Probing the Heme Iron Coordination Structure of Alkaline Chloroperoxidase†

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ABSTRACT: The mechanism by which the heme-containing peroxidase, chloroperoxidase, is able to chlorinate substrates is poorly understood. One approach to advance our understanding of the mechanism of the enzyme is to determine those factors which contribute to its stability. In particular, under alkaline conditions, chloroperoxidase undergoes a transition to a new, spectrally distinct form, with accompanying loss of enzymatic activity. In the present investigation, ferric and ferrous alkaline chloroperoxidase (C420) have been characterized by electronic absorption, magnetic circular dichroism, and electron paramagnetic resonance spectroscopy. The heme iron oxidation state influences the transition to C420; the pK_a for the alkaline transition is 7.5 for the ferric protein and 9.5 for the ferrous protein. The five-coordinate, high-spin ferric native protein converts to a six-coordinate low-spin species (C420) as the pH is raised above 7.5. The inability of ferric C420 to bind exogenous ligands, as well as the dramatically increased reactivity of the proximal Cys29 heme ligand toward modification by the sulfhydryl reagent *p*-mercuribenzoate, suggests that a conformational change has occurred during conversion to C420 that restricts access to the peroxide binding site while increasing the accessibility of Cys29. However, it does appear that Cys29-derived ligation is at least partially retained by ferric C420, potentially in a thiolate/imidazole coordination sphere. Ferrous C420, on the other hand, appears not to possess a thiolate ligand but instead likely has a bis-imidazole (histidine) coordination structure. The axial ligand *trans* to carbon monoxide in ferrous−CO C420 may be a histidine imidazole. Since chloroperoxidase functions normally through the ferric and higher oxidation states, the fact that the proximal thiolate ligand is largely retained in ferric C420 clearly indicates that additional factors such as the absence of a vacant sixth coordination site sufficiently accessible for peroxide binding may be the cause of catalytic inactivity.

Chloroperoxidase from *Caldariomyces fumago* and the P450 cytochromes are distinct from most heme proteins in possessing a cysteine-derived thiolate ligand to the heme iron (Ortiz de Montellano, 1995; Sundaramoorthy et al., 1995). This feature is responsible for the unusually red-shifted Soret band in the ferrous−CO complex of these heme proteins. While chloroperoxidase and cytochrome P450 share a number of physiochemical properties (Hollenberg & Hager, 1973; Dawson & Sono, 1987), clear differences exist between these two enzymes. Under acidic conditions, chloroperoxidase harnesses the oxidizing equivalents of hydrogen peroxide to catalyze one- and two-electron classical peroxidation reactions, oxygen evolution (the catalase reaction), and the halogenation of organic acceptor molecules. Cytochrome P450, on the other hand, is a monooxygenase that activates dioxygen to catalyze the hydroxylation of camphor. Thus, it is important to define those fine molecular details that differentiate the chemical reactivities demonstrated by chloroperoxidase and cytochrome P450.

A plethora of approaches have provided insight into the pertinent features of the chloroperoxidase active site (Dawson & Sono, 1987; Dawson, 1988). Spectroscopic and chemical modification studies identify Cys29 as the proximal heme iron ligand of chloroperoxidase (Blanke & Hager, 1988), a fact recently confirmed crystallographically by Poulos and co-workers (Sundaramoorthy et al., 1995). Kinetics studies as well as equilibrium binding experiments with a variety of exogenous ligands and halides over a broad pH range of 2−7 support the presence of an ionizable group on the distal side of the active site (Campbell et al., 1982; Lambeir & Dunford, 1983; Sono et al., 1986; Holzwarth et al., 1988). The crystal structure suggests that this residue is Glu183. Chemical modification of chloroperoxidase with diethyl pyrocarbonate implicates the presence of a histidine residue in the distal pocket that may be important in governing ligand binding to the heme as well as catalytic activity of the enzyme (Blanke & Hager, 1990). Collectively, the evidence to date suggests that, while chloroperoxidase shares the distinction of thiolate axial ligation with the P450 cytochromes, the enzyme may have evolved a more polar distal heme pocket analogous to that of the hydroperoxidases (Dawson, 1988; Sundaramoorthy et al., 1995).

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Pursuant to characterizing the structural details that differentiate the chemical activities of chloroperoxidase from cytochrome $P450_{cam}$, we have characterized alterations in the chloroperoxidase structure induced under alkaline conditions. Titration of the enzyme to pH values greater than 7.0 induces a rapid transition resulting in a new stable but inactive protein form (Hollenberg & Hager, 1973). A red shift in the Soret absorbance from 400 to 420 nm characterizes this alkaline form of ferric chloroperoxidase, designated C420.[1] Perhaps more pertinent is the shift in the position of the Soret absorption band of the ferrous−CO complex from 446 to 420 nm (Hollenberg & Hager, 1973), which is identical to that observed when cytochrome P450 is inactivated to a state called cytochrome P420 (Imai & Soto, 1967; Yu & Gunsalus, 1974). These and other observations suggest that C420 may have a coordination sphere analogous to P420 (Martinis et al., 1996).

The primary objective of the present study is to further characterize the transition of native chloroperoxidase to C420 and to extensively probe the perturbations to the heme environment that render an efficient and versatile catalyst an inactive but stable holoprotein. Electronic absorption, magnetic circular dichroism (MCD), and electron paramagnetic resonance (EPR) spectroscopy have been used to investigate the heme coordination sphere of C420. MCD and EPR, in particular, have frequently been employed to determine the coordination structures of heme iron sites in proteins (Dawson & Dooley, 1989; Sono et al., 1991). In addition, ferric chloroperoxidase and C420 have both been titrated with the thiol-specific modification reagent *p*-mercuribenzoate (*p*-mb) to probe the accessibility of Cys29 in the active site of these proteins. These experiments reveal that the transition from chloroperoxidase to C420 induces changes in the spin and coordination states, as well as in ligand-binding properties. Furthermore, reduction of C420 to the ferrous state yields a distinct structure from the ferric state.

EXPERIMENTAL PROCEDURES

Materials and Sample Preparation. Chloroperoxidase was produced in a semicontinuous flow bioreactor (Blanke et al., 1989) and purified as described earlier (Hollenberg & Hager, 1973) to a high specific activity ($> 2.0 \times 10^{-5}$ kat/mg) with an R_z value (OD_{400nm}/OD_{280nm}) greater than 1.4. All reagents were purchased from standard commercial sources. Enzyme concentrations were determined from the absorption at 399 nm using an extinction coefficient of 91 mM^{-1} cm^{-1} (pH 6.0, 4 °C) for the native enzyme (Hollenberg et al., 1980). Chloroperoxidase concentrations varied between 64 and 72 μM. The reduced enzyme samples were prepared from the corresponding ferric samples by the addition of a slight excess of sodium dithionite under a nitrogen atmosphere. The ferrous−CO enzyme complexes were prepared by gently bubbling CO into the ferrous enzyme samples. Electronic absorption spectra of the ferrous and the ferrous−CO enzyme samples were monitored with time to demonstrate complete conversion.

Spectroscopy. A Varian/Cary 210 spectrophotometer computer interfaced to an IBM PC was used for electronic absorption measurements. MCD spectra were recorded with a JASCO J-500A spectrapolarimeter equipped with a JASCO MCD-1B electromagnet (14.7 kG) and computer interfaced to an IBM PS/2 model 50 PC. Complete formation of each enzyme derivative and sample stability were checked by recording absorption spectra before and after each MCD measurement; changes of less than 5% were considered acceptable. Calibration procedures, data acquisition, and handling were done as described elsewhere (Huff et al., 1993). EPR measurements were recorded on a Bruker ER 22OD-SRC instrument equipped with a Bruker microwave bridge ERO42MRH, helium dewar, Varian gaussmeter, and an EIP microwave 58A frequency meter.

Active Site Titration with p-mb. The assay was typically performed by sequential additions of 4 nmol of *p*-mb to 20 nmol of protein. The titration was extended until the absorbance increase at 255 nm, reflecting formation of the mercuri−mercaptide linkage, had reached a plateau, indicating covalent modification of accessible sulfhydryl groups.

RESULTS

Electronic Absorption and MCD Spectral Changes during the Alkaline Transition of Ferric Chloroperoxidase. The changes that occur in the electronic absorption spectra during the alkaline transition of ferric chloroperoxidase to C420 are displayed in Figure 1A. As the first alkaline transition occurs above pH 7.0, the large Soret band at 399 nm for native chloroperoxidase shifts and splits to yield a broad band at 360 nm and a sharper Soret band at 427 nm. In the visible region (500−700 nm) of the spectrum, the high-spin marker bands at 515 and 650 nm diminish as the heme iron changes to a low-spin state. The spectra at pH 6.5, 7.0, 8.0, and 9.0 exhibit an isosbestic point near 419 nm that is not shared by the pH 10.0 sample, suggesting that only two states are involved in this first alkaline transition between pH 6.5 and 9.0. Above pH 10.0, a second alkaline transition occurs to yield a third distinct species (Lambeir & Dunford, 1983).

The MCD spectra of the ferric protein over the same pH range show an increased signal in the Soret region (300−500 nm) for the alkaline C420 form (Figure 1B), as compared to native ferric chloroperoxidase (pH 6.5). The signal intensity increase correlates with a change in spin state (Dawson & Dooley, 1989) from the native high-spin to the low-spin state above the alkaline transition. The strong negative signal just below 400 nm in the native spectra, indicative of axial thiolate ligation (Dawson et al., 1976), completely disappears and is replaced by large positive pseudo-A term centered at 420 nm in the alkaline form. A shoulder near 400 nm exists at pH values greater than 8.0, indicating overlapping electronic transitions. Lastly, the band centered at 560 nm for native chloroperoxidase decreases during the alkaline transition and is replaced by other bands in the visible region, spanning the 500−625 nm range. On the basis of the changes in absorption and MCD intensity during the alkaline titration, the pK_a for the alkaline transition is approximately 7.5 (Figure 1C).

Electronic Absorption and MCD Spectral Changes during the Alkaline Transition of Ferrous Chloroperoxidase. The electronic absorption spectra of ferrous chloroperoxidase at pH 6.5−11.0 are displayed in Figure 2A. The Soret region of the spectrum (pH 6.5 and 7.0 samples) is complex with a peak at 408 nm having two shoulders, one at 425 nm and

[1] Abbreviations: C420, the inactive alkaline form of chloroperoxidase; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; *p*-mb, *p*-mercuribenzoate.

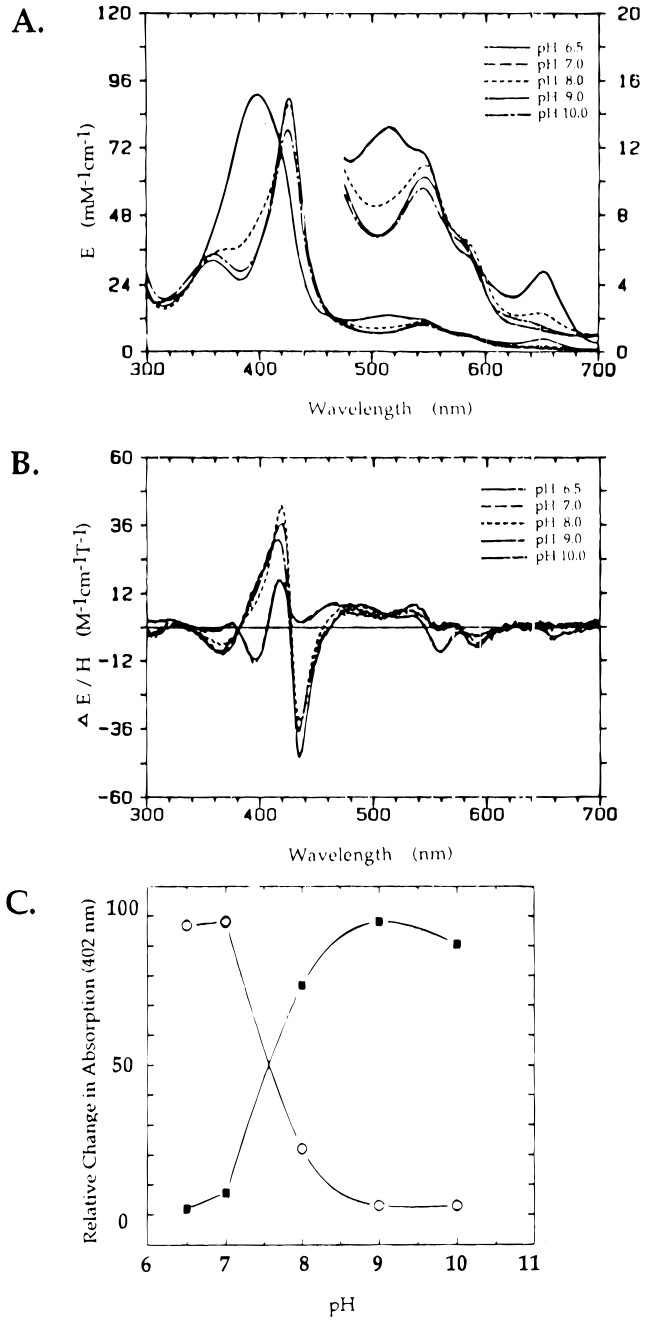

FIGURE 1: Electronic absorption (A) and magnetic circular dichroism (B) spectra of ferric chloroperoxidase and C420 at pH 6.5−10.0. The spectra were obtained at 4 °C with 64−72 μM enzyme in 0.5 M potassium phosphate buffer at pH 6.5 (—), 7.0 (− −), 8.0 (- - -), 9.0 (bold line), and 10.0 (− - −). The spectra for pH 6.5 and 7.0 samples are nearly superimposed. (C) The relative change in absorption (■) and magnetic circular dichroism (●) of ferric chloroperoxidase during the alkaline transition to C420 at 402 nm for the pH range 6.5−10.0.

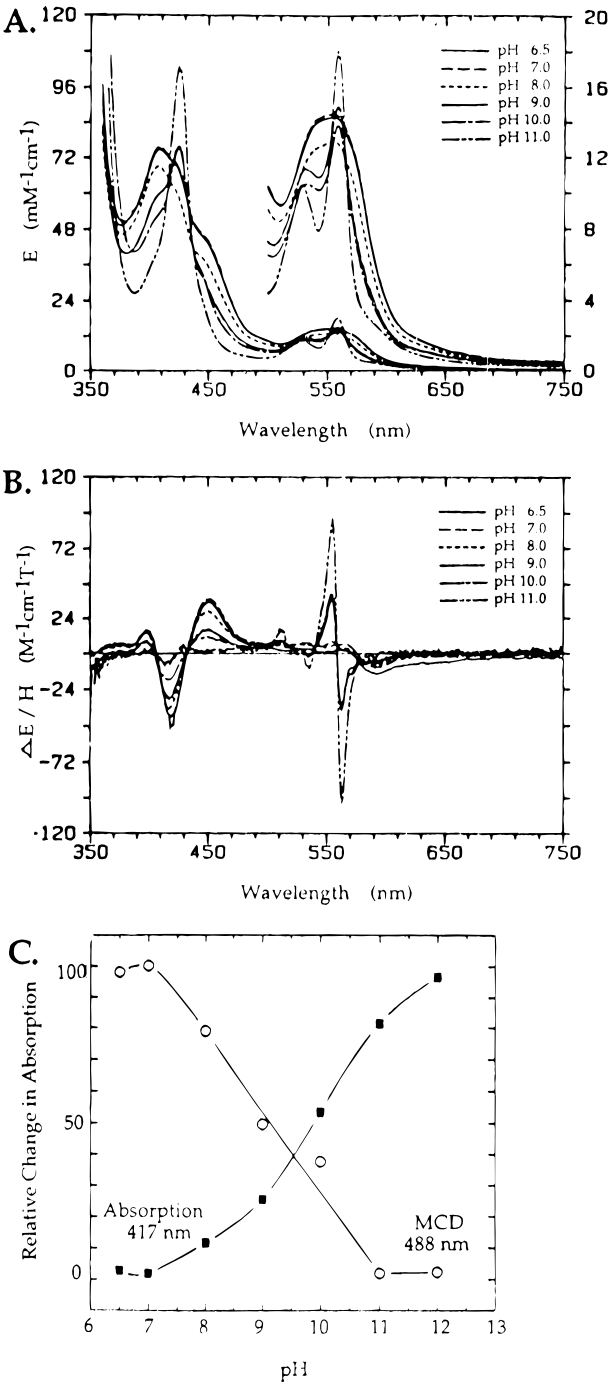

FIGURE 2: Electronic absorption (A) and magnetic circular dichroism (B) spectra of ferrous chloroperoxidase and C420 at pH 6.5−11.0. The spectra were obtained at 4 °C with 64−72 μM enzyme in 0.5 M potassium phosphate buffer at pH 6.5 (—), 7.0 (− −), 8.0 (- - -), 9.0 (bold line), 10.0 (− - −), and 11.0 (− - - −). The spectra for pH 6.5 and 7.0 samples are nearly superimposed. (C) The relative change in absorption at 417 nm (■) and magnetic circular dichroism at 488 nm (●) of ferrous chloroperoxidase during the alkaline transition to C420 for the pH range 6.5−12.0.

the other near 450 nm. During the first alkaline transition, the Soret absorption shifts to a single sharp band with a maximum at 425 nm and increases in intensity, and the single broad visible band of native ferrous chloroperoxidase splits into α and β bands.

The MCD spectra of ferrous chloroperoxidase at pH values spanning the first alkaline transition are displayed in Figure 2B. During this transition, the features in the Soret region of the MCD spectra decrease in intensity and the visible region features become dominant. The MCD spectrum of the alkaline form at pH 11.0 in the visible region shows some fine structure between 500 and 540 nm, followed by a large positive derivative-shaped band centered at 559.5 nm. Isosbestic points are found at 408, 434, and 581 nm. Notably, the pK_a for the alkaline transition of the ferrous protein is shifted to 9.5 (Figure 2C).

Electronic Absorption and MCD Spectral Changes during the Alkaline Transition of Ferrous−CO Chloroperoxidase. Ferrous−CO chloroperoxidase has a strong Soret transition at 443 nm and a single visible region peak at 550 nm (Figure

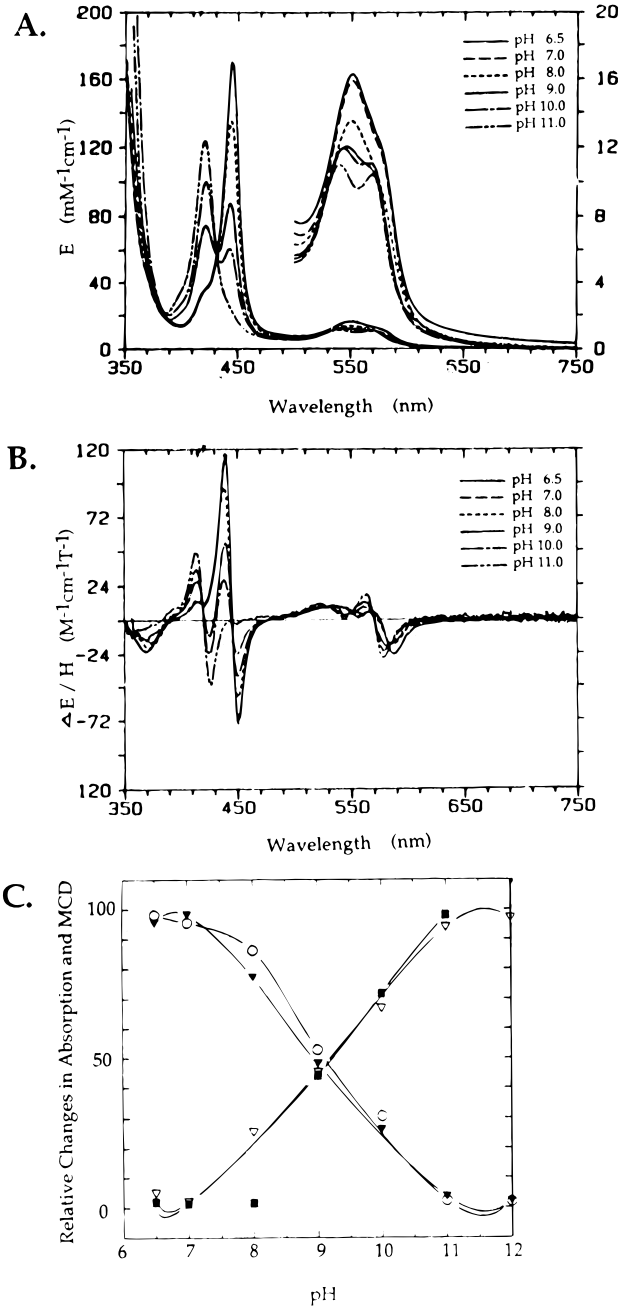

FIGURE 3: Electronic absorption (A) and magnetic circular dichroism (B) spectra of ferrous−CO chloroperoxidase and C420 at pH 6.5−11. The spectra were obtained at 4 °C with 64−72 μM enzyme in 0.5 M potassium phosphate buffer at pH 6.5 (—), 7.0 (− −), 8.0 (- - -), 9.0 (bold line), 10 (− - −), and 11.0 (− - - −). The spectra for pH 6.5 and 7.0 samples are nearly superimposed. (c) The relative change in absorption at 420 nm (■) and at 445 nm (▼) and magnetic circular dichroism at 440 nm (○) and at 451 nm (▽) of ferrous−CO chloroperoxidase during the alkaline transition to C420 for the pH range 6.5−12.0.

3A). As the pH is raised above neutrality, the Soret band shifts to 420 nm with an isosbestic point to 432 nm, and the single visible signal splits into distinct α and β bands.

The MCD spectra of ferrous−CO chloroperoxidase during the first alkaline transition are shown in Figure 3B. The spectra of the pH 6.5 and 7.0 samples show three features in the Soret region, a trough centered at 370 nm, a small peak at 415 nm, and a large positive derivative-shaped feature centered at 446 nm. The visible region of the spectra has a very broad peak from 475 to 550 nm, followed by a

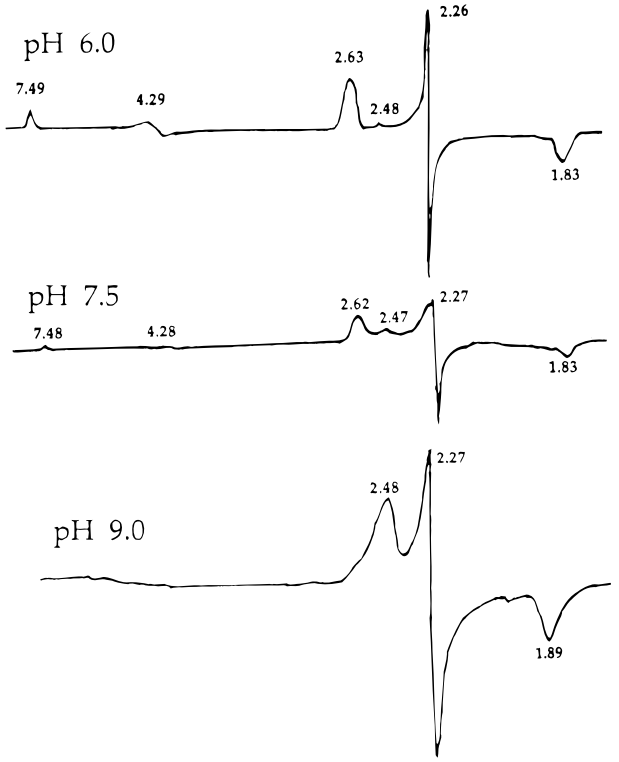

FIGURE 4: EPR spectral changes during the transition of ferric chloroperoxidase to C420 at 20 K. The samples were prepared in 1.0 M potassium phosphate buffer at the indicated pH value; spectra were collected at the following pH values: 6.0, 7.5, and 9.0. Experimental conditions: time constant, 1.25 ms; modulation amplitude, 8.0 G; microwave power, 4.0 mW; and modulation frequencies, 9.534 GHz (pH 6.0), 9.535 GHz (pH 7.5), and 9.531 GHz (pH 9.0).

derivative-shaped curve centered at 570 nm. Dramatic changes occur during the alkaline transition in both the Soret and visible regions of the MCD spectra with isosbestic points at 420, 446, 527, and 584 nm. A new derivative-shaped curve centered at 421 nm develops, and the MCD bands in the visible region narrow and shift to higher energy, revealing a positive band at 523 nm and a derivative-shaped curve centered at 570 nm. The spectra measured at intermediate pH values consist of mixtures of the native and alkaline protein, while the spectrum of the pH 11.0 sample shows no evidence of the native form. The pK_a for the alkaline transition of the ferrous−CO protein occurs at pH 9.0, essentially the same value as in the ferrous case.

Electron Paramagnetic Resonance Spectroscopy of C420. As reported previously (Hollenberg et al., 1980), at cryogenic temperatures, ferric chloroperoxidase (pH 6.0) exhibits both low-spin EPR signals at $g = 2.63$, 2.26, and 1.83 and high-spin signals at $g = 7.49$ and 4.29 (Figure 4). The g values observed for ferric C420 are clearly different than those collected for the native enzyme. At pH 9.0, the low-spin signal pattern narrows to $g = 2.48$, 2.27, and 1.89. These low-spin g values are similar to those of the *N*-phenylimidazole adducts of cytochrome P450 (2.47, 2.26, and 1.90) (Lipscomb, 1980) and chloroperoxidase (2.51, 2.28, and 1.87) (Sono et al., 1991). At pH 7.5, the pK_a for the ferric alkaline transition, the EPR spectrum reveals the presence of both species. In contrast, an earlier investigation reported identical EPR spectra for both acidic and alkaline chloroperoxidase (Hollenberg et al., 1980). This difference may be attributed to inadequate times of conversion from chloroperoxidase to

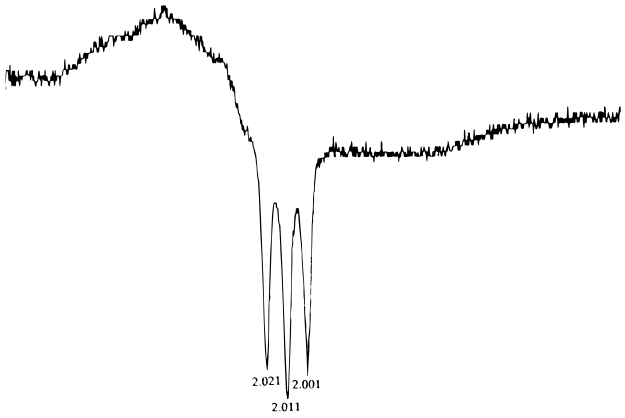

FIGURE 5: EPR spectrum of ferrous−NO C420 at 77 K. The sample was prepared in 1.0 M potassium phosphate buffer at pH 7.0. Experimental conditions: time constant, 80 ms; modulation amplitude, 1.0 G; microwave power, 5.0 mW; and modulation frequency, 9.29 GHz.

C420 in the earlier study (Hollenberg et al., 1980). We have observed that, at very high concentrations (>100 μM), chloroperoxidase is relatively stable to increases in pH that at lower concentrations result in rapid conversion to C420. Indeed, 1 mM chloroperoxidase was not converted to C420 overnight at room temperature at pH 8.0. At lower chloroperoxidase concentrations (2−20 μM), there was no detectable dependence of transition rate on enzyme concentration. In the present study, conversion to C420 was always confirmed by absorption spectroscopy prior to EPR measurement.

Ferrous−NO C420 was prepared to further scrutinize the electronic structure of the heme prosthetic group in C420. The presence of superhyperfine splitting of the g_z signal in the EPR spectra of ferrous−NO heme protein complexes is indicative of the presence of a bound imidazole ligand *trans* to NO; however, the absence of superhyperfine splitting does not necessarily preclude the presence of imidazole axial ligation (Ebel et al., 1975). The EPR spectrum of ferrous−NO C420 (Figure 5) was identical to that of ferrous−NO P420 (Martinis et al., 1996). The g_z signal is split to g = 2.021, 2.011, and 2.001. This is similar to that of sodium dodecyl sulfate-denatured hemoglobin and may denote that the bond to the axial ligand *trans* to the bound NO may be radically distorted or even broken (Ebel et al., 1975).

Active Site Titration of C420 with p-mb. To directly address whether Cys29, the single non-disulfide-linked cysteine of chloroperoxidase, is retained as a heme ligand (Blanke & Hager, 1988; Sundaramoorthy et al., 1995) upon conversion to C420, the protein was titrated with *p*-mb. At pH 4.5, there was no detectable modification when chloroperoxidase was titrated with *p*-mb (Figure 6A), even after long incubation times. Unlike P450 (Yu & Gunsalus, 1974), chloroperoxidase was not inactivated when incubated overnight with *p*-mb. The thiolate moiety of Cys29 appears not to be accessible to titration with *p*-mb at this pH.

In contrast, at pH 9.0, C420 contained approximately 0.6 mol of titratable sulfhydryl groups per mole of protein (Figure 6A). Notably, the optical spectrum of *p*-mb-modified C420 was perturbed with a shift in the Soret absorbance to 414 nm (Figure 6B). These data indicate that the alkaline transition to C420 perturbs the active site coordination sphere, increasing the accessibility of Cys29 to modification with *p*-mb.

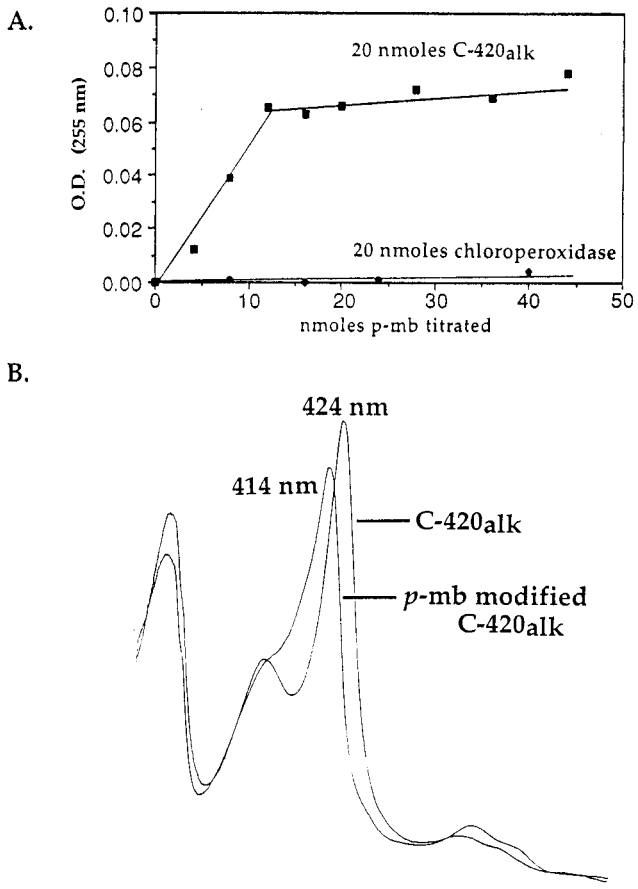

FIGURE 6: Modification of ferric C420 with *p*-mercuribenzoate. (A) The titration of 20 nmol of chloroperoxidase at pH 4.5 and 20 nmol of C420 at pH 9.0. (B) Electronic absorption spectra of ferric *p*-mercuribenzoate-modified C420 and ferric C420 at pH 9.0.

Ligand Complexes of Ferric C420. Ferric chloroperoxidase readily forms ligand adducts that have been extensively characterized by optical, EPR, and MCD spectroscopy (Sono et al., 1986; 1991). Addition of large excesses (>100 mM) of cyanide, azide, imidazole, 2-mercaptoethanol, or the halides to ferric C420 produced minimal or no changes in the electronic absorption or EPR spectra. Incubation of C420 with 500 mM 2-mercaptoethanol produced no detectable changes in the electronic absorption spectra. Clearly, ligand binding to the C420 heme iron is difficult or impossible in many cases with common heme ligands. To further illustrate this observation, the cyanoferric adduct of native chloroperoxidase was prepared and the absorption spectrum was monitored as the pH was raised to 8.0. Instead of the formation of the cyanoferric adduct of C420, the spectrum of C420 itself appeared, indicating that the cyanide was no longer bound.

DISCUSSION

The coordination structures of heme proteins are highly dependent on environmental conditions. The study of heme proteins within defined pH and buffer conditions has revealed that proximal and distal interactions contribute to the energetics of ligand binding at the heme site (Srajer et al., 1988). Changes in local or global protein structure can directly lead to alterations in proximal or distal heme interactions and ultimately affect function.

Ferric chloroperoxidase, which is stable from neutral pH values down to pH 2.0, readily converts to a stable but

enzymatically inactive alkaline form. The pK_a for the alkaline transition is 7.5. On the basis of kinetics evidence, Lambeir and Dunford (1983) proposed that the initial ionization of a single residue induces a conformational change which leads to deprotonation of other amino acids. The alkaline transition of cytochrome *c* peroxidase may also involve an initial ionization event which triggers a cascade of ionizations that modify both the conformation and electrostatic nature of the active site (Dhaliwal et al., 1985). Lambeir and Dunford (1983) further postulated that the conformational change accompanying the transition of chloroperoxidase to C420 results in the interaction of one of the ionizable residues with the heme iron. Interaction with the heme iron was proposed to affect the pK_a of the bound residue. Consistent with this proposal, the heme iron oxidation state has been shown in the present study to influence the pK_a of the transition from chloroperoxidase to C420, providing further evidence for interaction of the heme iron with an ionizable residue in the alkaline transition.

Ferric C420. The proximal ligand of chloroperoxidase is Cys29 (Blanke & Hager, 1988; Sundaramoorthy et al., 1995). The chemical nature of this ligand contributes significantly to dictating enzyme function and is primarily responsible for the distinct optical spectra of chloroperoxidase. It is paramount to ascertain if the thiolate moiety of Cys29 continues to interact with the heme iron upon conversion to C420. The MCD spectrum of high-spin ferric chloroperoxidase (Figure 1B) is marked by the strong negative signal just below 400 nm that is also found in high-spin ferric P450 and thiolate-ligated model complexes (Dawson et al., 1976). The transition of chloroperoxidase to C420 results in a shift of the 400 nm signal, initially suggesting disruption of thiolate ligation. However, the electronic absorption and MCD spectral properties of C420 and the imidazole complex of ferric chloroperoxidase are very similar (Sono et al., 1986). Furthermore, when the EPR spectrum of ferric C420 (Figure 4, pH 9.0) is compared to data for a wide range of low-spin ferric heme proteins and complexes with biomimetic exogenous ligands (Sono et al., 1991), the *g* values for C420 are closest to those of the *N*-phenylimidazole complex of chloroperoxidase, providing further support for the thiolate/imidazole ligand assignment for ferric C420. Clearly, the optical properties of chloroperoxidase and C420 are sensitive to the chemical nature of both the fifth and sixth axial heme ligands. While titration of ferric chloroperoxidase with *p*-mb yielded no detectable cysteines, titration of ferric alkaline C420 resulted in the modification of Cys29. Thus, Cys29 in chloroperoxidase is not accessible to modification, while transition to C420 results in increased accessibility of this residue to modification. Does the transition to C420 disrupt the interaction of the Cys29 thiolate to the heme iron? The perturbation of the absorption spectrum of ferric C420−*p*-mb relative to that of C420 suggests that Cys29 remains at least partially bound as an axial ligand. Recent characterization of ferric P420 by resonance Raman spectroscopy (Wells et al., 1992) suggests that it exists as a mixture of six-coordinate thiolate-bound and five-coordinate non-thiolate-bound species. The similarities between the optical spectra of ferric C420 and P420 raise the possibility that C420 may also contain a mixture of coordination structures.

The change from the predominately high-spin, ferric heme iron of chloroperoxidase to low-spin in C420 was denoted both by the decreased intensity of the high-spin absorbance bands at 515 and 650 nm (Figure 2A) and by the increased intensity of the MCD Soret band (Figure 2B) (Dawson & Dooley, 1989). These data are consistent with the binding of a sixth ligand. Lambeir and Dunford (1983) suggested that the absorption spectrum of ferric C420 is consistent with the binding of an endogenous histidine residue to the heme iron *trans* to Cys29. Kinetic and equilibrium binding studies with exogenous ligands (Campbell et al., 1982; Lambeir & Dunford, 1983; Holzwarth et al., 1988; Sono et al., 1986), as well as the time-dependent modification of chloroperoxidase with diethyl pyrocarbonate (Blanke & Hager, 1990), have provided support for the presence of a distal histidine residue in the enzyme active site. The crystal structure suggests that this is His105 which is hydrogen bonded to Glu183 adjacent to the peroxide binding site (Sundaramoorthy et al., 1995). However, for His105 to bind, a substantial conformational change would be required.

Ferrous C420. The present results provide strong evidence that the ferric and ferrous forms of C420 feature two distinct heme coordination structures. Native ferrous chloroperoxidase, like ferrous P450, is a predominately high-spin, thiolate-ligated heme protein (Dawson & Sono, 1987). The changes in the electronic absorption and MCD spectra of ferrous chloroperoxidase that accompany the conversion to C420 are the result of a spin state change from high- to low-spin. Analogous to the ferric protein, the conversion to a low-spin species is suggestive of the binding of an additional ligand to the ferrous heme iron to form a six-coordinate complex. In contrast to ferric C420, however, ferrous C420 does not appear to retain a thiolate-derived proximal ligand. Ferrous low-spin, thiolate-ligated heme complexes typically exhibit Soret peaks at approximately 445 nm (Dawson et al., 1983) compared to the 425 nm Soret band exhibited by ferrous C420. The absorption spectrum of ferrous C420 more closely matches those of the bis-histidine-ligated cytochrome b_5 and bis-imidazole model complexes than that of the imidazole−thioether ligation sphere of ferrous cytochrome *c* (Dawson & Dooley, 1989). These latter MCD spectra all have the fine structural features in the 500−525 nm region observed in the spectrum of ferrous C420. Although spectra have not been collected for a ferrous heme complex with an imidazole/thiol ligand set, cytochrome *c* is likely to be a good model for such a ligand set because it contains imidazole/neutral sulfur ligation. The fact that the positive Soret band centered at about 410 nm in the MCD spectrum of ferrous cytochrome *c* differs significantly from the band pattern observed in this region in the MCD spectrum of ferrous C420 would seem to rule out a histidine/neutral sulfur donor ligand set for ferrous C420. This leaves a ligand set consisting of two nitrogenous ligands as the most likely structure for this state.

Ferrous C420 readily binds the small exogenous ligands CO and NO. The position of the Soret maximum for native ferrous−CO chloroperoxidase, at 445 nm, is typical for ferrous, low-spin, thiolate-ligated heme complexes. In contrast, the Soret band in both the electronic absorption and MCD spectra of ferrous−CO chloroperoxidase is blue shifted almost 25 nm during the first alkaline transition, indicating that ferrous−CO C420 is not thiolate-ligated. Ferrous−CO C420 has spectral properties similar to those of heme systems with either nitrogen-donor or neutral sulfur-donor ligands

trans to CO, as the electronic spectra of these two systems are virtually indistinguishable (Collman et al., 1976).

Why Is C420 Inactive? Although chloroperoxidase is an efficient, versatile catalyst, the C420 form has no detectable enzymatic activity. In these investigations, we have characterized changes in the active site of chloroperoxidase leading to loss of activity. Cys29, a major determinant in the enzyme's mode of action, is at least partially retained in ferric C420 and probably completely dissociated as an axial heme ligand in ferrous C420. Possible perturbations in the axial ligation of the ferric protein include protonation of the thiolate ligand (Hanson et al., 1976) or lengthening of the Fe−S bond (Jung et al., 1979). A mixed population of thiolate-bound and non-thiolate-bound coordination structures may better represent ferric C420, as has been found for P420 (Wells et al., 1992; Martinis et al., 1996). These structural perturbations would clearly affect the strong electron-donating contribution of the axial thiolate proposed to be important in catalysis and ligand binding (Poulos et al., 1987; Dawson, 1988; Blanke & Hager, 1990). The transition to C420 results in distal as well as proximal effects in the active site. The presence of an additional ligand in the sixth axial site results in a spin state transition and may prevent the protein from binding exogenous ligands and/or the enzyme's natural oxidant, hydrogen peroxide; ligand binding to native ferric chloroperoxidase also yields a low-spin species with inhibited activity. It is not yet known whether the inability of hydrogen peroxide to displace the sixth axial ligand is due to chemical constraints or to steric constraints blocking the substrate access channel.

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