Influence of Protein Environment on Magnetic Circular Dichroism Spectral Properties of Ferric and Ferrous Ligand Complexes of Yeast Cytochrome *c* Peroxidase

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ABSTRACT: The addition of exogenous ligands to the ferric and ferrous states of yeast cytochrome *c* peroxidase (CCP) is investigated with magnetic circular dichroism (MCD) at 4°C to determine the effect the protein environment may exercise on spectral properties. The MCD spectrum of each derivative is directly compared to those of analogous forms of horseradish peroxidase (HRP) and myoglobin (Mb), two well-characterized histidine-ligated heme proteins. The ferric azide adduct of CCP is a hexacoordinate, largely low-spin species with an MCD spectrum very similar to that of ferric azide HRP. This complex displays an MCD spectrum dissimilar from that of the Mb derivative, possibly because of the stabilizing interaction between the azide ligand and the distal arginine of CCP (Arg 48). For the ferric fluoride derivative all three proteins display varied MCD data, indicating that the differences in the distal pocket of each protein influences their respective MCD characteristics. The MCD data for the cyanoferric complexes are similar for all three proteins, demonstrating that a strong field ligand bound in the sixth axial position dominates the MCD characteristics of the derivative. Similarly, the ferric NO complexes of the three proteins show MCD spectra similar in feature position and shape, but vary somewhat in intensity. Reduction of CCP at neutral pH yields a typical pentacoordinate high-spin complex with an MCD spectrum similar to that of deoxyferrous HRP. Formation of the NO and cyanide complexes of ferrous CCP gives derivatives with MCD spectra similar to the analogous forms of HRP and Mb in both feature position and shape. Addition of CO to deoxyferrous CCP results in a ferrous-CO complex with MCD spectral similarity to that of ferrous-CO HRP but not Mb, indicating that interactions between the ligand and the distal residues affects the MCD characteristics. Examination of alkaline (pH 9.7) deoxyferrous CCP indicates that a pH dependent conformational change has occurred, leading to a coordination structure similar to that of ferrous cytochrome b_5, a known

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bis-histidine complex. Exposure of this complex to CO further confirms that a conformational change has taken place in that the MCD spectral characteristics of the resulting complex are similar to those of ferrous-CO Mb but not ferrous-CO HRP. © 1999 John Wiley & Sons, Inc. Biospectroscopy 5: S42–S52, 1999

Keywords: magnetic circular dichroism; spectral properties; ligand complexes; yeast; horseradish peroxidase; cytochrome *c* peroxidase

INTRODUCTION

Heme peroxidases can be found throughout nature and comprise a class of similar enzymes with diverse functionalities. These enzymes demonstrate a common capability of catalyzing the oxidation of organic and inorganic molecules with the concomitant reduction of hydrogen peroxide (H_2O_2).[1] Their diversity arises from the variety of substrates they oxidize including lignin,[2] cytochrome *c*,[3] and even halide ions.[1] These enzymes are believed to perform their respective catalysis via a common mechanism that includes the formation of a high valent intermediate, termed compound I, that is formed through the heterolytic cleavage of the bound peroxide.[1] The heterolytic cleavage of the O—O bond is facilitated by interactions between the peroxo-bound heme and several key protein residues within the active site. The lack of these interactions in globins such as myoglobin (Mb) is believed to be a contributing factor in the predominant homolytic cleavage of bound peroxide that results in the less reactive compound II.[1]

One significant feature observed in the crystal structure of cytochrome *c* peroxidase (CCP), a prototypical peroxidase, is a strongly hydrogen-bonded proximal histidine.[4,5] In CCP the carboxylate side chain of Asp 235 is involved in hydrogen bonds with both Trp 191 and the proximal His 175 ligand. This strong interaction is believed to increase the electron density on the histidine imidazole ring. This partial anionic character provides an electronic "push" up through the heme iron into the peroxide O—O bond[6] that helps to both form and stabilize the compound I high valent intermediate of CCP, which is termed compound ES.

In addition to the control exerted by the environment on the proximal side of the heme, two residues key to efficient enzyme catalysis can be found on the distal side of the heme. The conserved distal arginine/histidine couple found in several peroxidases[7,8] is vital to efficient catalytic turnover by stabilizing and heterolytically cleaving bound peroxide to form the high valent intermediate, compound I.[9] In CCP the distal arginine (Arg 48) increases the polarity of the distal environment and participates in an extensive hydrogen bonding network with fixed solvent molecules and other residues in the active site.[10] Additionally, crystallographic and resonance Raman data have shown that this residue is very flexible and can move toward or away from the ligand in the sixth position.[11,12] The distal histidine (His 52) acts as a general acid–base catalyst in the reaction of CCP with hydrogen peroxide by first deprotonating the iron-bound oxygen of peroxide and transferring the proton to the outer oxygen.[8,13] Mutation of this residue to leucine (H52L CCP[14]) or alanine or valine (H42A and H42V HRP[15]) has been shown to greatly reduce the rate of formation of compound I by 5 orders of magnitude. This residue also has a secondary role as a structural barrier by excluding substrate from the active site and the oxoferryl heme. In HRP the movement of His 42 over one position to 41 (H42L/F41H HRP) not only reproduces the rate of compound I formation but also increases the peroxygenase activity of the system.[16]

Spectroscopic characterization of mixed ligand adducts and comparison with the analogous species of other proteins can reveal the influence of the protein environment on complex formation. To this end, magnetic circular dichroism (MCD) is well suited for the investigation of the coordination environment of heme containing proteins. This technique was especially valuable in studying the structural and magnetic properties of iron porphyrin systems[17,18] and was extensively employed for studies of the active site structures of numerous heme proteins.[18] The MCD was previously used to investigate the ligand free ferric complex[19,20] and the high valent intermediates, compound ES and compound II,[21] in the CCP system.

In the present study MCD is utilized to probe the electronic and, therefore indirectly, the phys-

ical structure of native ferric and ferrous CCP and its complexes with various ligands in an effort to examine the effect of the protein environment on the formation of ligand complexes. MCD spectroscopic data at 4°C are reported for the ferric and ferrous derivatives of CCP with cyanide, azide, fluoride, carbon monoxide, and nitric oxide in comparison with the analogous forms of HRP and Mb. The manner in which the varying protein environment of a peroxidase versus a globin effects the MCD characteristics are discussed.

EXPERIMENTAL

Materials

Native wild-type CCP and recombinant CCP expressed in *Escherichia coli* were purified as described elsewhere.[22,23] HRP (Sigma, type VI) and horse heart Mb (Sigma) were obtained commercially. Carbon monoxide and nitric oxide gases were purchased from Matheson. The KCN, NaN_3, and KF were from Aldrich and the sodium dithionite was from Sigma.

Preparation of Samples

The native and recombinant forms of CCP were crystal suspensions in distilled water[22,23] and were stored at −70°C until needed. Sample concentration was determined based on $\varepsilon_{408} = 98$ (m*M* cm)$^{-1}$ for ferric CCP.[24] The ferric CCP adducts with cyanide, fluoride, and azide were formed by addition of microliter volumes of KCN, KF, and NaN_3, respectively, from 1*M* stock solutions for which the pH was adjusted with phosphoric acid when necessary. The ferric-NO and deoxyferrous species of CCP were prepared in a cuvette sealed with a rubber septum by exchanging the atmosphere in the cuvette with nitrogen followed by an atmosphere exchange with NO (1 atm) or by the addition of solid sodium dithionite, respectively. The ferrous-CO and ferrous-NO adducts were prepared by the addition of solid sodium dithionite to ferric enzyme under CO and N_2 atmospheres, respectively, and in the presence of 2 m*M* sodium nitrite for the latter adduct.[25] The alkaline ferrous and ferrous-CO states were formed in potassium phosphate buffer (pH 9.7) in the same manner as that of the formation of their analogous forms at pH 6.0. The MCD samples of HRP and Mb whose spectra were recorded in this study were prepared as described elsewhere.[26,27]

Spectroscopic Techniques

UV–visible absorption spectra were recorded at ~4°C with a Cary 210 spectrophotometer interfaced to an IBM PC. The MCD spectra were measured at ~4°C using 0.2- or 1.0-cm cuvettes at 1.41 T with a Jasco J500-A spectropolarimeter. This instrument was equipped with a Jasco MCD-1B electromagnet and interfaced to a Gateway 2000 4DX2-66V PC through a Jasco IF-500-2 interface unit. Data acquisition and manipulation are described elsewhere.[28] UV–visible absorption spectra were recorded before and after the MCD measurements to verify sample integrity.

RESULTS AND DISCUSSION

In the present study we investigated numerous ligand adducts of ferric and ferrous forms, as well as the deoxyferrous state of CCP using electronic absorption and MCD spectroscopies. With the exception of the alkaline forms of the enzyme, the spectra of CCP were obtained at pH 6.0 or 7.0, which is within the pH range of 4–8 in which the coordination environment of CCP remains invariant.[24] Deoxyferrous[29] and ferrous-CO[30] CCP are known to form species at alkaline pH (>9) that are spectrally distinct from the neutral pH forms. For this reason these species were also examined. The MCD data (Figs. 1–4) for most of these CCP forms are overplotted with previously reported spectra of parallel complexes of Mb[26,31] and horseradish peroxidase (HRP),[26,32] both of which have a proximal histidine ligand like CCP. The electronic absorption and MCD spectral data of the ferric and ferrous states of CCP in this study are summarized Tables I and II, respectively. Electronic absorption peak positions and extinction coefficient values determined in this study are similar to those reported previously.[29–32]

The electronic character of the proximal histidine ligand and the residues located in the distal pocket influence the function of the protein, which was established by numerous mutagenic, spectroscopic, and crystallographic studies of peroxidases and globins.[13,33,34] For Mb a neutral proximal histidine ligand and the positioning of a distal histidine residue over the heme iron result in an excellent oxygen carrier protein with a very

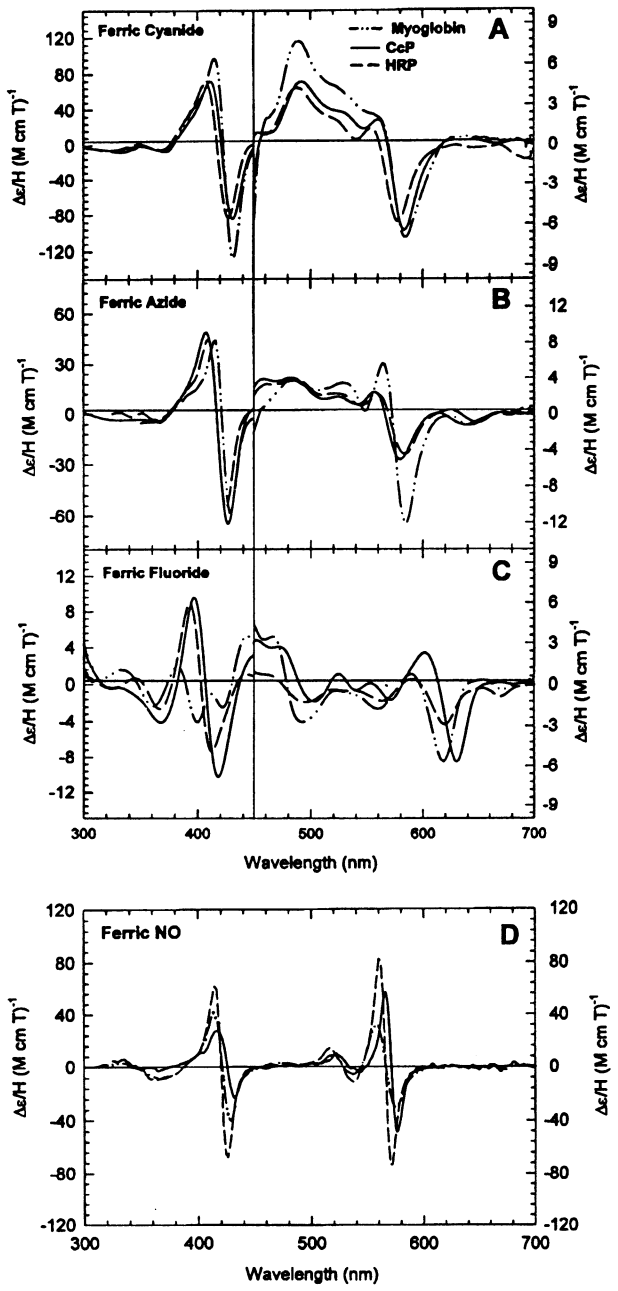

Figure 1. MCD spectra of the (A) ferric-azide (pH 6.0), (B) ferric-fluoride (pH 6.0), (C) ferric-cyanide (pH 6.0), and (D) ferric-NO (pH 7.0) complexes of CCP, HRP, and Mb at 4°C. (A) [NaN_3] = 183 m*M* for CCP. The spectra for the HRP- and Mb-azide complexes are similar to those reported previously.[26,31,32] (B) [KF] = 4.2 m*M* for CCP, HRP, and Mb. The spectra of HRP- and Mb-fluoride complexes are similar to those reported previously.[26,31,32] (C) [KCN] = 3.3 m*M* for CCP and HRP. The spectrum for the HRP-cyanide complex is similar to that reported previously[26,32] and that of the Mb-cyanide complex is taken from Ref. 26.

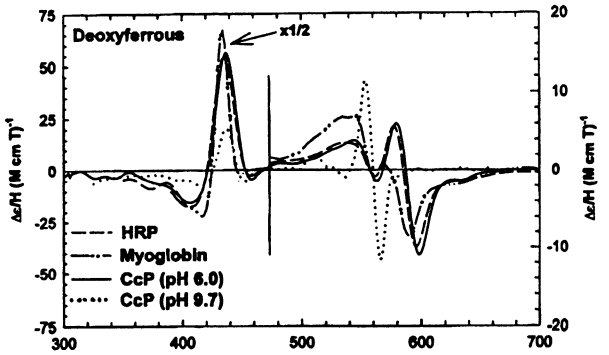

Figure 2. MCD spectra of the deoxyferrous states of CCP (pH 6.0), alkaline CCP (pH 9.7), HRP, and Mb at 4°C. Note that the *y*-axis scale for deoxyferrous Mb in the Soret region (300–475 nm) is reduced by a factor of 2. The spectra for HRP and Mb are similar to those reported previously.[23,31,32] See the Experimental section for sample preparation details.

stable ferrous oxy complex. For CCP and HRP a partially anionic proximal histidine ligand coupled with the presence and location of a histidine and an arginine residue in the distal pocket facilitate the formation and stabilization of the high valent oxo intermediates necessary for peroxidase activity. These differences in protein environment in the active sites of peroxidases versus globins affect not only their respective activity but also their respective interaction with various sixth ligands in both the ferric and ferrous oxidation states.

Ferric Derivatives

Addition of an exogenous sixth ligand (whether cyanide, azide, fluoride, or nitric oxide) to native ferric CCP results in a coordination state change from penta- to hexacoordination. This coordination state variation can be seen in a sharpening of the Soret peak and in a loss of the shoulder at 370 nm in the electronic absorption spectra of the four complexes (Table I). A comparison of the MCD data of these four complexes with the MCD spectra of the analogous forms of HRP and Mb is shown in Figure 1(A–D). As can be seen in Figure 1(A) (Table I), the addition of a strong field ligand such as cyanide yields complexes with MCD spectral characteristics similar among all three proteins. This finding indicates that interactions between the ligand and the distal environment do not significantly affect the MCD spectral charac-

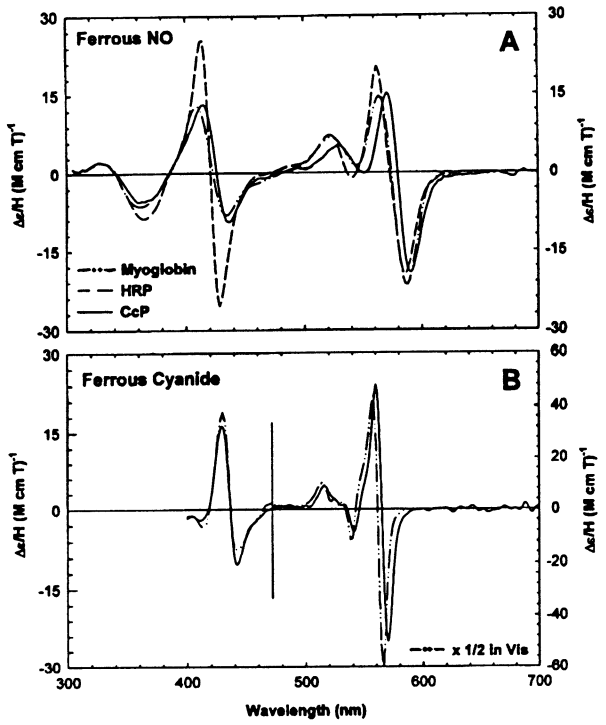

Figure 3. MCD spectra of (A) ferrous-NO complexes (pH 6.0) of CCP, HRP, and Mb at 4°C. The spectra for HRP and Mb are similar to those previously reported.[26,31,32] (B) MCD spectra of ferrous CN complexes of CCP (pH 7.0, [KCN] = 5 m*M*) and Mb. Note that the spectrum of Mb is similar to that seen in Ref. 31 and is reduced by a factor of 2 in the visible region (476–700 nm). See the Experimental section for sample preparation details.

teristics of the heme complex when a strong field ligand is bound in the sixth axial position.

Addition of a weak field ligand such as azide or fluoride to ferric CCP results in a complex with MCD spectral characteristics more similar to those of the analogous forms of HRP rather than Mb [Fig. 1(B,C), Table I]. The variation of the MCD spectral characteristics of the two peroxidases from those of Mb are likely due to the differing interactions between the sixth ligand and the protein environment, specifically the distal residues. In CCP the distal histidine (His 52) is located ~5.5 Å directly above the heme iron and acts as a general acid–base catalyst in the heterolytic cleavage of bound peroxide.[5] Furthermore, a positively charged arginine residue (Arg 48) is located nearby and has been determined to play a role in the stabilization of anionic sixth ligands.[12] In Mb there is no distal arginine residue; the distal histidine (His 64), which is located only 4.3 Å from the heme iron,[35] acts as a general base in the stabilization of ligands. Because azide is negatively charged at neutral pH (pK_a = 4.5), complex formation should be more favorable in the CCP environment than in that of Mb. This conjecture is supported by mutation experiments with Mb that determined that removal (H64L Mb) or movement of the distal histidine to a position similar to that seen for the peroxidases (H64L/F43H Mb) caused a 10-fold increase in the association rate constant of azide from 2.9 to ~31 mM^{-1} s^{-1}.[36,37] As indicated by these results, the position of the distal histidine in Mb causes a destabilization of the ferric azide complex by forcing the ligand to bind in a sterically unfavorable conformation.[38] The difference in the conformation of the azide ligand and the lack of a stabilizing arginine residue in Mb perturbs the electronic character of the complex that in turn causes variations in its MCD characteristics.

A closer examination of the MCD data of the ferric fluoride adducts of CCP and HRP indicates that in addition to being spectrally variant from the analogous form of Mb, there may also be subtle differences between the two peroxidases when fluoride is present as a sixth ligand. Neri and coworkers suggested that electronic absorption features, particularly the charge transfer (CT) band found between 600 and 650 nm, of high-spin ferric histidine-ligated heme complexes are very sensitive to the degree of hydrogen bonding interactions between the bound sixth ligand and the distal environment.[11] This absorption band is attributed to the CT from the porphyrin to the iron

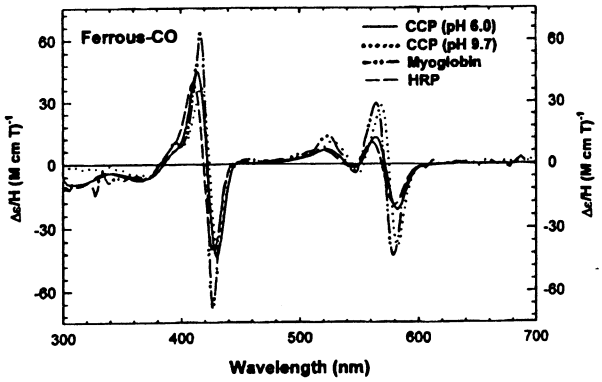

Figure 4. MCD spectra of the ferrous carbon monoxide complexes of CCP (pH 6.0), alkaline CCP (pH 9.7), HRP, and Mb at 4°C. The spectra for HRP and Mb are similar to those reported previously.[31,32] See the Experimental section for sample preparation details.

Table I. Electronic Absorption and MCD Spectral Features of Ferric Ligand Complexes of CCP

	Absorption		MCD	
Derivative	λ max (nm)	ε (mM cm)$^{-1}$	λ (nm)	$\Delta\varepsilon$/H (M cm T)$^{-1}$
Ferric-cyanide (pH 6)	362	29.4	411	71.6
			421	0
	423	109	431	−84.5
			492	4.7
	543	12	560	2
			569	0
	580 (sh)	7.4	583	7
Ferric-azide (pH 6.0)	415	118	407	49
			417	0
	528	9	427	−64.8
			483	4.1
	566 (sh)	6.8	557	2.4
			569	0
	642	1.6	580	−5.1
			642	−1.2
Ferric-fluoride (pH 6.0)	404.5	141	397	9.6
			409	0
	492.5	9.1	418	−10.3
			451	3.2
	564	5.1	601	2.3
			613	0
	616.5	7.9	630	−5.7
Ferric-NO (pH 7.0)	423.5	122	417	27.8
			425	0
	537	12.5	432	−23.2
			522	10.1
	570	15.9	543	−4.2
			566	57.0
			571	0
			576	−48.0

and has been recently shown to blue shift when the p and/or π donor capability of the ligand increases.[11] Hydrogen bonding interactions affect the p donor capability by altering the charge donation from the ligand to the iron.[11] If the ligand acts as a hydrogen bond acceptor, then the stronger the hydrogen bond, the less charge donation to the iron. This decrease in charge donation to the iron results in a higher wavelength for the CT band.[11,39] Because this CT band can be seen as a trough in the same wavelength range in the MCD data, we can use the position of the MCD trough in the spectrum of the ferric fluoride complex to determine any variation in hydrogen bonding interactions between the ligand and the distal environment of the three proteins compared here.

The crystal structure of the ferric fluoride adduct of CCP demonstrated that the Arg 48 side chain moves toward the ligand about 2.5 Å in order to maximize interactions with the bound fluoride.[12] Additional hydrogen bonding interactions directly with the NH of the distal Trp 51 and indirectly with His 52 via a solvent molecule further stabilize this complex in CCP.[11] These three interactions cause a decrease in the charge donation from the ligand to the iron, which in turn results in a CT band at 616.5 nm and an MCD trough at 630 nm. In HRP the interactions of the fluoride with His 42 and Arg 38 mimic those seen in CCP, but there is no third interaction because HRP has a phenylalanine (Phe 43) residue in the position analogous to Trp 51 in CCP.[11] The decrease in the hydrogen bonding interactions increases the charge donation from the fluoride to

Table II. Electronic Absorption and MCD Spectral Features of Ferrous Ligand Complexes of CCP

	Absorption		MCD	
Derivative	λ max (nm)	ε (mM cm)$^{-1}$	λ (nm)	$\Delta\varepsilon$/H (M cm T)$^{-1}$
Deoxyferrous (pH 6.0)	438.5	103	410	−17.9
			439	63.2
	558	14.1	516	3.1
			582	7.9
	586	8.2	590	0
			600	−11
Ferrous-CO (pH 6.0)	424	160	417	37.7
			424	0
	542	13.6	431	−40.8
			569	28.4
	572.5	12.4	575	0
			582	−38.5
Ferrous-NO (pH 6.0)[a]	423	118	415	13.2
			436	−9.4
			477	0
	544	12.0	530	5.4
			551	−0.14
			571	15.2
	572	11.0	580	0
			590	−19.1
Ferrous-cyanide (pH 7.0)	431	146	430	36.9
			436	0
	533	14.6	442	−23.6
			575	20.7
	567	18.9	541	−19.9
			560	109
			565	0
			570	−113
Deoxyferrous (pH 9,7)	426	96	427	0
			440	30.4
	530	8.4	457	0
			511	6.5
	560	14.1	536	−0.8
			553	32.8
	586 (sh)	4.4	560	0
			566	−31.6
Ferrous-CO (pH 9.7)	420	181	413	41
			420	0
	539	12.6	426	−47.1
			565	17.2
			571	0
	571	11.7	579	−29.1

[a] No detectable change at pH 10.0.

the iron, which causes a blue shift in both the UV–visible absorption CT band (612 nm) and the trough in the MCD data (620 nm). In Mb the bound fluoride is stabilized only by an indirect hydrogen bonding interaction with His 64. Removal of this residue (H64L Mb) completely eliminates fluoride binding in Mb.[40] Because there is only one hydrogen bonding interaction, the ferric fluoride complex of Mb has the most blue-shifted CT band (611 nm) and MCD trough (618 nm).

Interestingly, the ferric fluoride complex of ascorbate peroxidase (APX), a peroxidase with a distal pocket highly analogous to that of CCP, has a CT band at 616 nm and an MCD trough at 629 nm, which is nearly identical to the values for CCP.[41] Obviously, the hydrogen bonding interactions between the sixth ligand and the distal environment play an important role in the determination of the UV–visible absorption and MCD spectral characteristics of hexacoordinate high-spin heme complexes.

EPR and crystallographic studies of ferric-NO CCP showed that the bound NO transfers its unpaired electron to the ferric iron and that this unpaired electron density is further delocalized to the proximal His 175 and Trp 191 residues.[12,25] This electron push from the distal ligand to the proximal pocket causes Trp 191 to move ~0.2 Å with similar adjustments from its neighboring residues.[12] Further, the cationic character of the bound NO causes the distal Arg 48 to move 0.6 Å away from the ligand, an effect that is opposite from that seen for the anionic fluoride ligand.[12] It is likely that the movement of the latter residue is duplicated in HRP upon NO binding to the ferric state of the enzyme. Consequently, the MCD spectra of the ferric NO complex of CCP and HRP are similar in feature shape and position, but they vary in intensity [Fig. 1(D), Table I]. However, the *ratio* of the intensities of the Soret region derivative-shaped feature to that in the visible region (1:2) is similar for the two peroxidases. The variation in feature intensity could be due to a difference in how CCP distributes the electron density from the unpaired electron of NO compared to HRP because the latter peroxidase lacks a tryptophan residue in its proximal pocket.

Neutral Ferrous Derivatives

Reduction of native ferric CCP at neutral pH results in the formation of a high-spin complex with imidazole as the fifth ligand as evidenced by a red-shifted Soret absorbance band at 438.5 nm and a converging of the visible features into a prominent band at 558 nm with a shoulder at 586 nm (Table II). Similar to the trend seen for the ferric complexes with azide, fluoride, and NO, the MCD of deoxyferrous CCP resembles that of HRP rather than Mb (Fig. 2). Because all three proteins are five-coordinate high spin with a proximal histidine ligand, the difference in the electronic character giving rise to the varied MCD spectra must be attributable to the greater anionic character of the proximal histidine ligand of the peroxidases.

The NO and cyanide adducts of CCP were also examined in the ferrous oxidation state. In the UV–visible absorption spectrum of the ferrous-NO complex of CCP, the Soret absorbance band appears at 423 nm with two features at 544 and 572 nm in the visible region (Table II). Surprisingly, the MCD spectrum of this CCP complex is more similar to that of the analogous form of Mb than to HRP [Fig. 3(A)]. Here HRP has a more defined derivative-shaped feature where CCP and Mb have a derivative-shaped feature that is mostly positive. To determine whether the MCD data for CCP was abnormal for a peroxidase, a comparison of its MCD data was made to that of the ferrous-NO adduct of lacquer peroxidase.[42] The similarity of their respective MCD data in band shape, position, and intensity with CCP being slightly more intense in the visible region indicates that HRP not CCP exhibits somewhat abnormal MCD data for its ferrous-NO complex.

The low-spin complex of ferrous CCP with cyanide displays an MCD spectrum that is very similar to those of the corresponding derivatives of HRP (data not shown)[32] and Mb [Fig. 3(B)] in band position and shape, but that of Mb is much more intense. As seen with the ferric-NO and ferrous-NO complexes, the band intensity appears to vary from protein to protein but the band position and shape remain constant. These similarities indicate that, as seen with the ferric-CN complex, the varied hydrogen bonding interactions between the ligand and the distal environment do not perturb the MCD data significantly, yielding similar MCD spectra for all three proteins.

Addition of CO to the neutral deoxyferrous complex of CCP results in the formation of a six-coordinate complex as evidenced by a blue shift in the Soret band to 424 nm and the appearance of two visible bands at 542 and 572.5 nm (Table II). Again, the MCD spectrum of the ferrous-CO complex of CCP is more similar to the MCD spectrum of the analogous form of HRP than to Mb (Fig. 4, Table II). Resonance Raman and IR spectroscopic studies of the ferrous-CO complexes of Mb,[43,44] HRP,[45–47] and CCP[10,48] offer insight into the interactions responsible for the varied MCD data. Measurement of the Fe—CO (ν_{FeC}) and C—O (ν_{CO}) stretching frequencies and their comparison with those of other heme proteins and model sys-

tems can monitor the influence of the protein environment on the ferrous-CO complex. A strong proximal ligand such as that in the peroxidases affects the ν_{FeC} frequency by pushing electron density into the heme and weakening the *trans* Fe—C bond.[46] The interaction of the bound CO with positively charged and/or hydrogen bonding groups in the distal pocket enhances the electron backdonation from the iron dπ to the CO π^* orbital that in turn weakens the C—O bond and strengthens the Fe—C bond.[46] For Mb a neutral proximal histidine coupled with a nonbonding interaction between the bound CO and His 64 results in a ν_{FeC} of 507 cm^{-1} and a ν_{CO} of 1947 cm^{-1}.[43] CCP, which has a partially anionic proximal ligand and potential interactions between the bound CO and His 52 and Arg 48, has a ν_{FeC} stretch at a higher frequency of 530 cm^{-1} and a ν_{CO} at a lower frequency of 1922 cm^{-1}.[10,48] These stretching frequencies for the two proteins indicate that the protein environment stabilizes the ferrous-CO complex in the peroxidase and destabilizes the complex in Mb. Because the strength of the Fe—CO interaction contributes to the electronic character of the complex, the difference in this strength between CCP and Mb yields varied MCD signals for their respective ferrous-CO complexes.

Interestingly, the ν_{FeC} and ν_{CO} frequencies for CCP and HRP also vary from one another, indicating differences in their respective ferrous-CO complexes.[10,45–48] In fact, resonance Raman studies of the ferrous-CO complex of HRP showed that this derivative exists in two conformers with two distinctive sets of stretching frequencies.[45,47] These two conformers are due to separate interactions with each of the distal residues, His 42 and Arg 38.[47] Similar studies of wild-type CCP and the H52L[14,48] and R48L CCP[10,48] mutants demonstrated that the bound CO interacts only with the distal arginine. The X-ray crystal structure of the ferrous-CO complex of CCP confirmed this assignment, showing that CO is tilted 12° relative to the heme normal and hydrogen bonds to a water molecule that in turn hydrogen bonds to Arg 48.[12] Moreover, the Arg 48 terminal nitrogen atoms move away from the bound CO by about 0.6 Å with respect to ferric CCP. Despite these variations in ligand conformation and electronic interactions, the MCD spectra of the ferrous-CO complexes of CCP and HRP are very similar, indicating that these variations are not sufficient to perturb the respective MCD characteristics of the peroxidases.

Alkaline Ferrous Derivatives

CCP undergoes an alkaline transition at pH values above 8.0,[14] which results in spectrally distinct complexes of the ferric, deoxyferrous,[29] and ferrous-CO[30] derivatives but not for the ferrous-NO species (this study). This pH dependent alteration is believed to cause a conformational change that is associated with the titration of two protonatable groups in the ferrous form as revealed by the pH dependence of the absorption spectra, CO rebinding, and CO dissociation rates.[10,48] His 181 is clearly implicated as one of the protonatable groups because its mutation to a glycine residue resulted in a decrease from two to one in the titratable groups.[48] Based on the crystal structure, the imidazole side chain of His 181 appears to be hydrogen bonded to a heme propionate group.[5] The disruption of this interaction by deprotonation of His 181 was implicated in the conformational change observed in the protein at alkaline pH.[10,14] This conformational change is thought to affect the proximal and distal environments by weakening their respective hydrogen bonding networks. Earlier MCD[20] and resonance Raman studies[10,48] on alkaline ferric CCP indicated that the distal histidine, which is 5 Å away from the iron in the neutral species, binds to the heme at high pH, suggesting substantial displacement of the peptide backbone. Additional resonance Raman studies of wild-type and H52L CCP in the deoxyferrous state at alkaline pH[10,48] demonstrated that the distal histidine serves as the sixth axial ligand in this oxidation state and yields a six-coordinate complex that is spectroscopically similar to ferrous cytochrome b_5, a known bis-histidine protein. Here the generation of deoxyferrous CCP at pH 9.7 resulted in an electronic absorption spectrum that is considerably varied from that of the pH 6 form, exhibiting major bands at 426, 530, and 560 nm (Table II). The MCD spectrum of the deoxyferrous adduct of alkaline CCP (Fig. 2, Table II) shows that the most prominent feature is now a derivative-shaped band in the visible region that is similar to the MCD spectrum of ferrous cytochrome b_5.[49] The MCD spectrum of alkaline deoxyferrous CCP has a relatively red-shifted positive Soret feature centered at 440 nm compared to that of ferrous cytochrome b_5 centered at 425 nm. The similarity of

the visible region MCD spectral features of alkaline deoxyferrous CCP and ferrous cytochrome b_5 indicates that the former protein contains six-coordinate bis-histidine ligated species at alkaline pH. However, the variation in the position and intensity of their respective Soret features suggests that the alkaline deoxyferrous CCP derivative is a mixture of five-coordinate histidine-ligated and six-coordinate bis-histidine ligated species.

The neutral and alkaline (pH 9.7) ferrous-CO adducts of CCP display MCD spectra (Fig. 4, Table II) that are similar to each other in shape and in Soret region intensity, but they are distinguishable from one another in detailed features by slight red shifts and a somewhat greater visible region intensity for the alkaline form. Resonance Raman studies of alkaline ferrous-CO CCP indicated that transition to the alkaline conformation results in a complex in which His 52 inhibits the interaction of Arg 48 with the bound CO.[48] Additionally, the alkaline conformational variation causes a weakening of the hydrogen bonding network in the proximal pocket, potentially yielding a less anionic histidine ligand.[48] The resulting complex should be similar to the ferrous-CO adduct of Mb with its neutral imidazole ligand and no distal arginine. Comparison of the MCD of the ferrous-CO adduct of CCP at alkaline pH to that of the analogous form of Mb at neutral pH is very interesting, especially in the visible region. Aside from a red shift of the derivative-shaped feature of the CCP complex, the two MCD spectra are very similar in intensity and band shape, indicating a similar electronic structure. The differences in intensity of the derivative-shaped feature in the Soret region of the MCD spectra may indicate the presence of some residual neutral species. Nevertheless, the MCD spectral similarities between the ferrous-CO complexes of alkaline CCP and neutral Mb indicate that the conformational change in CCP that is due to the alkaline transition yields a complex with spectroscopic characteristics similar to a globin rather than a peroxidase.

CONCLUSIONS

In the present study we examined the ferric and ferrous derivatives of CCP with MCD and compared the resulting spectra with those of the analogous forms of HRP and Mb. For the mixed ligand complexes in the ferric state, the three proteins exhibited varied MCD spectra when a weak field ligand such as azide or fluoride was present at the sixth axial position. Conversely, the presence of a strong field ligand such as cyanide or NO yielded similar MCD spectra for all three proteins. In the ferrous oxidation state the two peroxidases had similar MCD spectral characteristics in the deoxyferrous, ferrous-CN, ferrous-NO, and ferrous-CO states at neutral pH. At pH 9.7 the deoxyferrous CCP had an MCD spectrum similar to that of ferrous cytochrome b_5 while ferrous-CO CCP had MCD intensity patterns that were similar to those of ferrous-CO Mb rather than HRP.

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