sono et al., 1985a), strongly suggesting endogenous ligation of a thiolate anion to the heme iron of chloroperoxidase as has been well established for P-450 (Dawson & Eble, 1985). However, chloroperoxidase is quite distinct from P-450 in its catalytic properties: in addition to classic peroxidase and catalase activities, it can catalyze the peroxide-dependent halogenation of organic substrates utilizing halide anions such as Cl^-, Br^-, and I^- as the source of the halogen atom (Hager et al., 1966; Thomas et al., 1970a). Only a very limited number of reactions have been found to be carried out by both enzymes (Kedderis et al., 1980; McCarthy & White, 1983; Padbury & Sligar, 1985) despite extensive investigations of their catalytic reactivities. Although many studies of chloroperoxidase focusing on its molecular, catalytic, and spectroscopic properties have been reported since its discovery (Morris & Hager, 1966), the relationship between its active site structure and its catalytic activity, vis-à-vis P-450, remains unclear.

In order to obtain new information about the active site structure and heme environment of chloroperoxidase, we have performed detailed studies of the exogenous ligand and halide anion binding properties of the enzyme over a wide pH range (pH 2–7) using more than twenty ligands with different donor atoms, electronic properties, and structures. The ligand binding properties of heme proteins have frequently been used

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[1] Abbreviations: P-450, cytochrome P-450; MCD, magnetic circular dichroism; CD, circular dichroism; EPR, electron paramagnetic resonance; CAM, camphor.

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to probe their active site structures [see, for example, Sono et al. (1982)]. In the course of our comparative spectroscopic studies of chloroperoxidase and P-450 (Dawson et al., 1983; Sono et al., 1984), Dunford and co-workers have reported on the chloride, cyanide, and nitrate binding properties of the ferric enzyme (Lambeir & Dunford, 1983a,b; Lambeir et al., 1983). Our present results have shown that, consistent with the results for those three ligands, all of the ligands examined in this study including the four halides bind directly to the heme iron of ferric chloroperoxidase. More importantly, our studies reveal that chloroperoxidase possesses typical peroxidase-type ligand binding properties and, therefore, a peroxidase-like active site heme environment, although its heme iron coordination structure and spectroscopic properties resemble those of P-450. Possible halide binding sites of chloroperoxidase during its catalytic reactions are also discussed.

Experimental Procedures

Materials. Chloroperoxidase, purified from *Caldariomyces fumago* grown on fructose (Picard, 1980) as described by Morris & Hager (1966) and Palic et al. (1980), was greater than 85% pure as judged by electrophoresis and had an R_z (A_{399}/A_{280}) value of 1.47 or greater at pH 6.0 and 4 °C. Cytochrome P-450 was purified to electrophoretic homogeneity ($A_{391}/A_{280} > 1.5$) from *Pseudomonas putida* grown on *d*-camphor following slight modification (Dawson et al., 1982) of the original methods of Peterson and co-workers (O'Keeffe et al., 1978) and of Gunsalus & Wagner (1978). All chemicals were of reagent grade, were purchased from Fisher or Aldrich, and were used without further purification.

Titrations of Chloroperoxidase with Ligands and Halides and Preparations of Homogeneous Complexes. Spectrophotometric titrations were generally performed by stepwise additions of microliter volumes of ligand or halide stock solutions to the enzyme solutions (1–2 mL) in 0.1 M potassium phosphate between pH 2 and 7. Since the ionic strength (μ) of 0.1 M potassium phosphate varies little between pH 3 and 6 (μ = 0.08 at pH 3.0 and μ = 0.12 at pH 6.0) and because ionic strength has very little effect on ligand binding affinity as reported by Thomas et al. (1970b) and confirmed in this work, adjustment of the ionic strength was done only below pH 3 by additions of sodium sulfate. Although phosphate is not a good buffer between pH 3.5 and 5, other buffers were not used in order to avoid any possible effect of different buffers such as is discussed below for acetate. Titrations of the ferrous enzyme were carried out in the presence of a slight excess of sodium dithionite under a nitrogen atmosphere. In the titrations of the ferrous enzyme with *n*-butyl isocyanide below pH 4, stepwise additions of small amounts of the ligand that were expected to yield 10–20% ligand saturation of the enzyme showed anomalous and nonreproducible results for unknown reasons. Consequently, determinatrion of K_d values of the ferrous enzyme–alkyl isocyanate compelx below pH 4 were done as follows. First, ferric enzyme–alkyl isocyanide complexes were prepared, and then the enzyme was reduced with dithionite under a nitrogen atmosphere. K_d values were then calculated from the fractional ligand saturation (Y) of the enzyme (10^{-6}–10^{-5} M) as a function of the ligand (L) concentrations (10^{-3}–10^{-2} M): $K_d = [L](1 - Y)/Y$. The spectrum of the ligand-saturated enzyme below pH 4 could not be obtained because of relatively low affinity and solubility of the alkyl isocyanide and was assumed to be identical with that obtained above pH 5. Ligand stock solutions were prepared in water or 0.1–0.5 M potassium phosphate buffer, and the pH was adjusted, when necessary, with phosphoric acid. For water-insoluble ligands [alkyl isocyanides, *N*-phenylimidazole, and phosphines (under nitrogen)], stock solutions (0.01–1 M) were prepared in ethanol. The pH of KF stock solutions (1 M) was adjusted with phosphoric acid according to the Henderson–Hasselbach equation ($pK_a^{HF} = 3.18$, $pK_a^{H_3PO_4} = 2.15$) because a pH meter is not accurate for acidic fluoride solutions. To check the accuracy of this method, potassium formate ($pK_a = 3.75$) solutions of various pH values were prepared in a similar fashion, and the resultant pH values were confirmed to agree with the calculated pH values by direct measurement. In some cases, solid salts of potassium halides (KCl, KBr, KI) were directly added to sample solutions to avoid volume changes with low-affinity halides (KCl and KBr) and to avoid autoxidation of iodide in stock solutions. In those cases, the pH was adjusted with KOH and indirectly monitored by checking the pH values of control solutions containing no enzyme. HCl solutions were not used for pH adjustments since chloride is itself a ligand. For similar reasons, a pH meter was not used directly on titration samples because the electrode is stored in saturated KCl solution, possibly resulting in a small chloride contamination.

For spectroscopic measurements, it is important that homogeneous (i.e., fully saturated) ligand complexes be examined whenever possible. This was accomplished by use of total ligand (L) or halide (X) concentrations in large excess of the concentration value of the dissociation constant: in most cases $[L]/K_d^L$ and $[X]/K_d^X > 20$. In halide solutions above 1 M, the enzyme was unstable even at 4 °C, as judged by a decrease with time in Soret absorbance concomitant with a blue shift of its position. At pH values below 3, lower halide concentrations (<1 M) were also found to cause denaturation of the enzyme. The samples after these spectral changes contained a considerable amount of the denatured, P-420-type form of chloroperoxidase (Hollenberg & Hager, 1973) when checked by reduction with dithionite in the presence of CO. For this reason, the spectra of homogeneous complexes with chloride and bromide could not be obtained and were instead generated by the use of a computer (Sono et al., 1984). When K_d values were not determined, ligand saturation was estimated from the absence of subsequent spectral changes upon further ligand addition.

Other Measurements. Spectrophotometric measurements were performed on Varian Cary 219 and 210 spectrophotometers equipped with a Lauda circulator for temperature control (4 ± 1 °C, unless otherwise stated). MCD/CD spectra were recorded on an upgraded JASCO J-40 spectropolarimeter as previously described (Dawson et al., 1982). The pH measurements were carried out at room temperature with a Brinkman 101 pH meter (Metrohm combination electrode).

Results

Equilibrium, Spectral, and Spin-State Properties of Ferric Chloroperoxidase–Ligand Complexes. Over twenty ligands, including C, N, O, P, and S donors, have been found to form complexes with ferric chloroperoxidase. The optical absorption spectral data, dissociation constants, and spin states of these complexes are summarized in Table I in the order of decreasing wavelength of the Soret peaks of the complexes. Acetate, used as a buffer in past studies of chloroperoxidase (Hollenberg et al., 1980), has been found to bind to the heme iron of the enzyme. Except for nitrite (vide infra) and nitrate, the ligands listed in Table I also form adducts with ferric P-450-CAM (Sono & Dawson, 1982; Dawson et al., 1982). The Soret absorption peak positions of most ferric chloroperoxidase complexes (Table I) are red-shifted by 4–18 nm from those of the analogous P-450-CAM complexes. The pyridine complex shows the largest difference (439 nm vs. 421 nm). The

Table I: Optical Absorption Data, Dissociation Constants, and Spin States of Ferric Chloroperoxidase–Ligand Complexes[a]

ligand	pK_a of ligand[b]	absorption maxima[c] [λ (ϵ_{mM})] δ	Soret	β	α		K_d^{app} (mM) pH 3	pH 6	K_d^{int}[d] (M)	spin state
CH_3SH[e]	10.4	372 (65)	455 (63)	554 (13.6)			*f*	90	9.0×10^{-2}	low
$(HOMe)_2MeP$[g]		376 (56.5)	450 (64)	553 (12.0)			*f*	2	*f*	low
CH_3COSH	3.5	378 (55)	446 (62)	554 (11)		648 (3.2)	20	*f*	1.5×10^{-2}	low
KCN	9.14	439 (92)	557 (13.3)	594[h] (6.5)			0.017	0.019	1.8×10^{-5}	low
pyridine[i]	5.19	366 (51)	439 (82)	557 (13.1)	600[h] (6.3)		*f*	160	1.4×10^{-2}	low
KCN[j] (alkaline)	9.14	364 (42)	437 (79)	556 (11.5)						low
NO		362 (*f*)	437 (114)	545 (17.5)	577 (18.1)		*f*	*f*	*f*	low
n-butyl isocyanide		368 (39)	435 (106)	551 (15.4)	584[h] (8.1)		0.24	0.23	2.3×10^{-4}	low
benzyl isocyanide		374	433	548			*f*	3.5	3.5×10^{-3}	low
KN_3	4.72	360 (43)	432 (110)	549 (12.3)	584 (9.4)	644 (2.5)	0.2	4.2	1.8×10^{-4}	low
KSeCN		*f*	432 (80)	550 (11.6)	588[h] (7.0)	648 (2.5)	*f*	10	*f*	low
KSCN	−1.9	365 (37)	429 (112)	548 (13.6)	584 (8.4)	646 (2.5)	0.007	10	*f*	low
imidazole[i]	7.0	360 (35)	429 (100)	546 (12.2)	580 (7.7)	640 (1.9)	*f*	250	2.3×10^{-2}	low
N-phenylimidazole[i]		363 (36)	428 (102)	545 (12.4)	578 (7.7)	640 (1.9)	*f*	8		low
$NaNO_2$	3.23	365 (33)	426 (107)	544 (12.3)	577 (9.6)	645 (2.6)	0.089	28	5.6×10^{-5}	low
KOCN	3.46	*f*	427	546	582	648	*f*	24	4×10^{-4}	low
alkaline form[j]		359 (36)	425 (102)	545 (11.6)	578[h] (7.5)	639 (1.8)				low
HCOOK	3.75	360 (33.1)	425 (121)	545 (11.3)	581 (10.1)	638 (3.3)	0.135	28	1.1×10^{-4}	low
CH_3COOK[k]	4.77	360 (32.8)	413 (97)	540 (8.3)	583 (7.0)	638 (5.5)	93	1250	9.0×10^{-2}	mix
KNO_3[l]	−1.3		400 (94)	544 (11.3)	584 (5.6)	651 (5.4)	0.55	*f*	*f*	high

[a] All data were obtained in 0.1 M potassium phosphate buffer at pH 6 or 3 and 4 °C except for those for the alkaline form and its cyanide complex. [b] pK_a values for the ligands are taken from the following literature: (i) Danehy & Parameswaran (1968); (ii) Perrin et al. (1981); (iii) Streitwieser & Heathcock (1981). [c] The λ and ϵ_{mM} values, expressed in nm and mM^{-1} cm^{-1}, respectively, are pH-independent between pH 3 and 6. [d] Intrinsic (int) affinity for the neutral form of the ligands (see text). [e] Sono et al, 1984. [f] Not determined. [g] Bis(hydroxymethyl)methylphosphine (Sono et al., 1985a). [h] Shoulders. [i] Unlike the other anionic ligands listed, a deprotonated (i.e., neutral) species of the conjugated acid binds to the enzyme. Therefore, $K_d^{app} = K_d^{int}(1 + [H^+]/K_a)$; cf. eq 4 in the Appendix. [j] Determined in 0.1 M glycine–KOH buffer at pH 9.5 and 4 °C. [k] A charge-transfer band is observed at 507 nm ($\epsilon_{mM} = 9.7$). [l] A charge-transfer band is observed at 513 nm ($\epsilon_{mM} = 13.4$).

Table II: Optical Absorption Maxima and Apparent Dissociation Constants (K_d^{app}) of Ferrous Chloroperoxidase–Ligand Complexes[a]

ligand	K_d^{app} (mM)	absorption maxima [λ (ϵ_{mM})][k] δ	Soret	β	α
Me_2PhP[b,c]	*d*	*d*	459 (*d*)	552 (*d*)	576 (*d*)
$(HOMe)_2MeP$[e]	7.7	337 (37) 365 (35)	457.5 (125)	551 (21.8)	578 (9.1)
cyanide[c]	~900	*d*	454 (140)	550 (*d*)	578.5 (*d*)
n-BuNC[f]	0.15	356 (34)	452 (140)	547 (23.2)	576 (17.7)
CO	0.03[g]	362 (50)	445 (167)	550 (15.9)	576[h] (11.6)
NO	*d*	366 (42)	441 (96)	560 (15.3)	585[h] (11.4)
O_2[i]	*d*	354 (41)	430 (94)	554 (16.5)	587 (12.5)
CO[j] (pH 10)	*d*	*d*	421 (167)	540 (14.9)	569 (13.8)

[a] Unless otherwise indicated, all data were obtained in this work in 0.1 M potassium phosphate buffer at pH 6.0 and 4 °C. [b] Dimethylphenylphosphine. [c] Complex formation was incomplete because of low ligand solubilities or relatively low ligand affinity. [d] Not determined. [e] Bis(hydroxymethyl)methylphosphine (Sono et al., 1985a). [f] *n*-Butyl isocyanide (Sono et al., 1985b). [g] Reported by Campbell et al. (1982), determined at pH 6.3 and 23 °C. [h] Shoulders. [i] Determined by the present authors at −30 °C in 65% (v/v) glycerol–0.035 M potassium phosphate (pH 7.5) (Sono et al., 1985b). [j] Alkaline enzyme + CO + dithionite, determined in 0.1 M glycine–KOH at pH 10. [k] The λ and ϵ_{mM} values are expressed in nm and mM^{-1} cm^{-1}, respectively.

thiolate-bound proteins are exceptional in that the P-450-CAM complexes exhibit more red-shifted Soret peak positions than do the chloroperoxidase adducts. The cyanide adducts of the two enzymes have the same Soret peak position at 439 nm. As will be presented later, the ferrous ligand complexes of the two enzymes have nearly identical Soret peak positions (Table II; Dawson et al., 1983). With the exception of the acetate and nitrate cases, all of the ferric chloroperoxidase–ligand complexes are low spin, as has been observed for ferric P-450-CAM–ligand complexes (Sono & Dawson, 1982).

Since chloroperoxidase is stable even at quite low pH, we have been able to examine the effect of pH on the spectral and equilibrium properties of the ferric enzyme–ligand complexes over a wide pH range (pH 2–7). The UV–visible absorption spectrum of the native ferric enzyme changes only very slightly ($\Delta\epsilon_{mM} < 2$), if at all, between pH 3 and 6 (Thomas et al., 1970b). This has been confirmed in this study with both UV–visible absorption and MCD spectroscopy (data not shown). Above pH 7, the ferric enzyme undergoes an irreversible spectral change to an alkaline form (Hollenberg & Hager, 1973).

The ligands in Table I fall into three categories depending on the pH profiles of their binding constants. The *first* group contains weakly acidic ligands such as are shown in Figure 1. These ligands have pK_a values between 3.2 and 9.14 and exhibit decreases in their binding affinity in the pH range *above* their pK_a values (Figure 1A). Cyanide also belongs to this group although it is an exceptionally weak acid (pK_a = 9.14) as compared with the other anionic ligands. In fact, no effects of pH are detectable for cyanide binding to chloroperoxidase between pH 2 and 7. The apparent K_d values (K_d^{app}) for these weakly acidic ligands, when recalculated to an intrinsic K_d (K_d^{int}) on the basis of the concentrations of the protonated (neutral) form actually present at various pH values (see the Appendix), become independent of pH values (Figure 1B). Fluoride (pK_a = 3.17) also falls in this category. The *second* group includes strongly acidic ligands such as thiocyanate (pK_a = −1.9), nitrate (pK_a = −1.3), and the halides other than fluoride. The affinities of these ligands show marked pH effects over the entire pH range down to pH 2 (Figure 2). The apparent K_d values decrease in proportion to proton concentration, i.e., a plot of the pK_d^{app} value as a

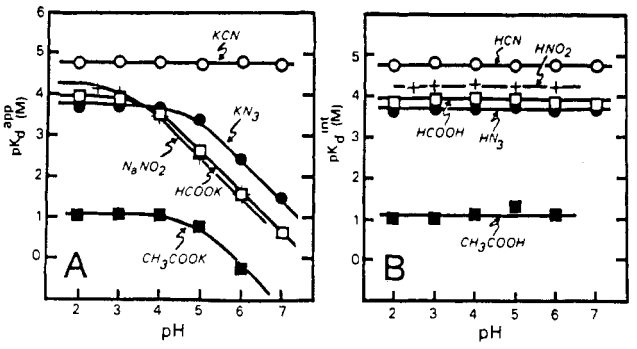

FIGURE 1: Effects of pH on the affinities of ferric chloroperoxidase for weakly acidic anionic ligands: (A) apparent equilibrium dissociation constants (K_d^{app}) and (B) intrinsic K_d (K_d^{int}) values as a function of pH. The K_d^{int} values were calculated on the basis of protonated (neutral) ligand concentrations. The solid lines in (A) are theoretical curves calculated according to eq 4 in the Appendix. All measurements were done at 4 °C in 0.1 M potassium phosphate at the indicated pH. Ligands used and their symbols are indicated in the figure. See Experimental Procedures for further details.

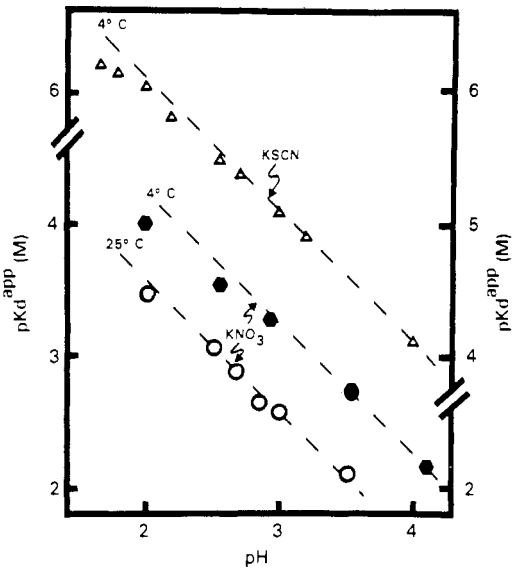

FIGURE 2: Effects of pH on the affinities of ferric chloroperoxidase for strongly acidic anionic ligands. The igands used, their symbols, and the temperature of study are indicated. The dashed lines with slopes of −1 are simply drawn to show the linear correlation between pK_a^{app} and pH values above 2.5. All measurements were performed in 0.1 M potassium phosphate at 4 °C at the indicated pH.

function of medium pH gives a straight line with a slope of −1. Although these data could be analyzed in the same way as was done for the weakly acidic ligands, such an interpretation leads to unrealistically low calculated values of K_d^{int} in the range below nanomolar. Therefore, we suggest that these ligands bind in their anionic (deprotonated) forms to a protonated form of the enzyme. In order to see whether similar pH effects are seen for anionic ligand binding to ferric P-450, we have examined the binding of thiocyanate to camphor-free P-450-CAM from pH 6 to 9: the binding affinity varies insignificantly between pH 7 and 8 (K_d = 30–50 mM), while at more acidic and alkaline pH values, the affinity is lower (K_d = 200 mM at pH 6, 170 mM at pH 9). The *third* group includes neutral ligands. A typical example of this group is *n*-butyl isocyanide. No pH effects are observed on its affinity for the ferric enzyme (Table I).

Halide Binding Properties of Ferric Chloroperoxidase. The formation of spectrally distinct complexes of ferric chloroperoxidase with chloride, bromide, and iodide anions was first studied by difference spectroscopy (Thomas et al., 1970b).

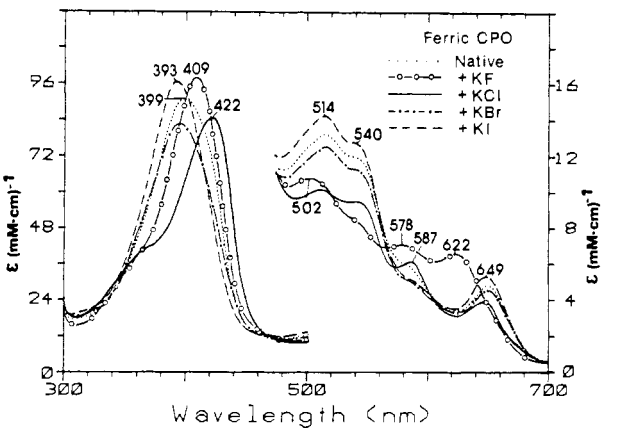

FIGURE 3: UV–visible absorption spectra of native ferric chloroperoxidase (CPO) and its halide complexes. The spectra were obtained with 10–15 μM enzyme in 0.1 M potassium phosphate buffer, pH 3.0 at 4 °C, in the presence of 200 mM KF, 800 mM KCl, 600 mM KBr, and 200 mM KI. Under these conditions, the extent of halide saturation of the enzyme was >95% with KF and KI, 80% with KCl, and about 83% with KBr (see Table III). The spectrum of the KCl complex presented here has been normalized to 100% saturation by the use of a computer (see Experimental Procedures). Similar normalization for the bromide complex has not been done because the spectral change of the native enzyme upon bromide binding was too small for accurate normalization.

Table III: pH Effects on Halide Affinity for Ferric Chloroperoxidase and Soret Absorption Peaks of the Enzyme–Halide Complexes[a]

halide	pK_a[b]	Soret peak [λ (ϵ_{mM})][c]	K_d^{app}[d] (mM) pH 2.0	pH 2.5	pH 3.0	pH 3.5
F^-	3.18	409 (98)	6.6	7.8	8.8	21.5
Cl^-	−2.2	422 (85)	34	85	200	660
Br^-	−4.7	394 (83)	20	50	125	400
I^-	−5.2	393 (97)	1.2	3.4	7.6	28

[a] The apparent dissociation constrants (K_d^{app}) and the absorption data were derermined in 0.1 M potassium phosphate buffer at 4 °C. See Experimental Procedures for details. [b] pK_a values for hydrogen halides, taken from the literature. See footnote *b* to Table I. [c] The λ values are expressed in nm, and the ϵ_{mM} values are accurate to ±2 and are expressed in mM^{-1} cm^{-1}. [d] The values were reproducible within ±10% errors.

Apparent dissociation constants for these adducts at pH 2.8 and at room temperature were also reported. Later, Makino et al. (1976) and Hollenberg et al. (1980) displayed absolute spectra (360–500 nm) of the complexes at room temperature and tabulated additional data between 300 and 700 nm at both 20 °C and 77 K. Because of the central role of the halide complexes in this paper and since our spectral data between 300 and 700 nm differ somewhat from those previously reported, we represent the absolute UV–visible absorption spectra of the four halide complexes in Figure 3, together with that of native ferric chloroperoxidase. The spectral data in the Soret region are summarized in Table III. Among the three halide anion substrates, chloride forms a complex that exhibits the most red-shifted Soret peak (422 nm) and the weakest charge-transfer bands around 500 and 650 nm together with a prominent α band at 587 nm. The spectral features of the fluoride adduct in the visible region are distinct from those of the other halide complexes (Figure 3).

The dissociation constants for the halides determined at 4 °C between pH 2 and 3.5 are summarized in Table III. Data for fluoride have not been reported previously. The values for the other halides at pH 3 are in good agreement with those reported by Thomas et al. (1970b) at pH 2.8. The constants for chloride are essentially identical with those determined by Lambeir & Dunford (1983b) at 25 °C between pH 2.36 and

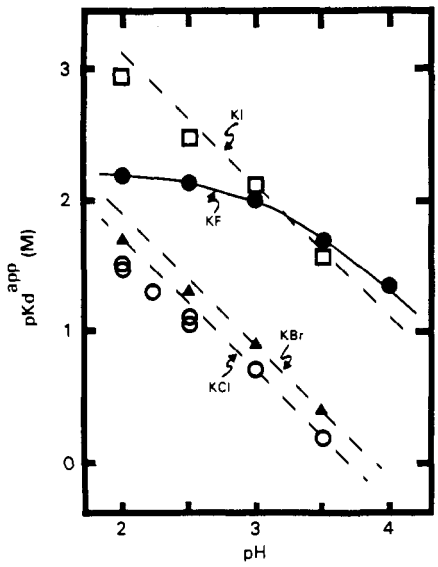

FIGURE 4: Effects of pH on halide affinities of native ferric chloroperoxidase. The K_d values were determined either from direct halide titrations (KF, KI, KCl) or from the competition experiments with cyanide (see Experimental Procedures) in 0.1 M potassium phosphate buffer at 4 °C and at the indicated pH. The solid line for the fluoride complex was drawn according to eq 4 (in the Appendix). The dashed lines for the other halides are only drawn to show a linear correlation with a slope of −1 between the pK_d^{app} and pH values.

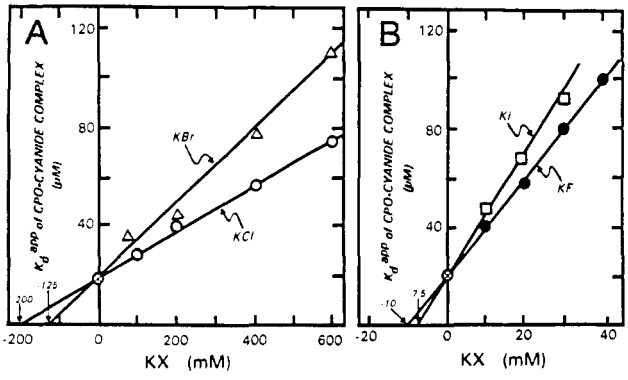

FIGURE 5: Competition between halide anions and cyanide for binding to ferric chloroperoxidase. See the text for further details.

3.40. As mentioned earlier, the effect of pH on the K_d values of the chloride, bromide, and iodide adducts (Figure 4) shows the same trend as observed with the strongly acidic ligands (Figure 2): the pK_d values increase linearly with decreasing pH with small downward deviation from the theoretical straight line seen only below pH 2.5. Fluoride behaves as a weakly acidic ligand, and the theoretical curve (solid line in Figure 4) that has been drawn is based on eq 4 (see the Appendix) using $pK_a = 3.18$ and $K_d^{int} = 6.3$ mM to fit the experimental data.

In order to see whether the heme iron is the binding site for all halides as has been demonstrated for chloride by Lambeir & Dunford (1983b) in kinetics competition experiments with cyanide, we have performed equilibrium competition experiments in which cyanide titrations of the ferric enzyme have been carried out in the absence and presence of varying concentrations of halides. As shown in Figure 5, the results clearly demonstrate that not only chloride but also the other three halides are competitive with cyanide for binding to the enzyme as judged from the straight line in the plots of K_d^{app} values for cyanide as a function of halide concentrations [see Sono et al. (1982) for data analysis]. The x intercepts of the plots for chloride, bromide, iodide, and fluoride at pH 3.0 are −200, −125, −7.5, and −10 mM, respectively, at pH 3.0, which are in excellent agreement with the $-K_d$ values (see Table II).

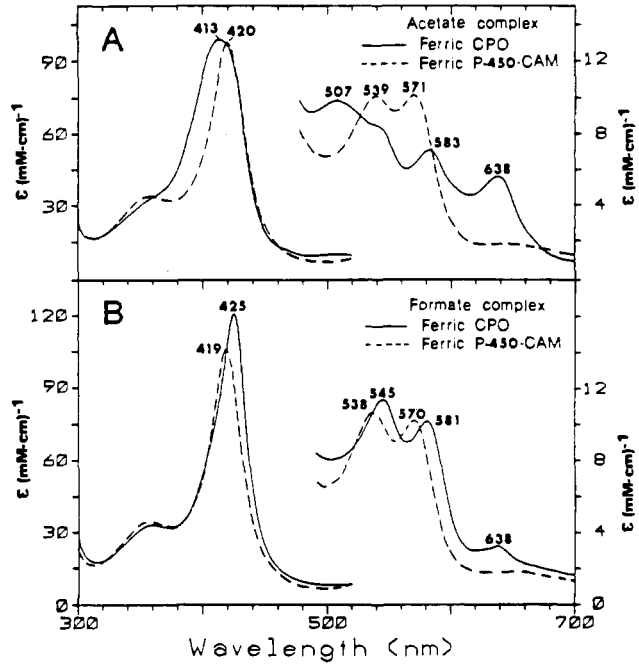

FIGURE 6: UV-visible absorption spectra of the acetate (A) and formate (B) complexes of ferric chloroperoxidase (CPO) (solid lines) and P-450-CAM (dashed lines). The spectra were obtained in 0.1 M potassium phosphate buffer at 4 °C with 10–15 μM enzyme concentrations. The ligand concentrations and pH values were as follows: for CPO, 2 M acetate at pH 3.0 and 1 M formate a pH 6.0; for P-450-CAM, 5 M acetate and 4 M formate, both at pH 7.0, in absence of camphor.

Spectral and Spin-State Properties of Carboxylate Complexes of Ferric Chloroperoxidase. Nearly all of the ligands that bind to boh ferric chloroperoxidase and P-450-CAM form complexes that have similar spectral properties for the same ligands (Table I; Dawson et al., 1982) and are predominantly low spin (Sono & Dawson, 1982). However, a unique difference is observed for the acetate complex of these heme proteins. With P-450-CAM, acetate forms a low-spin complex. In contrast, the acetate adduct of ferric chloroperoxidase exhibits an absorption spectrum (Figure 6A) indicative of a mixed spin state even though the enzyme is saturated ([acetate] > $20K_d$) with this ligand. Interestingly, such differences are not seen with the formate complexes of these enzymes (Figure 6B). The major evidence for the inherent mixed-spin nature of the acetate complex of chloroperoxidase arises from the presence of a *new* high-spin marker band[2] at 638 nm,

[2] Native chloroperoxidase (Figure 3) and camphor-bound cytochrome P-450-CAM (Dawson & Eble, 1986) have electronic transitions at 650 and 646 nm, respectively, and are predominantly (>80%) high spin at ambient temperature as judged by Mössbauer (Champion et al., 1973; Sharrock et al., 1973) or, for chloroperoxidase, proton nuclear magnetic resonance (Goff et al., 1985) spectroscopy. These transitions completely disappear upon conversion of the two proteins to the low-spin state by coordination of cyanide ion, for example. The correlation between the presence of an electronic transition in the vicinity of 650 nm and the high-spin state leads us to refer to this peak as a high-spin marker band. A similar correlation exists for myoglobin with a peak at approximately 630 nm serving as the high-spin marker band (Beetlestone & George, 1964; Smith & Williams, 1968). Although a small peak is observed at 638 nm in the chloroperoxidase–formate complex, it is noticeably less intense than in the acetate-bound case, and so we consider the former complex to be predominantly low spin. Additional support for this assignment for the formate adduct comes from the presence of discrete δ (360 nm), β (545 nm), and α (581 nm) bands and the absence of the feature at 507 nm seen in the acetate complex that is analogous to the 514-nm transition seen in high-spin native chloroperoxidase.

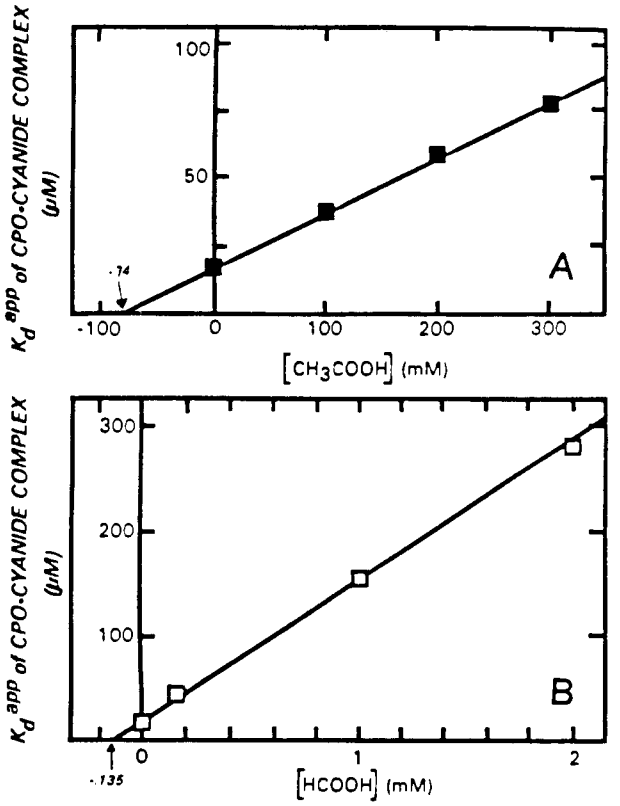

FIGURE 7: Competition between acetate and cyanide (A) and between formate and cyanide (B) for binding to ferric chloroperoxidase. The experiments were performed in 0.1 M potassium phosphate buffer, pH 3.0 at 4 °C. See the text for analysis of the data.

distinct from the analogous transition at 650 nm in the native high-spin ferric enzyme. Propionate also gives a rise to the 638-nm band upon binding to the native ferric enzyme although a homogeneous complex could not be formed due to low ligand affinity. In order to check the possibility that acetate might bind to the enzyme at a site other than the heme iron, competition experiments using cyanide have been performed. As shown in Figure 7A, a plot of the apparent K_d values for cyanide as a function of acetate concentration gives a straight line with an x intercept of −74 mM at pH 3.0, indicating that acetate binds directly to the heme iron of the enzyme. The direct acetate titration of the enzyme gives a nearly identical K_d value (Table I, K_d = 94 mM). Similar results were also obtained from parallel experiments with formate and cyanide (Figure 7B). Thus, although the heme iron is the binding site for both acetate and formate, the resulting adducts of these carboxylates exhibit different spin-state compositions.

Chemical Properties of Nitrite-Bound Ferric Chloroperoxidase and P-450. During the course of the titration of ferric chloroperoxidase with nitrite, a shift of the initial isosbestic points for the spectra of the native enzyme and the primary complex has been noted. The secondary spectral change starts to occur when the fractional ligand saturation of the enzyme exceeds approximately 50%. Up to half-saturation, the spectral change is uniform with a single set of isosbestic points (348, 415, 466, 539, 603, and 675 nm), allowing one to calculate dissociation constants (Table I) for the primary complex. The secondary spectral change is dependent on both the concentration of the ligand and the incubation time. The final spectrum obtained after complete formation of the secondary complex is essentially identical with that of the ferric enzyme–NO complex (Table I). A similar, anomalous reaction has previously been observed by Sono & Dawson (1982) upon

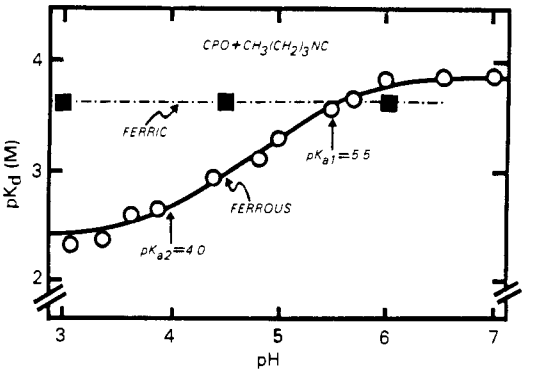

FIGURE 8: Effects of pH on the *n*-butyl isocyanide affinities of ferrous (—) and ferric (-·-) chloroperoxidase (CPO). The measurements were done in 0.1 M potassium phosphate at 4 °C. The solid line was drawn according to eq 12 by using the following constants determined by the methods described in the Appendix: pK_{a1} = 5.5, pK_{a2} = 4.0, $K_L{}^b$ = 0.13 mM, and $K_L{}^a$ = 4.0 mM. See Experimental Procedures for additional details.

nitrite binding to ferric P-450-CAM. We can schematically describe this biphasic reaction process as follows:

RS–Fe(III) heme + NO_2^- →

[RS–Fe(III) heme–NO_2^-] → RS–Fe(III) heme–NO

Spectral and Equilibrium Properties of Ferrous Chloroperoxidase–Ligand Complexes. The UV–visible absorption spectral data for complexes of ferrous chloroperoxidase with several exogenous ligands are listed in Table II. In addition to previously reported complexes with CO (Hollenberg & Hager, 1973) and NO (Chiang et al., 1975), we have recently been able to prepare homogeneous adducts of ferrous chloroperoxidase with cyanide, phosphines, alkyl isocyanides, and dioxygen. Additional properties of these latter three ligand complexes will be published elsewhere (Sono et al., 1985b). Table III also includes the ferrous–CO complex of alkaline chloroperoxidase (pH 9.5) that is an analogue to cytochrome P-420, a denatured form of P-450. All of the complexes listed in Table II are in the low-spin state, their spectroscopic properties are remarkably similar to those of the analogous ferrous P-450 adducts (Dawson et al., 1983). However, the dioxygen adducts of these two enzymes display considerable dissimilarities as will be discussed elsewhere (Sono et al., 1985b).

The effect of pH on the dissociation constant of ferrous chloroperoxidase–*n*-butyl isocyanide has been examined in detail betwen pH 3 and 7 (Figure 8). The K_d value for this alkyl isocyanide varies markedly vs. pH with the affinity decresing at lower pH. This result is in sharp contrast to the lack of pH effect observed for binding of the same alkyl isocyanide to the ferric enzyme (Figure 8). The titration results from the ferrous enzyme have been analyzed according to eq 12 and 13 of the Appendix, and the pK_a values of the ionizable group in the enzyme have been determined to be 5.5 and 4.0 ± 0.1 for ligand-free and *n*-butyl isocyanide bound enzymes, respectively. The value of 5.5 coincides with that previously reported by Campbell et al. (1982) from the pH dependence of the kinetic rate constants for CO binding to the ferrous enzyme.

Spectroscopic Characterization of Alkaline Ferric and Ferrous Chloroperoxidase. It has been reported that native ferric chloroperoxidase undergoes a spectral transition from native to alkaline form above pH 7 at 20 °C (Hollenberg & Hager, 1973; Lambeir & Dunford, 1983a). We have prepared a stable alkaline form of chloroperoxidase at pH 9.5 and 4 °C and have characterized it with UV–visible absorption, MCD,

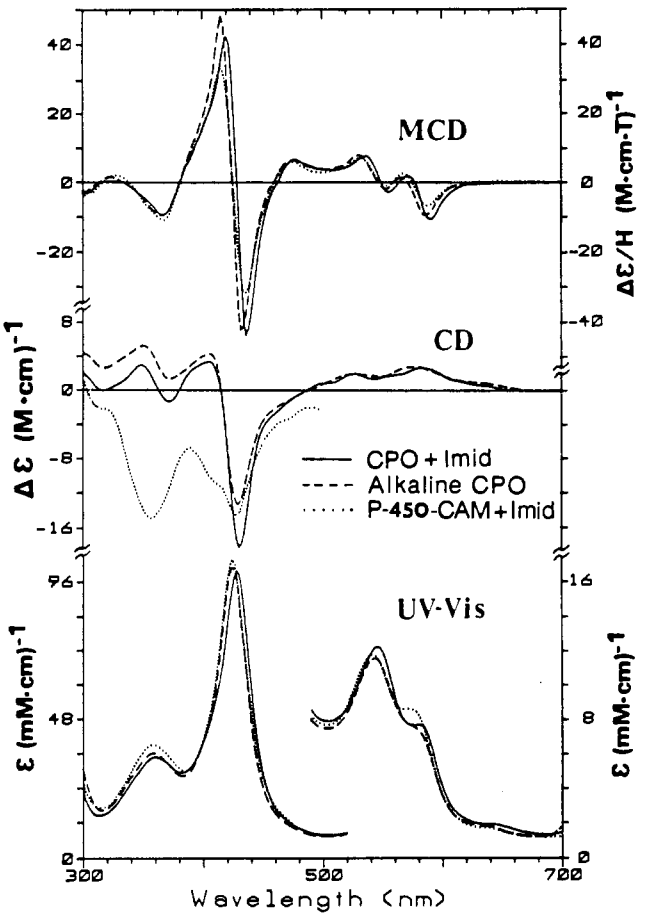

FIGURE 9: MCD (top), CD (middle), and UV–visible absorption (bottom) spectra of alkaline ferric chloroperoxidase (CPO) and the imidazole complexes of native ferric CPO and of ferric P-450-CAM. The spectra of the imidazole complexes of CPO (—) (1 M imidazole), and P-450-CAM (···) (88 mM imidazole, 22 μM camphor) were obtained in 0.1 M potassium phosphate buffer, pH 6.0 and pH 7.0, respectively, and that of alkaline CPO (---) was obtained in 0.1 M glycine–KOH buffer, pH 9.5, all at 4 °C. The enzyme concentrations used were 10–15 μM. The CD spectrum of the P-450-CAM–imidazole complex is shown only in the Soret region between 300 and 500 nm. For further details, see the text.

and CD spectroscopy. Since the UV–visible absorption and MCD spectra of the alkaline ferric enzyme are quite similar to those of the imidazole complexes of native ferric chloroperoxidase below pH 7 and of ferric P-450-CAM, we have overplotted the spectra of the three protein derivatives in Figure 9. The CD spectra of the native ferric chloroperoxidase–imidazole adduct and of the alkaline enzyme (Figure 9) are nearly identical in their trough (430 nm) and peak (350 and 405 nm) positions in the Soret region, although differences in intensity are seen. The CD spectrum of the imidazole complex of ferric P-450-CAM (Figure 9) is considerably different for those of the other two species.

Alkaline chloroperoxidase forms a low-spin complex with cyanide at pH 9.5 with an apparent K_d value of 4 mM at 4 °C. The UV–visible absorption peak positions of the alkaline enzyme–cyanide complex are only 1–2 nm blue-shifted from those of the cyanide complex of the native ferric enzyme prepared at acidic pH (Table I) but are more red-shifted by about 15 nm from those found with cyanide adducts of histidine-ligated heme proteins such as ferric myoglobin (Antonini & Brunori, 1971) and horseradish peroxidase (Keilin & Hartree, 1951).

Reduction of the alkaline form (pH 9.5) of ferric chloroperoxidase yields a species whose UV–visible absorption

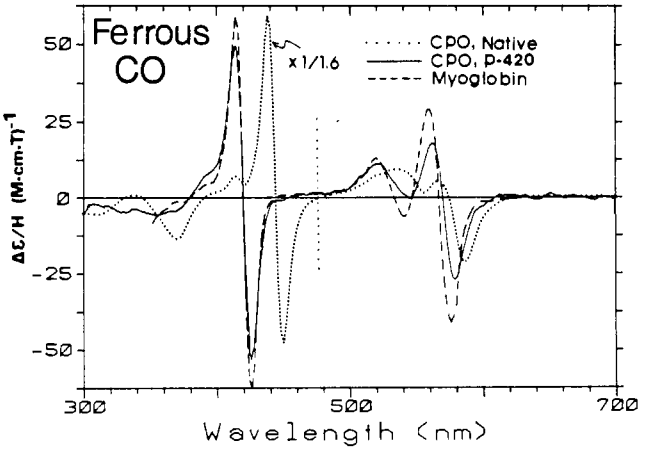

FIGURE 10: MCD spectra of the ferrous CO complexes of native chloroperoxidase (CPO) (···), alkaline CPO (—), and myoglobin (Mb) (---). The spectra were obtained in 0.1 M potassium phosphate buffer, pH 6.0 for the CPO adduct and pH 7.0 for the Mb adduct, and in 0.1 M glycine–KOH, pH 9.5, for the alkaline CPO adduct, all at 4 °C. The enzyme concentrations were 10–15 μM. The spectrum of the CPO adduct at pH 6 between 300 and 480 nm is reduced by a factor of 1.6.

spectrum is indicative of a mixture of high-spin and low-spin ferrous forms. In the presence of CO, however, a homogeneous low-spin CO complex is formed which has a Soret peak at 421 nm (Table II). The MCD spectrum of alkaline ferrous–CO chloroperoxidase is shown in Figure 10 in comparison with the spectra of ferrous–CO myoglobin at pH 7.0 and of ferrous–CO chloroperoxidase at pH 6.0. The MCD spectra of CO-bound alkaline chloroperoxidase and myoglobin closely resemble each other and are noticeably different from that of ferrous–CO chloroperoxidase at pH 6.0.

Discussion

In order to probe the active site structure and the heme environment of chloroperoxidase, we have carried out extensive ligand binding studies of both the ferric and ferrous enzyme as a function of solution pH and have compared the results with available data for the spectroscopically related enzyme cytochrome P-450 as well as for other hydroperoxidase enzymes. With UV–visible absorption spectroscopy, we have found that the close spectroscopic similarities previously observed between analogous derivatives of chloroperoxidase and P-450 (vide supra) can be extended to include a number of additional ligand complexes of the two enzymes in both the ferric and ferrous states. In most cases, the relatively small differences in the spectral properties of parallel derivatives of the two enzymes likely reflect minor differences in the hydrophobic nature of the active site heme environments in the two enzymes. In addition, we have found that almost all of the ligand adducts of ferric chloroperoxidase are low spin, as have previously been observed for all ligand complexes of ferric P-450 (Sono & Dawson, 1982). This suggests that the ligand field strength of the endogenous axial ligand trans to the ligand binding site in chloroperoxidase is unusually strong. Among the potential amino acid derived ligands, a thiolate sulfur donor is the most likely candidate, further supporting the presence of an endogenous thiolate ligand bound to the chloroperoxidase heme iron.

The case of the acetate complex of ferric chloroperoxidase, which exhibits a mixed spin state, is especially intriguing in light of the low-spin nature of the formate adduct of the chloroperoxidase and of all carboxylate complexes of ferric P-450 (Sono & Dawson, 1982). The spin states of the ferric chloroperoxidase complexes with halides and nitrate are either

mixed (chloride) or exclusively high spin as judged from their EPR (Hollenberg et al., 1980) and UV–visible absorption spectra (Tables I and III). Unfortunately, analogous complexes of ferric P-450 with this latter group of ligands do not form in the pH range accessible for P-450. Together, these data seem to suggest that the thiolate sulfur donor atom of chloroperoxidase does have a somewhat lower ligand field strength relative to the thiolate ligand in P-450 and that, as a result, high-spin ferric chloroperoxidase complexes can be formed with extremely weak-field ligands such as those mentioned above.

The binding of halide ions directly to the heme iron of ferric chloroperoxidase is a property that the enzyme has in common with other peroxidase enzymes. Recently, Araiso & Dunford (1981) have presented evidence that chloride binds to the heme iron of horseradish peroxidase below pH 4. For halide-utilizing peroxidases (Morrison & Schonbaun, 1976) such as chloroperoxidase, myeloperoxidase, and lactoperoxidase, halide binding to the enzyme active site could be an obligatory step in their catalytic mechanism. However, it seems unlikely that the heme iron is the direct binding site during the catalysis. Logically, halide binding directly to the heme iron should be unfavorable since compound I, with an oxygen atom bound to the heme iron (Hewson & Hager, 1978), is thought to precede formation of the active halogenating species (Libby et al., 1982; Lambeir & Dunford, 1983b). In fact, an inhibitory effect of chloride has been demonstrated by Lambeir & Dunford (1983b). Thus, the catalytically important halide binding site can involve either a covalent interaction with the compound I oxygen to yield a heme iron bound halogenating intermediate (Libby et al., 1982) or an ionic interaction with a protonated protein residue (or residues) near the active site. The ionizable group with a pK_a of <2 could play a role in the binding of chloride to the compound I form of the enzyme. On the other hand, nonspecific chloride binding to chloroperoxidase below pH 4 has been demonstrated by the use of broad-line nuclear magnetic resonance spectroscopy (Krejcarek et al., 1976). Unfortunately, the present results do not distinguish these two possibilities.

The pH profiles of the anionic ligand affinities of ferric chloroperoxidase (Figures 1 and 2) are identical with those observed with catalase between pH 3 and 6 by Chance (1952a,b) using cyanide, azide, formate, fluoride, and acetate as ligands. The present results with chloroperoxidase suggest that only the protonated forms of the weakly acidic ligands bind to the enzyme (Figure 1). Similar conclusions have previously been reported for ferric horseradish peroxidase and cytochrome *c* peroxidase (Dunford & Alberty, 1967; Ellis & Dunford, 1967; Erman, 1974a,b). For the strongly acidic anionic ligands (Figure 2) including the halides other than fluoride (Figure 3), it appears that only deprotonated ligands bind to the protonated ($pK_a < 2$) form the enzyme. This pK_a value (<2) is somewhat lower than the value (≤3) estimated previously by Lambeir et al. (1983) from studies of nitrate binding. In any case, only a single proton is involved in determining the dissociation contants for the complexes of ferric chloroperoxidase with anionic ligands: for weakly acidic ligands, the ligand proton, and for strongly acidic ligands, the enzyme-bound proton. The pH effects on the ligand binding equilibrium constants for non-peroxidase heme proteins such as ferric P-450 and myoglobin are different from those observed for peroxidase enzymes. An example is the binding of thiol(ate) to chloroperoxidase (Sono et al., 1984) and to P-450-CAM or myoglobin (Sono et al., 1982). No pH effects on the K_d values for the complexes of chloroperoxidase are observed between pH 3 and 7, while the medium pH has a considerable effect on the ligand affinity for the latter two heme proteins. This is taken with the present results for the thiocyanate binding properties of chloroperoxidase and P-450-CAM (see Results) to indicate that chloroperoxidase and P-450 have totally different active site heme environments. Even weakly acidic ligands can bind directly to P-450 in their anionic forms, while neutralization of the negative charge on the anionic ligand seems to be required for binding to chloroperoxidase. With P-450-CAM, enzyme heme iron bound thiols can undergo thiol–thiolate interconversion depending on the medium pH value (Sono et al., 1982). In contrast, no such pH effects are seen with chloroperoxidase–thiol adducts (Sono et al., 1984). These observations, taken together, lead to the interpretation that the active site of P-450-CAM is directly accessible to small anions and protons, while only neutral molecules can enter the "gate" of the chloroperoxidase active site. This property of chloroperoxidase is common to catalase and to other peroxidase enzymes (Davies et al., 1976). Thus, chloroperoxidase clearly belongs to the hydroperoxidase class of enzymes in terms of its ligand binding properties. Interestingly, the inaccessibility of the chloroperoxidase active site to small ions in its ferric form is lost upon reduction to the ferrous form. This is shown in the marked pH dependence observed for the affinity of a neutral ligand (e.g., alkyl isocyanide) for the ferrous enzyme (Figure 7). The spectral similarities observed between the complexes of ferric chloroperoxidase and P-450 with analogous anionic ligands indicate that the heme iron bound ligands have the same protonation state in both enzymes, most likely deprotonated (anionic). This requires that anionic ligands bind to chloroperoxidase in their protonated state, followed by deprotonation upon ligation to the heme iron. The observed ligand pK_a dependence and medium pH dependence of the anionic ligand binding equilibrium constants (Figure 1A) also require that the proton thus dissociated from the enzyme-bound ligand is not released to the medium and is presumably picked up by a nearby basic amino acid such as histidine imidazole. The ionizable group with the pK_a of 5.5 that is detected upon alkyl isocyanide binding to ferrous chloroperoxidase is likely the same residue as that revealed by Campbell et al. (1982) in studies of the kinetics of CO binding. This ionizable group has been strongly suggested to be histidine. The results with ferrous chloroperoxidase presented herein, considered together with the structural characterization of alkaline chloroperoxidase in which a distal nitrogen-containing residue (probably histidine) ligates to the sixth coordination position of the heme iron (Figure 9; Lambeir & Dunford, 1983a), provide additional support for the presence of a histidine residue in the enzyme active site. The possible role of an active site histidine residue in the cytochrome *c* peroxidase reaction has been suggested on the basis of the crystal structure of that enzyme (Poulos & Kraut, 1980).

Chloroperoxidase and P-450 belong to totally different classes of enzymes: the former is a hydroperoxidase (Hewson & Hager, 1978), while the latter is a monooxygenase (White & Coon, 1978). These enzymes have only a limited set of enzymatic activities in common such as the N-demethylation of certain organic substrates (Kedderis et al., 1980; Padbury & Sligar, 1985) and the epoxidation and hydroxylation of cyclohexene (McCarthy & White, 1983). The unusual reactivity of the nitrite complex of chloroperoxidase observed in this work is also observed with P-450 and may result from the presence of a thiolate ligand bound to the heme iron of the two enzymes. Histidine-ligated heme proteins such as

ferric myoglobin do not have this reactivity. In summary, we have shown in this study that chloroperoxidase behaves like a hydroperoxidase in terms of its detailed ligand binding properties despite having a P-450-like active site coordination structure.

ACKNOWLEDGMENTS

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APPENDIX

Data Analysis. The determination of K_d values through ordinary as well as competitive titration experiments was accomplished according to procedures described in our previous paper (Sono et al., 1982). Analyses of the pH effects on the apparent K_d values for the complexes of ferric chloroperoxidase with weakly acidic ligands (including fluoride) were performed as follows.

An acidic ligand (HL) exists as an equilibrium mixture of its protonated (neutral) and deprotonated (anionic) forms

$$HL \overset{K_a}{\rightleftharpoons} H^+ + L^-$$

and its proton dissociation constant (K_a) is defined by

$$K_a = [H^+][L^-]/[HL] \quad (1)$$

When only the protonated ligand binds to the enzyme (E), the apparent (K_d^{app}) and intrinsic (K_d^{int}) dissociation constants for the complex are defined by eq 2 and 3 according to the scheme

$$\begin{array}{ccc} HL + E & \overset{K_d^{int}}{\rightleftharpoons} & E{\cdot}HL \\ {\scriptstyle H^+}\downarrow\uparrow{\scriptstyle K_a} & & \\ L^- & & \end{array}$$

$$K_d^{int} = [HL][E]/[E{\cdot}HL] \quad (2)$$

$$K_d^{app} = ([HL] + [L^-])[E]/[E{\cdot}HL] \quad (3)$$

Combining eq 1–3, we can obtain

$$K_d^{app} = K_d^{int}(1 + K_a/[H^+]) \quad (4)$$

Equation 4 indicates that a plot of K_d^{app} as a function of $1/[H^+]$ would give a straight line with a slope and x and y intercepts of $K_d^{int}K_a$, $-1/K_a$, and K_d^{int}, respectively. Accordingly, theoretical K_d^{app} values can be determined as a function of pH.

For the case of ferrous chloroperoxidase, where an ionizable group on the protein is involved in ligand (L) binding, the proposed scheme is

$$\begin{array}{ccccc} H^+ + E^- & \underset{-L}{\overset{+L}{\rightleftharpoons}} & E^-{\cdot}L + H^+ \\ & {\scriptstyle K_L^b} & \\ {\scriptstyle K_{a1}}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_{a2}} \\ EH & \underset{-L}{\overset{+L}{\rightleftharpoons}} & EH{\cdot}L \\ & {\scriptstyle K_L^a} & \end{array}$$

where EH and E^- represent the states of the enzyme in which the ionizable group is protonated and deprotonated, respectively. From the above scheme, four microscopic equilibrium dissociation constants can be defined:

$$K_L^a = [EH][L]/[EH{\cdot}L] \quad (5)$$

$$K_L^b = [E^-][L]/[E^-{\cdot}L] \quad (6)$$

$$K_{a1} = [E^-][H^+]/[EH] \quad (7)$$

$$K_{a2} = [E^-{\cdot}L][H^+]/[EH{\cdot}L] \quad (8)$$

Combination of these four equations leads to

$$K_L^a K_{a1} = K_L^b K_{a2} \quad (9)$$

$$K_L^a/K_L^b = K_{a2}/K_{a1} \quad (10)$$

Apparent dissociation constants (K_d^{app}) for an enzyme–ligand complex at given pH values can then be defined by eq 11. Alternatively, K_d^{app} can also be expressed by eq 12, using the other constants in eq 5–8, and $[H^+]$. In general, values of

$$K_d^{app} = \frac{([EH] + [E^-])[L]}{[EH{\cdot}L] + [E^-{\cdot}L]} \quad (11)$$

$$K_d^{app} = \frac{K_L^b(1 + [H^+]/K_{a1})}{1 + [H^+]/K_{a2}} \quad (12)$$

K_{a1} and K_{a2} can be determined from experimental K_d^{app} values as a function of pH, with a computer being used to obtain the best fit. However, under some conditions, such as those described below, these constants can be determined by the following convenient method.

If $K_{a1} \ll K_{a2}$ (i.e., $pK_{a1} > pK_{a2}$), $[H^+]/K_{a2} \sim 0$ when $[H^+] < K_{a1}$ (i.e., $pH > pK_{a1}$). Thus, in the pH region above pK_{a1}, eq 12 can be simplified to give

$$K_d^{app} = K_L^b(1 + [H^+]/K_{a1}) \quad (13)$$

Equation 13 indicates that a plot of K_d^{app} values as a function of $[H^+]$ would give a straight line with a slope and x and y intercepts of K_L^b/K_{a1}, $-K_{a1}$, and K_L^b, respectively.

The same method can be applied for the determination of pK_{a2} and K_L^a values from the experimental data of K_d^{app} as a function of pH in the pH region below pK_{a2}.

Registry No. P-450, 9035-51-2; MeSH, 74-93-1; $(HOMe)_2MeP$, 5958-52-1; MeCOSH, 507-09-5; CN^-, 57-12-5; NO, 10102-43-9; BuNC, 2769-64-4; $PhCH_2NC$, 10340-91-7; N_3^-, 14343-69-2; $SeCN^-$, 5749-48-4; SCN^-, 302-04-5; NO_2^-, 14797-65-0; OCN^-, 71000-82-3; $HCOO^-$, 71-47-6; $MeCOO^-$, 71-50-1; NO_3^-, 14797-55-8; Me_2PhP, 672-66-2; CO, 630-08-0; O_2, 7782-44-7; F^-, 16984-48-8; Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; Fe, 7439-89-6; pyridine, 110-86-1; imidazole, 288-32-4; *N*-phenylimidazole, 7164-98-9; peroxidase, 9003-99-0; heme, 14875-96-8; chloroperoxidase, 9055-20-3.

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