Protein Conformational Perturbations Affect the Photoreduction of Native Cytochrome *c* Peroxidase(III) at Alkaline pH†

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ABSTRACT: Ferric cytochrome *c* peroxidase (CCP) undergoes a ligation-state transition from a pentacoordinate, high-spin (5c/hs) heme to a hexacoordinate, low-spin (6c/1s) heme when titrated over a pH range of 7.30–9.70. This behavior is similar to that exhibited by the ferrous form of the enzyme. However, the photodissociation of the low-spin, axial ligand, exhibited by ferrous CCP at alkaline pH, is not observed for ferric CCP. Instead, a photoinduced reduction of the ferric heme is apparent in the pH range 7.90–9.70. In the absence of O_2 and redox mediators such as methyl viologen (MV^{2+}), the reoxidation of the photoreduced enzyme is very slow ($\tau_{1/2} \sim 3$ min). F^--bound CCP(III) (6c/hs) displays similar pH-dependent photoreduction. Horseradish peroxidase, however, does not. The formation of 6c/1s heme coincides with the onset of appreciable photoreduction (between laser pulses, >60 ms) of CCP (III) at alkaline pH, suggesting a global protein conformational rearrangement within or around its heme pocket. Photoreduction of alkaline CCP(III) most likely involves intramolecular electron transfer (ET) from the aromatic residue in the proximal heme pocket to the photoexcited heme. We speculate that the kinetics of electron transfer are affected by changes in the orientation of Trp-191.

Cytochrome *c* peroxidase (CCP) (Poulos & Finzel, 1984; Mauro et al., 1989; Anni & Yonetani, 1992), a mitochondrial heme protein found only in yeast (Yonetani & Ohnishi, 1966), catalyzes the oxidation of ferrocytochrome *c* to ferricytochrome *c* and the concomitant reduction of H_2O_2 to water. The enzyme is localized in the intermembrane space between the inner and outer membranes of yeast mitochondria (Erecinska et al., 1973; Williams & Stewart, 1976). Catalysis is initiated by the coordination of H_2O_2 to the heme iron. This is followed by the heterolytic cleavage of the O–O bond to form an intermediate (compound I) containing a ferryl heme iron (Poulos & Kraut, 1980; Poulos et al., 1980) and a cation radical at Trp-191 in the proximal site of its heme pocket (Goodin et al., 1987; Erman et al., 1989; Sivaraja et al., 1989). The location of the cation radical at Trp-191 has been further proven since both the enzyme activity and its EPR signals for the corresponding compound I radical are substantially diminished upon the mutation of Trp-191 to Phe (Mauro et al., 1988; Scholes et al., 1989). Miller et al. (1992) have recently demonstrated that Trp-191 and Asp-235 are directly involved in the rapid oxidation of ferrous CCP by dioxygen.

Recent studies have revealed that the CCP heme pocket is quite susceptible to environmental perturbations. For instance, Erman and co-workers (Vitello et al., 1990) confirmed the pH dependence of the kinetic activity of CCP with H_2O_2 (Loo & Erman, 1975), and low-spin heme species were detected at alkaline pH for ferric CCP (Wittenberg et al., 1968; Hashimoto et al., 1986) and for ferrous enzyme (Conroy et al., 1978; Smulevich et al., 1989; Spiro et al., 1990; Wang et al., 1991, 1992a,b). Satterlee and Erman (1983) further revealed that two pH-dependent transitions were evident in the proton NMR studies on CCP-cyanide. Physicochemical studies of CCP (Dowe & Erman, 1985) indicated extensive structural modifications upon exposure to alkaline pH. Meanwhile, a kinetic study on CCP (Dhaliwal & Erman, 1985) showed that the transition from high spin to low spin occurred at alkaline pH and altered its catalytic kinetics. This pH dependence evidently induces large-scale protein conformational changes and alters heme ligation state by disrupting the strong H-bonding network (His-175···Trp-191···Asp-235) in the heme pocket (Spiro et al., 1990; Wang et al., 1992a,b). The heme pocket of CCP can also be directly altered by site-specific mutagenesis. Recent EPR data further indicate the elimination of the compound I signal upon the mutation of Asp-235 to Asn. This coincides with a reorientation of the indole ring of Trp-191 and the subsequent disruption of His-175···Trp-191···Asp-235 H-bonding network (Fishel et al., 1991).

Resonance Raman spectroscopy (RRS) is particularly sensitive to changes in the local protein environment of the heme-active site (Spiro, 1987; Rousseau & Ondrias, 1984). For instance, Shelnutt et al. (1983) reported a substantial decrease in the π-electron density in the porphyrin ring of ferric CCP as the pH was lowered from 6.5 to 4.5. They further suggested that the pH-dependent variations in the interaction of the heme with its environment might result from the rotation of the heme's peripheral vinyl groups or the movement of distal tryptophan closer to the peroxide-binding site of the enzyme. Recently, Spiro and co-workers (Smulevich et al., 1989; 1991; Spiro et al., 1990) documented spin-state transitions in a variety of site-directed CCP mutants and concluded that protein conformational changes controlled the heme chemistry. Their studies revealed that the endogenous sixth ligand (possibly His-52) in alkaline-reduced CCP was photodissociable. The response of native CCP(II) to elevated pH has been characterized in our laboratory by RRS, optical absorption, and circular dichroism (CD) techniques (Wang et al., 1991, 1992a). We recently reported a reversible transition of CCP(II) from a pentacoordinate, high-spin (5c/hs) heme to a hexacoordinate, low-spin (6c/1s) heme ($pK_a \sim 7.7$) when titrated from pH 7.00 to 9.70. Our CD data clearly showed the disappearance of a single high-spin species and

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the occurrence of at least two low-spin species of CCP(II) at pH above 7.90. This argument has been strongly supported by the pH-dependent photolysis of the distal ligand His-52, with a maximum yield at pH = 8.50. Furthermore, the photodynamics of low-spin, ferrous CCP are quite distinctive relative to those involving the fast photodissociation and recombination of σ-ligands in other heme proteins (Jongeward et al., 1988). Recent dynamic studies on the photodissociation and recombination of alkaline CCP(II) (Wang et al., 1992b) show that the photodissociated His-52 rebinds in 500 ns–1 μs.

The present work extends our studies on alkaline CCP to its ferric form. We find that elevation in pH influences both the equilibrium structure and photodynamics of alkaline CCP-(III) and the photoinduced reduction of ferric CCP.

MATERIALS AND METHODS

Twice-crystallized CCP was isolated and purified from bakers' yeast (Red Star) by a modification of the procedure of Yonetani and Nelson et al. that was developed by Moench (Moench, 1986; Wang, 1992). Horseradish peroxidase (HRP) (Type VI from Sigma) was used without further purification. Care was exercised to use only fresh CCP in these investigations, and phosphate buffers were avoided (Dasgupta et al., 1989). The concentrations of ferric CCP and HRP were spectroscopically determined at 408 nm ($\epsilon \sim 93$ mM^{-1} cm^{-1}; Yonetani & Ray, 1965) and at 403 nm ($\epsilon \sim 91$ mM^{-1} cm^{-1}; Nozawa et al., 1976), respectively. Ferric samples of CCP and HRP were obtained by dissolving CCP or HRP crystals directly into 100 mM Mes (Sigma) or Tris-HCl (Sigma) buffer solutions at the required pH. The samples of F^-- and imidazole- (Imd-) bound CCP were prepared by adding KF and imidazole stock buffer solutions into ferric CCP samples. Methyl viologen (MV^{2+}) was dissolved in 100 mM Tris-HCl stock solutions. CCP/MV^{2+} samples were made by adding a MV^{2+} stock solution into a CCP(III) solution (~3%), followed by degassing with five cycles of vacuum/N_2. The reoxidation kinetics of alkaline CCP(III), which was photoreduced under anaerobic conditions, were quantified by monitoring the growth of the Soret band for ferric CCP (at 414 nm) via a Hewlett-Packard HP8452A diode array UV/vis spectrometer.

Resonance Raman data were collected by instrumentation described elsewhere (Wang, 1992). All transient RRS data were recorded with 10-ns pulses at 404, 408, or 410 nm. During the measurements, the samples were cooled to approximately 4 °C by blowing nitrogen gas over the sample cells. The laser flux at the sample was regulated by using focusing optics and/or neutral density filters to attenuate the beam, yielding laser fluxes from $\sim 1 \times 10^7$ to $\sim 3 \times 10^8$ W/cm^2. Optical absorption spectra were obtained with a Hewlett-Packard HP8452A diode array UV/vis spectrometer under the same conditions used in RRS measurements.

RESULTS

Spectra of Equilibrium CCP(III). In our previous characterizations of alkaline CCP(II) (Wang et al., 1992a), we reported a time-and temperature-dependent ligation transition from a 5c/hs heme to a 6C/1s heme. This kinetically slow process ($\tau_{1/2} > 1$ h at 4–10 °C) was also observed for ferric CCP at alkaline pH. Consequently, all the optical absorption and RRS data were recorded only after equilibrium was achieved. The absorption spectra (data not shown) clearly show a conversion from high-spin to low-spin heme as solution conditions are varied from neutral to alkaline pH. This conversion is evident in both Soret (408–414 nm) and visible

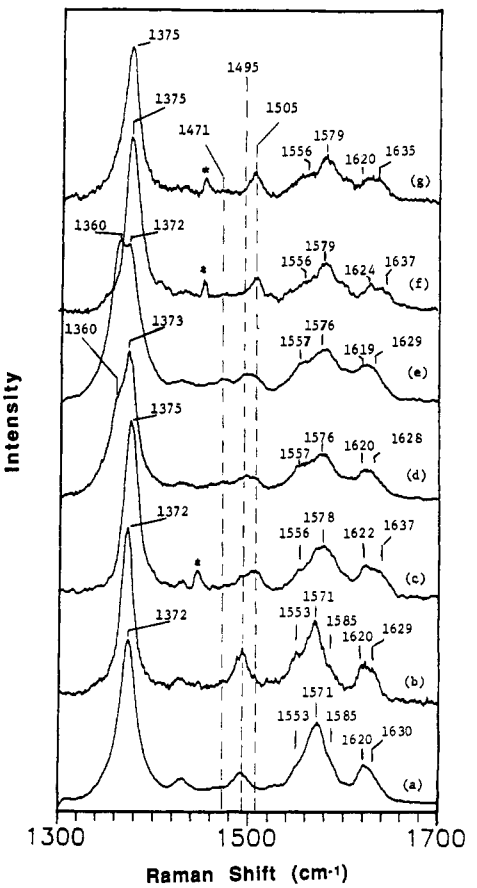


FIGURE 1: Resonance Raman spectra of fresh ferric cytochrome *c* peroxidase as a function of pH and laser flux. All spectra were obtained at 408–410-nm excitation with a spectral band pass of ≤6 cm^{-1} and are the unsmoothed sums of 3–6 scans at 10 cm^{-1}/min. The feature marked with an asterisk is an instrumental artifact. Samples were ~100 μm CCP(III) in 100 mM Mes (for pH 4.5) or 100 mM Tris (pH 7.0–9.7): (a) pH 4.5, high flux, (b) pH 7.0, high flux, (c) pH 8.5, low flux, (d) pH 8.5, medium flux, (e) pH 8.5, high flux, (f) pH 9.7, low flux, and (g) pH 7.3 + 10 mM imidazole, high flux. In this context, high, medium, and low flux were $\sim 1 \times 10^7$, $\sim 2 \times 10^8$, and $\sim 3 \times 10^8$ W/cm^2, respectively (see text for further details).

(504–534 nm) regions. This confirms the existence of a 6c/1s heme in alkaline medium, as observed by previous investigators (Dhaliwal & Erman, 1985; Smulevich et al., 1991).

At neutral pH, the fresh form of native ferric CCP possesses a 5c/hs heme characterized by a sharp ν_3 band (a ligation-state and core-size marker) at ~1494 cm^{-1}, while the aged enzyme displays a mixture of 5c/hs, 6c/1s, and 6c/hs (hexacoordinate high-spin) components (evidenced by a broad ν_3 at 1494 cm^{-1} with shoulders at 1505 and 1478 cm^{-1}, respectively) (Dasgupta et al., 1989). Our RRS data (Figure 1) clearly show the single species of a 5c/hs heme (Figure 1a,b) with ν_4 (an oxidation marker) at 1372 cm^{-1}, a narrow ν_3 band at 1495 cm^{-1}, ν_{11} at 1553 cm^{-1}, ν_2 at 1571 cm^{-1}, ν_{37} at 1585 cm^{-1}, ν_{10} at 1629 cm^{-1}, and a vinyl stretching vibration mode at 1620 cm^{-1}. A 6c/1s heme is obtained when imidazole binds at its sixth ligand position (see Figure 1g), showing a ν_4 at 1375 cm^{-1}, a sharp ν_3 band at 1505 cm^{-1}, ν_{11} at 1556 cm^{-1}, ν_2 at 1579 cm^{-1}, ν_{37} at 1594 cm^{-1}, ν_{vinyl} at 1620 cm^{-1}, and ν_{10} at 1635 cm^{-1}. RRS data of alkaline CCP(III) (Figure 1c,f) are quite distinct from those of CCP(III) at neutral pH when low laser flux (~1×10^7 W/cm^2) is employed. The increase in intensity of ν_3 at 1505 cm^{-1} and the 3-cm^{-1} shift of ν_4 (1372 to 1375 cm^{-1}) indicate the formation of low-spin heme iron at alkaline pH. The concomitant shifts of ν_2 (1571

Table I: Resonance Raman Data for Different Oxidation and Spin States

	ν_4 (oxidation state) (cm^{-1})	ν_3 (ligation state) (cm^{-1})	refs
ferric			
6c/ls	1375	1505	*a–e*
5c/hs	1372	1496	*a–e*
6c/hs	1372	1478	*a, b, e*
ferrous			
6c/ls	1360	1495	*a, f–h*
5c/hs	1356	1471	*a, b, d–h*

[a] This work. [b] Dasgupta et al. (1989). [c] Smulevich et al. (1986). [d] Hashimoto et al. (1986). [e] Wang et al. (1990); Want (1992). [f] Smulevich et al. (1989). [g] Spiro et al. (1990). [h] Wang et al. (1992a).

to ~1578 cm^{-1}) and ν_{10} (1629 to 1637 cm^{-1}) also support this argument. However, the shoulder of ν_3 at 1495 cm^{-1} clearly indicates that this transition is incomplete at pH 8.50 (Figure 1c).

Photodynamics of CCP(III) at Alkaline pH. Increasing the laser flux (from ~1×10^7 to ~3×10^8 W/cm^2) produces noticeable changes in the RRS spectra of ferric CCP at pH 8.50 (Figure 1d,e). First, ν_4 significantly broadens (from 9 up to 18 cm^{-1}) and shifts to lower energy due to the gradual growth of another component at 1360 cm^{-1}. Second, complicated alterations in the ν_3 region (1450–1520 cm^{-1}) occur. These include a drop in the intensity at 1505 cm^{-1} and an increase at 1495 cm^{-1} at a medium laser flux (~2×10^8 W/cm^2). [Unfortunately, 6c/1s ferrous heme and 5c/hs ferric heme exhibit the same position for ν_3 (see Table I).] As the laser flux is raised further (to ~3×10^8 W/cm^2), a weak ν_3 band develops at 1471 cm^{-1}. Finally, the bands in the 1520–1650-cm^{-1} region also vary as the laser flux is increased. For instance, the component at 1557 cm^{-1} slowly grows and ν_{10} at 1637 cm^{-1} for ferric low-spin heme disappears. These changes are all consistent with the photoreduction of the ferric heme and the creation of a photolabile 6c/1s ferrous species. It is noteworthy that no photoreduction was observed for HRP over a pH range of 7.30–8.50 (either in the position or half-width of ν_4) even when laser fluxes greater than ~2×10^8 W/cm^2 were used (Wang, 1992).

The photodynamic behavior of ferric CCP was further characterized by using both absorption and RRS spectroscopies. Figure 2 illustrates that, under anaerobic conditions, significant amounts of photoreduced CCP could be generated (at pH ~ 8.50) by pumping the sample with 408-nm excitation. This photoreduction evidently occurs with low yield but produces a measurable steady-state population of CCP(II). The accumulation of ferrous CCP bands in the absorption spectrum occurs chiefly as a result of the long ($\tau_{1/2}$ ~ 3 min) lifetime of the photoreduced species (see Figure 2B). Photoreduction of CCP(III) is also evident by the power-dependent behavior of ν_4 (Wang, 1992). At low powers, the low yield of CCP photoreduction prevents the net accumulation of measurable amounts of ferrous enzyme within the scattering volume. As the excitation flux is increased, photoreduction begins to compete with the rate of diffusion of ferric CCP into the scattering volume, and the ν_4 in the RR spectra for ferrous CCP at ~1360 cm^{-1} becomes evident.

In oxygenated solutions, the yield of photoreduced CCP(II) is reduced (see Figure 2B), but its lifetime remains about the same. When methyl viologen was added to aerobic samples of alkaline CCP(III) exposed to high laser flux (data not shown), however, most of the spectral changes associated with photoreduction either disappeared or were greatly diminished. The RR spectrum obtained with high laser flux conditions in the presence of MV^{2+}/O_2 [data not shown; see Wang (1992)] was quite similar to that obtained under anaerobic conditions at low laser flux (Figure 1c), exhibiting a narrow (~9 cm^{-1}) ν_4 at 1375 cm^{-1} and a predominant ν_3 at 1505 cm^{-1}. Thus it is unlikely that O_2 is simply binding to the ferric heme of alkaline CCP. Instead, O_2 most likely functions as the electron acceptor in the methyl viologen mediated oxidation of photoreduced cytochrome *c* peroxidase.

RRS investigations were also performed on the F^--bound CCP(III) at neutral and alkaline pHs (Wang, 1992). In the former, CCP(III) displays narrow ν_4 at 1372 cm^{-1} and ν_3 at 1478 cm^{-1}, clearly indicating the presence of a single 6c/hs species. This species exhibited no photolability at any pH used. However, as the pH was raised to 8.50, photoreduction was observed in the behavior of both ν_4 and ν_3 bands. Apparently, the heme ligation state has little influence on the photoreduction process of alkaline ferric CCP. It is noteworthy that the photodynamics of the low-spin, ferric CCP might be further complicated by photolysis of its sixth ligand. In order to address this, the power dependence of the CCP(III) in the presence of MV^{2+}/O_2 was examined (Figure 3). Under these conditions, the net photoreduction of CCP(III) is minimized, and the ligation state of the ferric species can easily be determined. No evidence for ligand photodissociation was found in CCP(III) at any pH.

Table II: Photodynamic Behavior of CCP at Neutral and Alkaline pHs

	pH = 4.50	pH = 7.00–7.30	pH = 8.50	pH = 9.70
CCP(II)				
equilibrium structure		~100% 5c/hs[a,b]	~25% 5c/hs, 75% 6c/ls[a,c]	~20% 5c/hs, ~80% 6c/ls[a]
photolysis		no[a,b]	significant increase in 5c/hs; generated by photolysis;[a,c] reversible within 1 μs[a,d]	no appreciable photolysis[a]
CCP(III)				
equilibrium structure	~100% 5c/hs[b,e]	~100% 5c/hs[b,e–h]	6c/ls is predominant[f,h]	~100% 6c/ls[f,g]
photoreduction	trace amount of photoreduction; reversible on millisecond time scale	small amount of photoreduction; reversible on millisecond time scale[e]	significant photoreduction; slowly reversible (lifetime ~3 min)[f]	significant photoreduction; irreverisible on millisecond time scale[f]
photolysis (ferric)			no ligand photolysis of ferric 6c/ls but significant ligand photolysis of photogenerated ferrous 6c/ls[f]	no ligand photolysis for either ferric or photogenerated ferrous 6c/ls heme[f]
HRP(III)				
photoreduction		no[f]	no[f]	

[a] Wang et al. (1992a). [b] Dasgupta et al. (1989). [c] Spiro et al. (1990). [d] Wang et al. (1992b). [e] Wang and Ondrias, unpublished data. [f] This work. [g] Hashimoto et al. (1986). [h] Smulevich et al. (1988a).

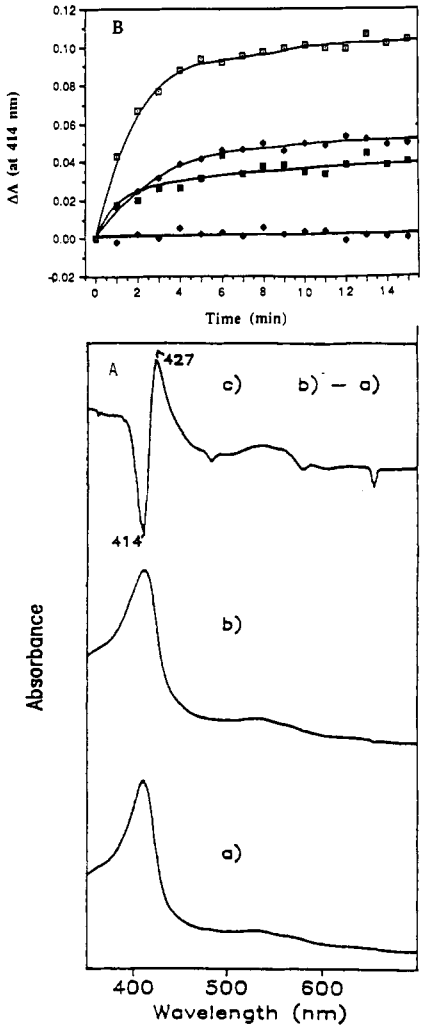


FIGURE 2: (Panel A) Absorption spectra of ferric cytochrome *c* peroxidase (~40 μm) obtained under anaerobic conditions (100 mM Tris, pH 8.5). Spectrum a represents the original CCP sample. Spectrum b was obtained immediately after photoreduction of the sample by high-power irradiation at 408 nm. Spectrum c is the normalized difference of (b) and (a). (Panel B) Kinetics of reoxidation of photoreduced CCP(III) monitored at 414 nm. CCP(III) (~10 μM) in 100 mM Tris, pH 8.5, under anaerobic conditions, ⊡; initial sample + O_2 (~1 mM), ◆; initial sample + methyl viologen (~1 mM), ■; initial sample + O_2 + methyl viologen, ◇.

pH Titration of the Photoreduction of Ferric Alkaline CCP. The pH dependence of CCP photodynamics was examined in some detail (see Figure 4). Great care was taken to ensure that all relevant parameters (concentration, cell configuration, laser flux, focusing optics, etc.) were the same for each sample. It is apparent that at least two photoinduced processes occur: (1) photoreduction of ferric CCP and (2) the subsequent binding and photodissociation of a strong-field ligand at the photoreduced heme (Wang et al., 1991, 1992a). Ferric CCP displays a complex series of spectral changes in the spin-state and ligation marker region (1450–1520 cm^{-1}) over the pH range of 7.30–9.10 (see Figure 4b). These changes can be divided into three parts: As the pH is raised from 7.30 to 8.20 (traces a–c), the behavior of ν_3 and ν_4 reflects both the spin-state conversion of the equilibrium heme species and its power-dependent photoreduction (see above). Further elevation of the pH from 8.20 to 8.80 (traces c–e), however, does not increase the signal at 1495 cm^{-1} even though the behavior of ν_4 shows that the net yield of photoreduction has increased (Figure 4a). Instead, an additional ν_3 band appears at 1471 cm^{-1}, a position characteristic of 5c/hs ferrous CCP. Finally,

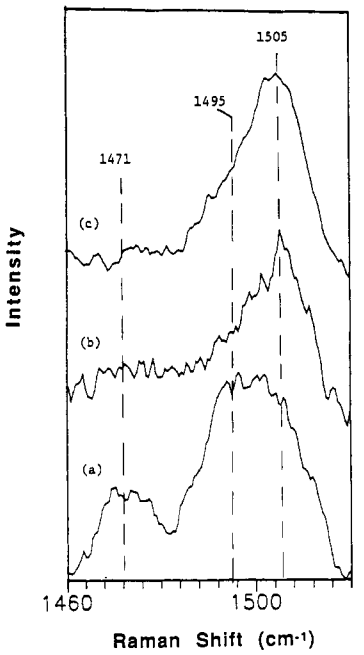


FIGURE 3: Resonance Raman spectra of fresh CCP(III) in 100 mM Tris-HCl, pH = 8.50, in ν_3 region. (a) CCP(III), with high (~3 × 10^8 W/cm^2) laser flux, and CCP(III)/Mv^{2+}/O_2, irradiated with high (b) and low (~1 × 10^7 W/cm^2) laser fluxes (c). Excitation wavelength, 410 nm (all other conditions the same as Figure 1).

as the pH is raised above 9.00 (Figure 4b,f), the intensity of the ν_3 band at ~1471 cm^{-1} significantly decreases, while those of the bands at ~1495 and ~1505 cm^{-1} remain constant. This indicates that, at these elevated pH values (pH > 9.00), the photoreduced CCP(II) exists mostly in the hexacoordinate, low-spin form.

DISCUSSION

pH Dependence of Equilibrium Ferric CCP. RRS spectra of ferric CCP obtained at pH 7.0 are completely consistent with the results of previous studies of CCP (Shelnutt et al., 1983; Dhaliwal & Erman, 1985; Hashimoto et al., 1986; Dasgupta et al., 1989) and establish that the heme is pentacoordinate and high-spin. Apparently, the water molecule located in the distal heme pocket by high-resolution crystallography (Finzel et al., 1984; Poulos & Finzel, 1984) is too far away from the heme (~2.4 Å) to influence its coordination state.

As the pH is raised, an equilibrium between 5c/hs (ν_3 ~ 1495 cm^{-1}) and 6c/1s (ν_3 ~ 1505 cm^{-1}) species is established. At pH > 9.40, the hemes are almost completely low-spin. Previous studies have demonstrate the existence of a pH-dependent transition in acidic CCP (pK_a ~ 5.5) that influenced the electron density at the heme (Shelnutt et al., 1983). This transition has little or no effect on the heme spin state (See Figure 1) and most probably perturbs the heme via its linkage to His-175. The alkaline transition observed in our data has a pK_a of 7.70 and is much more pervasive, producing wholesale changes in the equilibrium distal heme pocket and its dynamic behavior (see below). This pH-induced, spin-state alteration is spectroscopically distinct from the effects of "aging" the protein that were observed by Rousseau and co-workers (Dasgupta et al., 1989). Aged ferric CCP displays a mixture of 5c/hs, 6c/1s, and 6c/hs heme species even at neutral pH. The neutral–alkaline transition of CCP involves only 5c/hs and 6c/1s species. Analogous behavior has been observed for ferrous CCP (Wang et al., 1991, 1992a). The similarities in

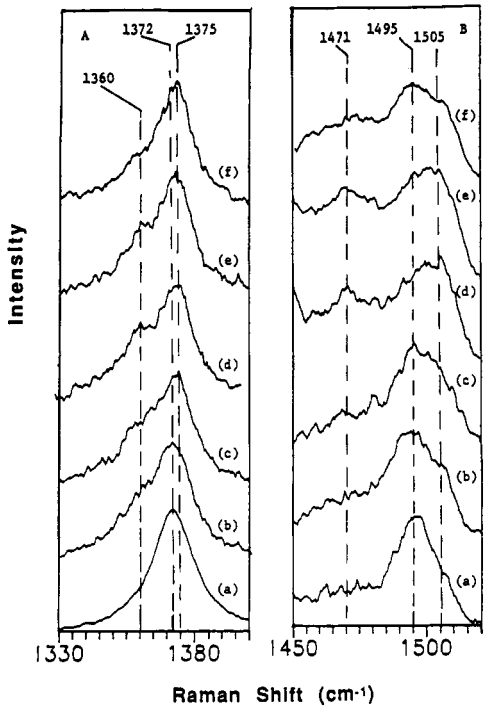


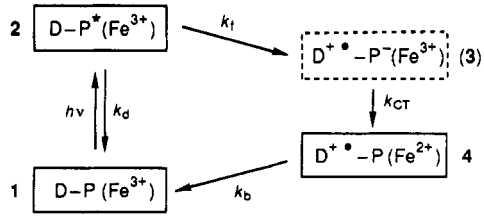
FIGURE 4: Photoreduction of CCP(III) as a function of pH measured by RRS in ν_4 (A) and ν_3 (B) regions. Samples were ~100 μM CCP(III) in 100 mM Tris-HCl at pH 7.30 (a), 7.90 (b), 8.20 (c), 8.50 (d), 8.80 (e), and 9.10 (f). Excitation was at 410 nm, with medium laser flux (~2×10^8 W/cm^2).

the pK_a for the high-spin to low-spin transition and the CD evidence for large-scale protein rearrangement in both ferrous and ferric CCP strongly suggest that similar pH-dependent protein changes are responsible for both. Interestingly, the conversion to the low-spin form is complete at pH 9.70 for the ferric enzyme, while ferrous CCP still displays a mixture of high-spin and low-spin hemes at that pH (Wang et al., 1992a,b). This indicates that the low-spin species is more stabilized (relative to its high-spin counterpart) in ferric CCP. Though the identity of the sixth ligand in ferric CCP cannot be unequivocally assigned, we suggest that His-52 is the most likely candidate.

Previous studies of an Asn-235 modified mutant of CCP in neutral pH also revealed the presence of 6c/1s heme (Smulevich et al., 1988; Satterlee et al., 1990; Wang et al., 1990b). Smulevich et al. (1991) have also observed that the replacement of His-52 by Leu significantly reduces the alkaline spin-state transition of the heme in CCP. Together, these results strongly suggest that His-52 is the distal low-spin ligand for alkaline CCP, and Asp-235 plays an extremely important role in retaining its hydrogen-bonding network in the proximal site of CCP heme pocket.

Photolability of Photoreduced CCP. The data obtained in this study indicate that the photodynamics of ferric CCP at high pH (>8.0) involve more than simple photoreduction of the heme. The behavior of ν_4 clearly shows that the equilibrium form of hexacoordinate ferric heme becomes photoreduced (see Figure 4A). However, the appearance of a ν_3 band at ~1471 cm^{-1} demonstrates that high-spin ferrous heme is created by photoexcitation (at least in pH > 8.0 solutions, See Figure 4B), while the flux-dependent increase in intensity at ~1495 cm^{-1} (Figure 1) indicates that ferric high-spin and/or ferrous low-spin hemes are also generated. In view of the demonstrated photolability of the ferrous hexacoordinate heme sites of alkaline CCP (Spiro et al., 1990; Smulevich et al., 1989, 1991; Wang et al., 1991, 1992a,b), rather complicated photodynamics involving both pH-dependent photoreduction and ligand photolysis of both ferric and ferrous species might be occurring.

Scheme I[a]



[a] D and P represent the endogenous donor and the porphyrin, respectively.

The heme of ferric CCP is evidently photoreducible irrespective of either its spin state or coordination number (see below). Thus, both high- and low-spin ferrous hemes might be generated via photoreduction of their ferric counterparts. Figure 3 (b and c) convincingly demonstrates that no significant ligand photolysis occurs within the laser excitation pulses for hexacoordinate ferric CCP at pH 8.50. This strongly suggests that the buildup of intensity at ~1495 cm^{-1} that occurs under high-flux conditions in the absence of MV^{2+} results from the creation of ferrous hexacoordinate, low-spin (not ferric pentacoordinate high-spin) hemes. This species is presumably generated by direct photoreduction of the hexacoordinate ferric sites. Under low- or medium-flux excitation (Figure 1b,c), little or on high-spin ferrous heme (ν_3 ~ 1471 cm^{-1}) is generated. We conclude that the appreciable intensity observed at 1471 cm^{-1} under high-flux conditions must result from ligand photolysis from the already photoreduced hexacoordinate, low-spin sites.

Photoreduction of Ferric Alkaline CCP: (a) General Considerations. Previous investigations have demonstrated that several heme proteins [Kitagawa et al., 1984; Wang et al. (1990a) and references therein] and metalloporphyrins [Fidler et al. (1991) and references therein] are photoreducible with visible light. In the majority of these cases, photoreduction occurs via excitation at or near the heme Soret transition and is irreversible under anaerobic conditions. Our data show that the photoreduction of ferric CCP is unique with respect to previously observed heme protein photoreduction. At pH < 7.30, only minimal photoreduction is observed when 408-nm excitation is used. Moreover, the small amount of photoreduction observed in acidic and neutral solutions is reversible on a millisecond time scale (Wang and Ondrias, unpublished results), in contrast to the photoreduction (with a half-life ~3 min) observed at higher pH values.

Our data clearly show that photoreduced CCP is relatively stable ($\tau_{1/2} \sim 10^2$ s) and its conversion back to the equilibrium ferric species is not influenced by O_2 in solution (except in the presence of a redox mediator). These observations strongly suggest that the photogenerated metastable ferrous species is created and destroyed via intramolecular electron transfer processes involving an endogenous electron donor. This can be depicted schematically as shown in Scheme I. The photoreduction process is initiated by the instantaneous generation of photoexcited metal–porphyrin complexes [species **2**: D–P*(Fe^{3+})]. These photoexcited states are short-lived and either relax back to the ground state or convert to species **3** and/or **4** by intramolecular electron transfer from a donor to the heme (with a rate of k_f). The last step involves the thermodynamically favored back-transport of the electron from the ferrous heme iron center to the donor.

The lack of an accumulation of species **4** in photoexcited CCP(III) samples at neutral pH indicates that (1) electron

transfer cannot effectively compete as a deactivation pathway for species **2** or (2) k_b is rapid on the ~70-ms repetition period of the excitation pulses. At higher pH values, this situation is altered and photoreduced CCP accumulates to a steady-state concentration of ≥10% within the illuminated volume under "high-power" conditions (see Results). Under these conditions, k_b is $\sim 4 \times 10^{-3}$ s^{-1}.

By using the previously published values (Dixon et al., 1985) for the deactivation rate of photoexcited ferric hemes ($t_{1/2} <$ 35 ps, $k_d \sim 10^{10}$ s^{-1}) as well as the electron back-transfer rate ($k_b \sim 4 \times 10^{-3}$ s^{-1}; see Figure 2B) recorded in our lab, we can estimate the quantum yield for the photoreduction of alkaline CCP(III):

$$(15\ s^{-1})\Phi[CCP(III)] = k_b[CCP(II)] \quad (1)$$

Under saturating conditions (i.e., photon flux ≫ [CCP(III)]) we assume that all CCP(III) molecules are excited at a rate of 15 s^{-1} (laser repetition rate). We further assume that k_{CT} (if applicable) ≫ k_f and estimate (from the high-power RR spectra) that [CCP(II)]/[CCP(III)] in the illuminated volume is ~25%. On the basis of these assumptions, a quantum yield, Φ, of 10^{-4}–10^{-5} and a value for k_f ($=\Phi k_d$) of 10^5–10^6 are obtained. Electron transfer rates from $\sim 3 \times 10^6$ s^{-1} to 3×10^1 s^{-1} have been observed (Nocera et al., 1984; Ho et al., 1985; McLendon & Miller, 1985; McLendon et al., 1985; Mayo et al., 1986; Liang et al., 1987, 1988; Durham et al., 1989; Meade et al., 1989; Hoffman et al., 1990; Everest et al., 1991; Chang et al., 1991; Wuttke & Gray, 1991) for intra- and interprotein ET within 17 Å. Thus, the estimated k_f rate involved in the photoreduction of alkaline CCP(III) is of reasonable magnitude.

(*b*) *Possible Mechanisms of Photoreduction.* The electron transfer dynamics of large biological systems is currently an area of considerable experimental activity. ET in heme proteins can be qualitatively modeled by using formalisms originally developed by Marcus and co-workers (Marcus & Sutin, 1985; Guarr & McLendon, 1985; Gray, 1986; Gray & Malmstrom, 1989; Hoffman et al., 1990; Beratan et al., 1991). For adiabatic, outer-sphere ET at fixed distances, the rate depends critically on the thermodynamic driving force ($\Delta G°$), the distance between donor and acceptor (r), and the reorganizational energy (λ):

$$k_{et} = Ae^{-\alpha(r-r_0)}\, e^{-(\Delta G° + \lambda)^2/4\lambda RT} \quad (2)$$

where T is temperature, $A \approx 10^{13}$, $r_0 \approx 3$ Å (the distance where ET becomes adiabatic), and distance dependence, α, depends on electron binding energy. To date, investigations of ET involving CCP have elegantly characterized the kinetics of long-range intermolecular ET between the catalytic site and either cytochrome *c* or exogenous electron donors (McLendon, 1988; Hoffman et al., 1990; Nocek et al., 1991; Tollin & Hazzard, 1991; Geren et al., 1991; Wang et al., unpublished results). In general, the intermolecular ET dynamics of CCP exhibit large reorganization energy ($\lambda \approx$ 1.0–1.5 eV) and distance dependence ($\alpha = 1.2$ Å^{-1}) with respect to other protein systems (McLendon & Miller, 1985, Fox et al., 1990).

The data obtained in our studies strongly suggest that existence of rapid intramolecular ET between the heme and an endogenous donor subsequent to photoexcitation of the heme. Clearly, the yield of photoreduced CCP depends on the relative magnitudes of k_d, k_{et}, and k_b. CCP is apparently unique (with respect to other heme proteins) in providing an electron transfer pathway that is competitive with heme electronic deactivation. It is noteworthy that the local heme environment of CCP is apparently designed to stabilize a cation radical in protein residues of the proximal pocket during the enzyme's catalytic cycle. Thus, the unique photodynamics of CCP may result, in part, from the ability of its heme pocket to stabilize a cation near the heme at alkaline pH. In this respect, the dynamics of photoreduction may be analogous to the proposed redistribution of electrons between the heme and protein residue(s) that must accompany the initial steps in the CCP catalytic mechanism (Mauro et al., 1988; Erman et al., 1989; Sivaraja et al., 1989).

The alkaline transition (with p$K_a \sim 7.7$) of CCP produces large changes in the protein secondary and tertiary structure (Wang et al., 1992a) that have been proposed to disrupt hydrogen-bonding interactions on the proximal (His-175) side of the heme. Recent studies of wild-type and site-directed mutants of CCP (Poulos & Finzel, 1984; Goodin et al., 1987; Mauro et al., 1988; Erman et al., 1989; Scholes et al., 1989; Sivaraja et al., 1989; Fishel et al., 1991) have convincingly demonstrated that Trp-191 is the site of the cation radical species observed in compound I. Furthermore, changing Asp-235 to Asn disrupts the proximal heme pocket hydrogen-bonding network, producing a hexacoordinate, low-spin heme (Smulevich et al., 1988; Spiro et al., 1990; Satterlee et al., 1990; Wang et al., 1990b) that exhibits significant photoreduction even at neutral pH (Wang et al., unpublished results). Mutation of Asp-235 to Asn also eliminates the broad component in the EPR signals of catalytic intermediate compound I. This has been interpreted to result from the reorientation of the Trp-191 indole ring and the loss of its H-bonding network mentioned above (Fishel et al., 1991). We suggest that native CCP(III) undergoes a pH-induced conformational reorganization similar to that occurring in the Asn-235 mutant enzyme at neutral pH.

Proximal heme pocket conformational reorganization could, in principle, produce a larger steady-state yield of photoreduced CCP by increasing k_f or decreasing k_b at high pH. However, a small amount of photoreduction is observed at neutral pH within high-flux ~10-ns excitation pulses (Wang et al., unpublished results). Thus, it appears that electron transfer to the heme is competitive with heme deactivation even at neutral pH. Only under alkaline conditions is k_b slow enough for the photoreduced species to reach an appreciable steady-state population. We therefore propose that the major effect of the alkaline protein transition is to inhibit the back-transfer from ferrous heme to the Trp-191 cation.

Heme pocket conformational perturbations of the magnitude exhibited by the CCP alkaline transition and Asp to Asn-235 mutation might be expected to affect k_b in several ways. Clearly, increasing either the heme to Trp-191 distance or the value of α will reduce $\exp[-\alpha(r - r_0)]$. Using Asn-235 as a model, it can be anticipated that r, the distance between heme and Trp-191, decreases as a result of rotation of the Trp-191 indole ring. Similarly, the net α of the CCP heme pocket might be expected to be quite sensitive to hydrogen-bonding interactions. However, any static changes in r or α should reduce both k_f and k_b proportionately (assuming the ET pathways are the same). Since the steady-state yield of photoreduction is ultimately related to k_{et}/k_b, it will be unaffected. This argues that changes activation energetics (i.e., $\exp[-(\Delta G° + \lambda)^2/4\lambda RT]$) must be responsible for the pH dependence of k_b.

The extremely low value for k_b dictates that $\Delta G°$ is either much more positive ("normal" region) or much more negative ("inverted" region) than λ. In view of the redox couples [Fe(II)/Fe(III) and Trp/Trp$^{\bullet+}$) involved, it is almost certain

that back-transfer occurs far into the inverted region. Thus, k_b is likely to be extremely sensitive to small, pH-induced changes in λ or the relative redox potentials of either redox couple. We speculate that the most likely cause for the inhibition of back-transfer kinetics is the thermodynamic destabilization of the Trp-191 cation.

In summary, the results of this work focused on the electron transfer dynamics of CCP(III) photoreduction at alkaline pH. We propose that the rapid reoxidation of photoreduced CCP at neutral pH is similar to the fast interconversion from the cation radical form to the oxyferryl form observed in CCP compound II (Coulson et al., 1971; Ho et al., 1983, 1984; Summers & Erman, 1988). Both occur via rapid electron transfer through the proximal hydrogen-bonding network involving His-175, Asp-235, and Trp-191. Mutation of Asp-235 to Asn and, by analogy, elevation in pH move the Trp-191 to an orientation nearer to, and parallel with, the porphyrin plane. We speculate that this pH-induced protein conformational rearrangement, especially the reorientation of Trp-191, significantly increases the activation energetics for the back electron transfer from the photoreduced heme center. As a result, the reoxidation kinetics of photoreduced alkaline CCP are dramatically slowed.

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