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Reaction of Ferrous Cytochrome *c* Peroxidase with Dioxygen: Site-Directed Mutagenesis Provides Evidence for Rapid Reduction of Dioxygen by Intramolecular Electron Transfer from the Compound I Radical Site†

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ABSTRACT: The reaction of dioxygen with the ferrous forms of the cloned cytochrome *c* peroxidase [CCP(MI)] and mutants of CCP(MI) prepared by site-directed mutagenesis was studied by photolysis of the respective ferrous–CO complexes in the presence of dioxygen. Reaction of ferrous CCP(MI) with dioxygen transiently formed a Fe^{II}–O_2 complex (bimolecular rate constant = $(3.8 \pm 0.3) \times 10^4$ M^{-1} s^{-1} at pH 6.0; 23 °C) that reacted further (first-order rate constant = 4 ± 1 s^{-1}) to form a product with an absorption spectrum and an EPR radical signal at $g = 2.00$ that were identical to those of compound I formed by the reaction of $CCP(MI)^{III}$ with peroxide. Thus, the product of the reaction of $CCP(MI)^{II}$ with dioxygen retained three of the four oxidizing equivalents of dioxygen. Gel electrophoresis of the $CCP(MI)^{II}$ + dioxygen reaction products showed that covalent dimeric and trimeric forms of CCP(MI) were produced by the reaction of $CCP(MI)^{II}$ with dioxygen. Photolysis of the $CCP(MI)^{II}$–CO complex in the presence of ferrous cytochrome *c* prevented the appearance of the cross-linked forms and resulted in the oxidation of 3 mol of cytochrome *c*/mol of $CCP(MI)^{II}$–CO added. The results provide evidence that reaction of $CCP(MI)^{II}$ with dioxygen causes transient oxidation of the enzyme by 1 equiv above the normal compound I oxidation state. Mutations that eliminate the broad EPR signal at $g = 2.00$ characteristic of the compound I radical also prevented the rapid oxidation of the ferrous enzyme by dioxygen. The Trp 191 → Phe, Gln, His and Asp 235 → Asn mutants readily formed a ferrous–dioxygen complex, but the rapid further reaction of this intermediate to an oxyferryl form was not observed. Instead, the Fe^{II}–O_2 enzymes returned slowly to the ferric state without detectable accumulation of intermediates (apparent rate constant = $(1 \pm 0.5) \times 10^{-4}$ s^{-1}). Other active site mutations did not prevent the rapid oxidation of the ferrous enzyme to compound I by dioxygen. The dramatic effects of mutations at Trp 191 and Asp 235 are interpreted as an indication that intramolecular electron transfer from the compound I radical site to the coordinated dioxygen ligand promotes the rapid oxidation of $CCP(MI)^{II}$ by dioxygen.

Cytochrome *c* peroxidase (cytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5; CCP[1]) is a monomeric heme protein that catalyzes the peroxide-dependent oxidation of ferrous cytochrome *c*. Although the normal catalytic cycle of CCP involves iron in the ferric and higher oxidation states, the enzyme can be reduced to the ferrous state under anaerobic conditions. The reactivity of ferrous CCP with dioxygen differs dramatically from that of other heme peroxidases. Photolysis of CCP^{II}–CO in the presence of dioxygen rapidly converts the enzyme to the oxyferryl state (Wittenberg et al., 1968). This product has been reported to be identical to compound I (or compound ES) produced by the reaction of the ferric enzyme with peroxide (Anni et al., 1985). In contrast, other ferrous

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[1] Abbreviations: CCP, cytochrome *c* peroxidase from bakers' yeast; CCP(MI), a cloned cytochrome *c* peroxidase expressed in *E. coli*; HRP, horseradish peroxidase; cyt *c*, cytochrome *c*; oxidation states of the respective enzymes are indicated by superscripts, i.e., $CCP(MI)^{II}$, ferrous CCP(MI); $trp^{\bullet}_{191}$; 1-N indolyl radical at Trp 191 of CCP; $por^{\bullet+}$, porphyrin π-cation radical; compound I, $CCP(MI)^{IV}{=}O(trp^{\bullet}_{191})$, the two-electron oxidation product of the reaction of ferric CCP(MI) with peroxide; compound I′, the product of $CCP(MI)^{II}$ oxidation by dioxygen; compound II_R, $CCP^{III}(trp^{\bullet}_{191})$, the transient product obtained by one-electron reduction of compound I by cyt c^{II}; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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peroxidases that have been characterized react with dioxygen under similar conditions to produce a Fe^{II}–O_2 complex (designated compound III) (Wittenberg et al., 1967; Renganathan et al., 1985) that returns slowly to the ferric state without detectable intermediates (Phelps et al., 1974; Tamura & Yamazaki, 1972).

The rapid conversion of $CCP(MI)^{II}$ to a higher oxidation state by dioxygen is atypical for a heme peroxidase, but it is not without precedent among ferrous heme proteins. Both heme oxidase and cytochrome oxidase form Fe^{II}–O_2 complexes that rapidly oxidize the respective enzymes to higher oxidation states, and in both cases the reaction involves reduction of the Fe^{II}–O_2 complex by an electron donor other than the heme. Further reaction of the Fe^{II}–O_2 complex of heme oxidase requires NADPH (Yoshida et al., 1980), while intramolecular electron transfer from Cu^{I} converts the Fe^{II}–O_2 complex of cytochrome oxidase to a peroxy intermediate that reacts further to produce the oxyferryl state (Wikstrom, 1989; Chan & Li, 1990; Han et al., 1990). By analogy, the rapid oxidation of CCP^{II} by dioxygen could be explained by the existence of an internal electron donor that promotes the rapid conversion of a transient Fe^{II}–O_2 complex to a higher oxidation state.

The presence of such an internal electron donor is indicated by the reaction of CCP^{III} with peroxide, which oxidizes the iron to the oxyferryl state and produces a free radical at Trp 191 (Edwards et al., 1987; Scholes et al., 1989; Sivaraja et al., 1989; Fishel et al., 1991; Yonetani et al., 1966; Erman & Yonetani, 1975; Hori & Yonetani, 1985). The rapid oxidation of Trp 191 during compound I formation indicates that a mechanism exists in CCP for rapid intramolecular transfer of one electron from the indole side chain of Trp 191 to the iron center. No equivalent donor exists in HRP, which instead reacts with peroxide to produce an oxyferryl iron center and a porphyrin π-cation radical that is stable for several minutes in solution (Dolphin et al., 1971). Thus, the two-electron oxidation of ferric HRP by peroxide removes both electrons from the heme.

Previous work has shown that intramolecular electron transfer from Trp 191 is eliminated when the indole ring of Trp 191 is replaced with a less readily oxidizable side chain in a cloned CCP [CCP(MI); Fishel et al., 1987]. The Trp 191 → Phe mutation prevents the formation of the broad EPR signal that is characteristic of $Trp^{\bullet}$ in compound I when the ferric enzyme is reacted with peroxide (Scholes et al., 1989). Instead of forming the compound I radical, the ferric Trp 191 → Phe enzyme reacts with peroxide to form an oxyferryl iron center and a transient porphyrin radical similar to that formed by the reaction of HRP^{III} with peroxide (Erman et al., 1989). A second mutation, Asp 235 → Asn, also prevents the formation of the compound I radical (Fishel et al., 1991). This substitution eliminates the interaction between the indole nitrogen of Trp 191 and the negatively charged carboxylate side chain of Asp 235 and causes the indole ring of Trp 191 to rotate 180° relative to its native orientation (Wang et al., 1990).

The present work examined the relationship between the ability of Trp 191 to serve as an electron donor in the reaction of the ferric enzyme with peroxide and the ability of the ferrous enzyme to undergo rapid autoxidation by dioxygen. The results indicate that while the ferrous forms of CCP(MI) and CCP(MI,F191) and CCP(MI,N235) all form a $CCP(MI)^{II}$–O_2 complex, conversion of this complex to form resembling compound I does not occur in the mutants that prevent formation of the compound I radical. The results suggest that rapid oxidation of $CCP(MI)^{II}$ by dioxygen is dependent upon intramolecular electron transfer from Trp 191 to the bound dioxygen.

Materials and Methods

Enzymes. The cloning and expression of the gene for yeast cytochrome *c* peroxidase in *Escherichia coli*, as well as detailed procedures for the introduction of single amino acid substitutions by site-directed mutagenesis, have been described (Fishel et al., 1987, and references therein). The cloned CCP(MI) and the mutant enzymes examined here were isolated from 15–20-L cultures of *E. coli*, purified, and converted completely to the holoenzyme by previously described techniques (Fishel et al., 1987; Mauro et al., 1988). Purified holoenzymes were recrystallized by exhaustive dialysis against H_2O and stored as crystalline suspensions in H_2O at −70 °C until use. Horse heart cytochrome *c* (Sigma, type VI) was used without further purification.

Reaction of Ferrous Enzymes with Dioxygen. Crystals of the enzymes were dissolved in 0.1 M potassium phosphate (pH 6.0). Enzyme concentrations were calculated from the molar extinction of the ferric enzymes at the Soret maximum, as determined by the pyridine hemochromogen method (Paul et al., 1953). The molar extinction coefficients for the ferric enzymes in 0.1 M potassium phosphate, pH 6.0, 23 °C, are CCP(MI), ϵ_{408} = 102 mM^{-1}; CCP(MI,F191), $\epsilon_{408.5}$ = 105 mM^{-1}; CCP(MI,H191), $\epsilon_{416.0}$ = 116 mM^{-1}; CCP(MI,Q191), $\epsilon_{414.0}$ = 119 mM^{-1}; and CCP(MI,N235), $\epsilon_{413.5}$ = 110 mM^{-1}. Ferric enzymes were converted to their Fe^{II}–CO derivatives by reduction with dithionite under 1 atm of CO as described (Miller et al., 1990). Following addition of dithionite, the Fe^{II}–CO enzymes were separated from other products of the dithionite reaction by passage over a Sephadex G-25 column (1 cm × 25 cm) equilibrated with air or dioxygen. The eluted Fe^{II}–CO enzymes were collected and diluted into buffer equilibrated with the required partial pressure of dioxygen or argon. These manipulations were conducted under conditions of reduced light to avoid photolysis of the CO complex and were complete within 15 min. Absorption spectra of the samples were recorded at 23 °C with a Perkin-Elmer Lambda 3b spectrophotometer before and after photolysis of the CO complex with a photographic flash. In some experiments, transient absorption spectra were recorded at 17-ms intervals after photolysis with a Princeton EG&G optical multichannel analyzer. For kinetic determinations, Fe^{II}–CO enzyme solutions equilibrated with 1 atm of dioxygen were diluted to 80 μM in a single 10-mL gas-tight syringe connected to a 2-mm path length observation cell. The dioxygen concentration was varied by dilution of the enzyme solution with appropriate volumes of buffers equilibrated with 1 atm of argon and/or dioxygen. The laser photolysis system utilized in these experiments has been described previously (Jongeward et al., 1988). Reactions were initiated with an output pulse of 16 ns width, and transient voltage changes were monitored at the appropriate wavelength. The sample in the cell was replaced by flow from the syringe between individual observations. Rate determinations at each wavelength represent the sum of 10–50 individual observations. The rate constants were derived by fitting the transient voltage traces with a nonlinear least-squares program.

Reductive Titrations with Cytochrome c. Cyt c^{II} was prepared as described previously (Fishel et al., 1987). The stoichiometry of cyt c^{II} oxidation by the product of the reaction of $CCP(MI)^{II}$ with dioxygen was determined essentially as described (Yonetani, 1965). Substoichiometric amounts of cyt c^{II} were added incrementally to the $CCP(MI)^{II}$ + O_2 product, and the absorption spectra were recorded. The initial

concentration of CCP(MI) was determined at Fe^{III}/Fe^{IV}=O isosbestic points near 454 nm (ϵ = 11.5 mM^{-1}) and 518 nm (ϵ = 11.5 mM^{-1}). Cyt c^{III} production was monitored as a linear increase in absorbance at these isosbestic points with incremental additions of cyt c^{II}. The end point of the titration was noted by a change in the slope of a plot of ΔA vs nmol of cyt c^{II} added and by the appearance of a peak at 550 nm, corresponding to excess cyt c^{II}. Spontaneous reduction of compound I was monitored in a parallel sample with no added cyt c^{II}. The stoichiometry of cyt c^{II} oxidation by the transient product of the dioxygen–ferrous CCP reaction was determined as follows: a stock solution of $CCP(MI)^{II}$–CO (prepared and equilibrated with air as described above) was diluted to a final concentration of up to 9 μM in cuvettes containing approximately 30 μM cyt c^{II} in 100 mM potassium phosphate, pH 6.0. After photolysis of the CO complex, the spectrum of the resulting mixture was recorded. The concentration of CCP(MI) was determined at Fe^{III}/Fe^{IV}=O isosbestic points near 454 nm and 518 nm in parallel samples with no added cyt c^{II}. The concentration of cyt c in each cuvette was taken as the mean of determinations at four cyt c^{II}/cyt c^{II} isosbestic wavelengths (near 502, 525, 540, and 556 nm), after the absorbance was subtracted from $CCP(MI)^{III}$. The absorbance due to cyt c^{II} + cyt c^{III} at 550 nm and the known [cyt c^{II}] + [cyt c^{III}] concentration were used to calculate net cyt c^{II} oxidation, assuming ϵ_{550} = 8 mM^{-1} for cyt c^{III}, and ϵ_{550} = 27.6 mM^{-1} for cyt c^{II}.

EPR Spectra of the $CCP(MI)^{II}$–Dioxygen Reaction Product. Crystals of the enzymes were thawed, and the crystalline suspension was placed under CO for 20 min. A small aliquot of sodium dithionite solution in 5 mM potassium phosphate (pH 6.0) was added to form the CO complex. The crystals were subsequently washed 4 times with 100 μL of anaerobic H_2O (4 °C) and finally dissolved in 100 μL of anaerobic 0.2 M potassium phosphate, pH 6.0. A small aliquot was removed to determine the concentration of the enzyme, and the ferrous–CO complex was mixed with an equal volume of air-saturated potassium phosphate (0.1 M; pH 6.0), placed in front of a 250-W tungsten/halogen lamp for 30 s, and immersed in liquid nitrogen. EPR spectra were recorded at 77K on a Varian E-3 spectrometer, operated at 9.15 GHz, with 100-kHz field modulation.

Results and Discussion

Rapid Oxidation of $CCP(MI)^{II}$ by Dioxygen. Photolysis of $CCP(MI)^{II}$–CO in the presence of dioxygen converted the enzyme to a product with an absorption spectrum that was essentially identical to that of compound I produced by the reaction of $CCP(MI)^{III}$ with peroxide (Figure 1); maxima in the visible region appeared at approximately 531 and 561 nm. Titration of the dioxygen + $CCP(MI)^{II}$ reaction product with cyt c^{II} revealed that 1.8 ± 0.1 equiv of cyt c^{II} was required to reduce the enzyme to the ferric state, as judged by changes in the visible absorption spectrum. This value represents a lower limit, since spontaneous conversion of the oxyferryl enzyme to the ferric form during the titration was approximately 10% in 30 min at 23 °C, on the basis of the changes in the absorption spectrum of the product in the absence of cyt c^{II}. EPR spectroscopy of the dioxygen + $CCP(MI)^{II}$ reaction product revealed the presence of a broad radical signal, centered at g = 2.00. The EPR spectrum of this product could not be distinguished from that produced by the reaction of $CCP(MI)^{III}$ with a 1.1-fold excess of hydrogen peroxide.

The stability of the $CCP(MI)^{II}$ + dioxygen reaction product under the present conditions was comparable to that of compound I produced by the reaction of the ferric enzyme with peroxide. When the column chromatography step prior to photolysis of the CO complex was omitted, however, the oxyferryl center of the dioxygen reaction product was rapidly reduced to the ferric state, as reported by others under similar conditions (Wittenberg et al., 1968; Anni et al., 1985). The column chromatography step apparently removes a product of the dithionite oxidation reaction that can rapidly reduce the oxyferryl center of CCP. The rapid reduction of the oxyferryl center of CCP–compound I by a product of dithionite oxidation was recently reported by Orii and Anni (1990), who found that the rate of reduction was proportional to the square root of dithionite concentration. This makes sulfite a likely candidate for the reductive species, since the 2 mol of SO_2 produced per 1 mol of dithionite oxidized will be rapidly converted to sulfite in aqueous solution. Preliminary results show that in the presence of a 10-fold molar excess of sodium sulfite $CCP(MI)$–compound I is reduced cleanly to the ferric enzyme; the reaction is complete in 6 min at 23 °C (not shown).

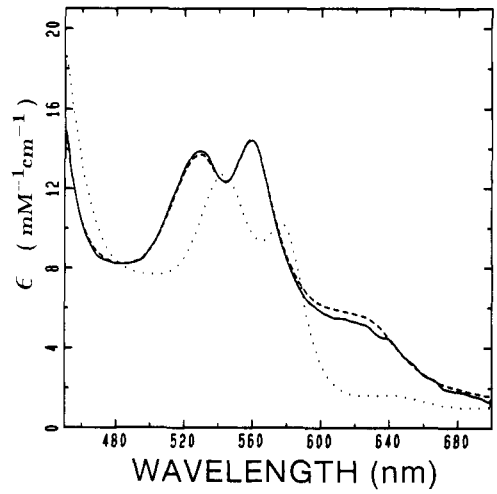

FIGURE 1: Absolute absorption spectra of reaction products of $CCP(MI)^{II}$ and $CCP(MI,F191)^{II}$ with dioxygen: reaction product of $CCP(MI)^{II}$ with dioxygen (solid line), maxima appear at 531 and 561 nm; reaction product of $CCP(MI,F191)^{II}$ with dioxygen (dotted line) maxima appear at 542 and 579 nm. Samples were prepared by photolysis of the Fe^{II}–CO complexes in 0.1 M potassium phosphate buffer, pH 6.0, 23 °C, as described under Materials and Methods. The absorption spectrum of CCP(MI)–compound I (dashed line), prepared by mixing equimolar amounts of hydrogen peroxide with ferric CCP(MI) under similar conditions, is also shown.

These observations confirm that $CCP(MI)^{II}$ is rapidly oxidized by dioxygen, as described by previous reports for bakers' yeast CCP (Wittenberg et al., 1968; Anni et al., 1985). The oxidation of $CCP(MI)^{II}$ by 2 equiv above the ferric state represents a net 3-equiv oxidation of the enzyme; this leaves 1 oxidizing equiv of dioxygen unaccounted for. Evidence presented below indicates that at least some fraction of the enzyme is modified by the transient presence of this extra equivalent during the reaction. For this reason, the product of the reaction of $CCP(MI)^{II}$ with dioxygen will be designated as compound I′ to distinguish it from the normal reaction product of the ferric enzyme with peroxide.

Oxidation of $CCP(MI)^{II}$ Proceeds by Further Reaction of a Ferrous–Dioxygen Complex. To further examine the mechanism of the reaction of $CCP(MI)^{II}$ with dioxygen, transient spectra were recorded after photolysis of $CCP(MI)^{II}$–CO in the presence of dioxygen. The changes in the absorption spectrum accompanying compound I′ formation indicate that two processes are involved. The initial process was characterized by decreasing absorbance at 440 and 560 nm and increasing absorbance at 416, 540, and 578 nm, with

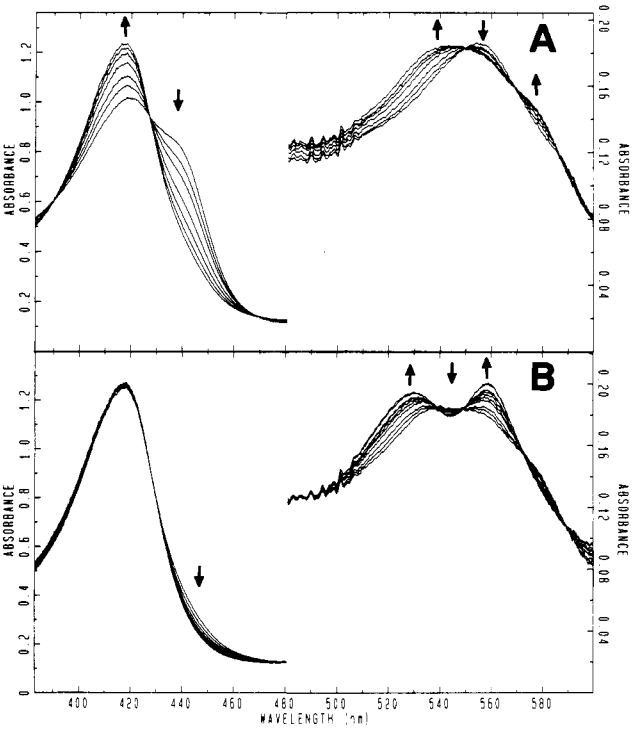

FIGURE 2: Transient absorption spectra recorded during the reaction of CCP(MI)^{II} with dioxygen. Spectra were recorded after photolysis of the CCP(MI)^{II}–CO complex at 23 °C as described under Materials and Methods. Direction of change in absorbance with time is indicated by the arrows. Panel A: t = 0.034, 0.051, 0.068, 0.102, 0.136, 0.17, and 0.204 s. Panel B: t = 0.26, 0.31, 0.37, 0.46, 0.54, 0.63, 0.73, 0.85, 1.7, and 5.1 s.

Table I: Absorption Maxima of Fe(II)–O_2 Complexes of CCP(MI) Mutants Compared with Those of Other Fe(II)–O_2 Heme Proteins

enzyme	Soret	β	α
CCP(MI,F191)[a]	417.4 (117)	545 (11.9)	579 (9.6)
CCP(MI,Q191)[a]	417.9 (119)	544 (12.2)	580 (10.6)
CCP(MI,H191)[a]	416.9 (118)	544 (12.3)	578 (10.9)
CCP(MI,N235)[a]	418.5 (117)	545 (13.2)	580 (11.5)
HRP*[b]	417 (108)	543 (11.5)	577 (10.0)
LiP*[c]	414 (106)	543 (8.8)	578 (7.6)
Hb(human)[d]	415 (125)	541 (13.8)	577 (14.6)
Hb(ascaris)[e]	412 (110)	542 (12.3)	577 (10.4)
Mb(sperm whale)[d]	418 (128)	543 (13.6)	581 (14.6)

[a] Present work. Complexes were prepared by photolysis of the respective Fe^{II}–CO complexes in 0.1 M potassium phosphate, pH 6.0, as described under Materials and Methods. [b] Wittenberg et al., 1967. [c] Wariishi & Gold, 1990. [d] Antonini & Brunori, 1971. [e] Wittenberg et al., 1965.

isosbestic points at 550 nm, 569 nm, and 586 nm (Figure 2A). These changes are typical for coordination of dioxygen to a pentacoordinate ferrous heme protein (Table I). The second process was characterized by a small increase in the intensity of the Soret band, decreasing absorbance at 540 and 578 nm, and increasing absorbance at 530 and 560 nm, the maxima characteristic of oxyferryl CCP(MI), with isosbestic points at 538, 549, 573, and 589 nm (Figure 2B). The clean isosbestic points for both processes indicate that additional intermediates do not accumulate during the reaction. A minimal mechanism that can account for these observations is formulated in the equation

$$\text{CCP(MI)}^{II} + O_2 \xrightarrow{k_1} \text{CCP(MI)}^{II}\text{–}O_2 \xrightarrow{k_2} \text{CCP(MI)}^{IV}\text{=O(trp}^{\bullet}_{191}\text{)} + O^{\bullet -} \quad (1)$$

The first reaction is written as essentially irreversible, on the basis of the observation that CO does not replace dioxygen in mutants that form a stable Fe(II)–O_2 complex (see below). The symbol $O^{\bullet -}$ is included to indicate the fourth reducing equivalent of dioxygen. The fate of this equivalent will be discussed below.

The progress of the first reaction was monitored at 435 nm, where the change in absorbance due to conversion of CCP(MI)^{II} to CCP(MI)^{II}–O_2 is large and the change due to the subsequent reaction to give the oxyferryl form is minimal (Figure 2). The initial reaction of CCP(MI)^{II} with dioxygen monitored at this wavelength was fit well by a single-exponential function under pseudo-first-order conditions with respect to dioxygen. The observed rate was linearly dependent on dioxygen concentration. The bimolecular rate constant $k_1 = (3.8 \pm 0.3) \times 10^4$ M^{-1} s^{-1} at 23 °C was obtained from the slope of the plot of k_{obs} versus dioxygen concentration, while the y-intercept of this plot was within an experimental error of zero.

The progress of both reactions was monitored at 455 nm, where changes in absorbance due to formation of CCP(MI)^{II}–O_2 from CCP(MI)^{II} and its subsequent conversion to compound I′ are approximately equal and of the same sign, and at 620 nm, where the oxyferryl form of CCP(MI) has a significant absorbance but other species in the reaction mixture do not. The concentrations of CCP(MI)^{II}–O_2 and compound I′ produced by the sequential mechanism suggested above are given by

$$[\text{CCP(MI)}^{II}\text{–}O_2] = [\text{CCP(MI)}^{II}]_0 \frac{k'_1}{k_2 - k'_1}(e^{-k'_1 t} - e^{-k_2 t}) \quad (2)$$

and

$$[\text{compound I}'] = [\text{CCP(MI)}^{II}]_0 \left(1 - e^{-k'_1 t} - \frac{k'_1}{k_2 - k'_1}(e^{-k'_1 t} - e^{-k_2 t})\right) \quad (3)$$

where $[\text{CCP(MI)}^{II}]_0$ is the initial concentration of CCP(MI)^{II}, and the pseudo-first-order rate constant $k'_1 = k_1[O_2]$.

The observed change in absorbance at 455 nm can be described by

$$A_t - A_\infty = (\Delta\epsilon_1[\text{CCP(MI)}^{II}]_t + \Delta\epsilon_2[\text{CCP(MI)}^{II}\text{–}O_2]_t) \quad (4)$$

where A_t and A_∞ are the absorbance at time t and at the completion of the reaction, respectively; $[\text{CCP(MI)}^{II}]_t = [\text{CCP(MI)}^{II}]_0 e^{-k'_1 t}$; $[\text{CCP(MI)}^{II}\text{–}O_2]_t$ is given by eq 2; $\Delta\epsilon_1$ and $\Delta\epsilon_2$ represent $(\epsilon_{\text{CCP(MI)}^{II}} - \epsilon_{\text{compound I}'})$ and $(\epsilon_{\text{CCP(MI)}^{II}\text{–}O_2} - \epsilon_{\text{compound I}'})$, respectively. Since the change in absorbance at 620 nm is due only to compound I′ formation, the data could be fit according to eq 3. On the basis of the data at 455 and 620 nm, a rate constant $k_2 = 4 \pm 1$ s^{-1} was derived for conversion of CCP(MI)^{II}–O_2 at compound I′. The rate of the second process was independent of dioxygen concentration in the concentration range examined here.

Rapid Conversion of CCP(MI)^{II}–O_2 to Compound I′ Is Eliminated by Mutations at Trp 191 and Asp 235. Photolysis of the CO derivative of CCP(MI,F191)^{II} in the presence of dioxygen gave a product with an absorption spectrum that is clearly distinct from that of compound I′ (Figure 1); this product did not react further with cyt c^{II}. A similar product, with maxima at approximately 417, 545, and 579 nm, was also obtained by photolysis of the CO derivatives of CCP(MI,Q191)^{II}, CCP(MI,H191)^{II}, and CCP(MI,N235)^{II}, in the presence of dioxygen. The absorption spectrum of the product formed by the reaction of these mutants with dioxygen is nearly identical to that reported for the Fe^{II}–O_2 complex (compound III) of HRP and other (imidazole)(O_2)-ligated protoheme proteins (Table I). The changes in the absorption

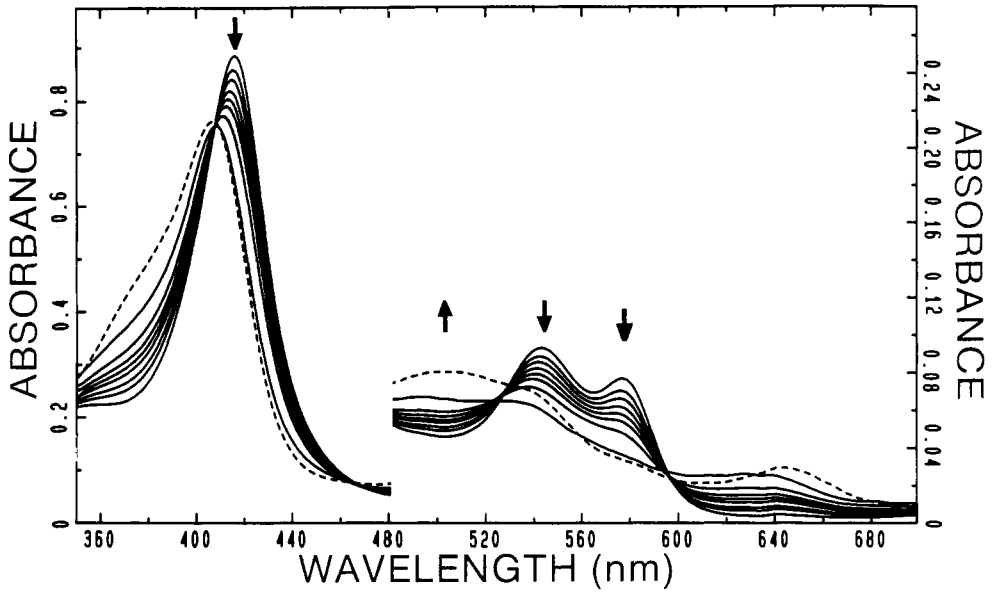

FIGURE 3: Autoxidation of CCP(MI,F191)^{II}–O_2. The solid lines represent spectra recorded at 1, 12, 25, 50, 76, 119, 183, 216, and 2136 min after photolysis of the CCP(MI,F191)^{II}–CO complex in the presence of dioxygen at pH 6.0, as described under Materials and Methods. The direction of change in absorbance is indicated by arrows. The initial spectrum of CCP(MI,F191)^{III} is indicated by the dashed line. The protein concentration was 6.2 μM.

spectrum that accompany the reaction of CCP(MI,F191)^{II} with dioxygen closely resemble the changes that accompany the initial reaction of CCP(MI)^{II} with dioxygen. The similarity between the absorption spectra of the CCP(MI,F191)^{II} + O_2 product and the transient CCP(MI)^{II}–O_2 intermediate is indicated by the similar isosbestic wavelengths for each of these products and CCP(MI)–compound I′ (540, 547, 575, and 590 nm, Figure 1; Figure 2B). On the basis of these observations, it is concluded that the mutations at Trp 191 and Asp 235 produce a Fe^{II}–O_2 complex that does not react further to produce compound I′.

Distal mutations Arg 48 → Lys, Trp 51 → Phe, and His 52 → Leu did not prevent the rapid further reaction of the Fe^{II}–O_2 complex, however. Absorption spectra of these mutants recorded 2 min after photolysis indicated the presence of a mixture of Fe^{III} and Fe^{IV}=O forms. The changes in the spectra with time indicated an isosbestic increase in the ferric form at the expense of the oxyferryl with increased time of incubation for each of these mutants. Similar changes in the spectra were observed following the reaction of the respective ferric enzymes with peroxide (Fishel et al., 1987; M. Miller and J. M. Mauro, unpublished observations).

Properties of CCP(MI,F191)^{II}–O_2. The reaction of CCP(MI,F191)^{II} with dioxygen was further characterized. After photolysis of the CCP(MI,F191)^{II}–CO complex, the change in absorbance with time at 435 and 455 nm fit well to a single-exponential function under pseudo-first-order conditions with respect to dioxygen and the apparent rate was linearly dependent on dioxygen concentration. The dependence of k_{obs} for reaction of CCP(MI,F191)^{II} with dioxygen gave a bimolecular rate constant k_1 = (8.6 ± 0.4) × 10^4 M^{-1} s^{-1}; this represents an approximately 2-fold increase in the apparent rate of dioxygen binding with respect to CCP(MI).

The CCP(MI,F191)^{II}–O_2 complex formed under these conditions returned slowly to the ferric enzyme as judged by the changes in the absorption spectrum (Figure 3). The constant isosbestic points indicate that no significant accumulation of intermediates occurs during this reaction. The autoxidation of CCP(MI,F191)^{II}–O_2 followed single-exponential kinetics, and a unimolecular rate constant k_{ox} = (1 ± 0.2) × 10^{-4} s^{-1} was derived from plots of ln $[A_0 - A_\infty/A_t - A_\infty]$ vs time. The apparent rate of autoxidation was independent of dioxygen concentration and was not increased by the presence of a 100-fold molar excess of CN^-. The rate of conversion of the dioxygen product to the ferric state was increased approximately 10-fold when the column chromatography step was omitted prior to photolysis. No conversion of the dioxygen reaction product to the CO adduct was detected under 1 atm of CO during the lifetime of the complex. No photolysis of the CCP(MI,F191)^{II}–dioxygen reaction product was detected in time regimes of 100 μs or greater. These characteristics are similar to those reported for HRP^{II}–O_2 (Wittenberg et al., 1967; Tamura & Yamazaki, 1972; Makino et al., 1976).

The absorption spectrum of the autoxidation product of CCP(MI,F191)^{II}–O_2 was not identical to that of the original ferric enzyme (Figure 3). Compared to the resting ferric enzyme, the final product was characterized by a loss in absorbance at 390 nm and blue shifts of the bands at 506 and 645 nm to 495 and 630 nm, respectively. In contrast, no significant changes were observed in the absorption spectrum of resting CCP(MI,F191)^{III} during incubation under similar conditions for up to 24 h. The changes in the absorption spectrum suggest that autoxidation increases the fraction of the ferric enzyme present in the hexacoordinate, high-spin form at the expense of the pentacoordinate, high-spin form (Yonetani & Anni, 1987; Smulevich et al., 1988). The spectrum of the autoxidation product (Figure 3) was essentially identical to that of the ferric His 181 → Gly mutant of CCP(MI) at pH 6.0 (Miller et al., 1988); this mutation causes a significant increase in the fraction of the enzyme present as the hexacoordinate, high-spin form relative to CCP(MI) (Smulevich et al., 1991).

The absorption spectra of the Fe^{II}–O_2 complexes formed by CCP(MI,N235), CCP(MI,Q191), and CCP(MI,H191) also underwent slow changes consistent with conversion to the respective ferric enzymes. During autoxidation of these enzymes, a nonisosbestic loss of absorbance in the Soret region was evident after approximately 2 h, making the rate constant for the reaction difficult to establish with precision. On the basis of the initial rate of disappearance of the bands at 542 and 578 nm, the rate of autoxidation of these mutants was

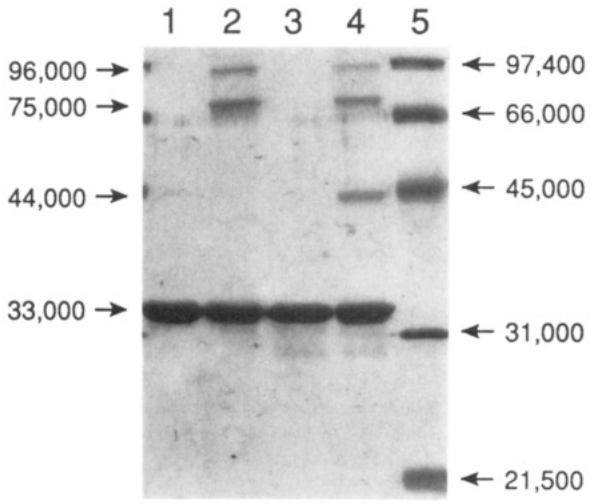

FIGURE 4: SDS–PAGE of the reaction products of CCP(MI)^{II} with dioxygen. Solutions of CCP(MI)^{III} or CCP(MI)^{II}–CO were prepared in the presence of dioxygen and diluted to 10 μM in the presence or absence of cytochrome *c*. After photolysis, aliquots were withdrawn from each reaction mixture and were mixed with an equal volume of reducing buffer. Individual wells on 14% acrylamide gels were loaded with 3.4 μg of CCP(MI) of the resulting solution and were run under reducing conditions. The gels were fixed and stained with Coomassie Brilliant Blue R. Lane 1: 10 μM CCP(MI)^{III} + 10 μM HOOH. Lane 2: 10 μM CCP(MI)^{II}–CO. Lane 3: 10 μM CCP(MI)^{II}–CO + 40 μM cyt c^{II}. Lane 4: 10 μM CCP(MI)^{II}–CO + 40 μM cyt c^{III}. Lane 5 contains molecular weight standards: phosphorylase *b* (97 400); bovine serum albumin (66 200); ovalbumin (45 000); carbonic anhydrase (31 000); and soybean trypsin inhibitor (21 500).

estimated to be within 50% of that observed for CCP(MI,F191).

Evidence for a Transient Intermediate That Retains All of the Oxidizing Equivalents of Dioxygen. The conversion of CCP(MI)^{II} to compound I′ requires only three oxidizing equivalents, indicating that one of the four oxidizing equivalents of dioxygen is rapidly reduced during this reaction. To determine whether or not a transient intermediate was formed that retained all of the equivalents of dioxygen, photolysis of CCP(MI)^{II}–CO was conducted in the presence of excess cyt c^{II} and dioxygen. Under these conditions, oxidation of cyt c^{II} was linearly dependent on the concentration of CCP(MI)^{II}–CO, and 3.2 ± 0.2 mol of cyt c^{II} was oxidized per 1 mol of CCP(MI)^{II}–CO converted to the ferric state. This result indicates that CCP(MI) can catalyze the quantitative reduction of three oxidizing equivalents of dioxygen by cyt c^{II}.

Conversion of CCP(MI)^{II} to Compound I′ Is Accompanied by Cross-Linking. Tew and Ortiz de Montellano (1988) showed that when metmyoglobin reacts with peroxide, 1 oxidizing equiv of peroxide converts the ferric iron to the oxyferryl (Fe^{IV}=O) state and the second equivalent is rapidly dissipated, producing radicals at surface Tyr residues that can ultimately form intermolecular dityrosine cross-links. To determine whether the rapid loss of 1 equiv of dioxygen during conversion of CCP(MI)^{II} to compound I′ also causes an intermolecular cross-linking reaction, the products of the dioxygen + CCP(MI)^{II} reaction were analyzed by SDS–PAGE (Figure 4). The results show that approximately 5–10% of the CCP(MI) present initially is converted to bands corresponding to dimeric (apparent MW ~ 74 000) and trimeric (apparent MW ~ 97 000) forms of the enzyme during the reaction of CCP(MI)^{II} with dioxygen (lane 2). The higher molecular weight bands were eliminated when photolysis was performed in the presence of 4 equiv of cyt c^{II} (lane 3) but not in the presence of an equivalent concentration of cyt c^{III} (lane 4). In the presence of cyt c^{III}, the appearance of an additional band (apparent MW ~ 45 000) was observed (lane 4), corresponding to the predicted molecular weight of a covalent CCP–cyt *c* heterodimer.

Previous results have shown that covalently cross-linked dimeric and trimeric forms of CCP are produced when the oxyferryl center of CCP–compound I returns slowly to the ferric state in the absence of added reductant (Spangler & Erman, 1986; Erman & Yonetani, 1975). This cross-linking reaction apparently results from the reduction of the oxyferryl center by an endogenous electron donor. The appearance of covalent dimeric and trimeric forms of CCP(MI) under the present conditions does not result from the endogenous reduction of compound I, however, since cross-linking of CCP(MI) was not observed when equimolar amounts of hydrogen peroxide and CCP(MI)^{III} were mixed under similar conditions (lane 1).

Mechanism of Conversion of CCP(MI)^{II}–O_2 to Compound I′. The results show that (1) the rapid conversion of CCP(MI)^{II}–O_2 to compound I′ is prevented by mutations at Trp 191 and Asp 235; mutations at these positions cause the Fe^{II}–O_2 complex to undergo slow autoxidation that is more characteristic of other heme peroxidases; (2) distal mutations at His 52 and Arg 48 do not prevent the rapid further reaction of CCP(MI)^{II}–O_2; (3) 1 equiv of dioxygen is reduced rapidly during the conversion of CCP(MI)^{II} to compound I′; the reduction of this equivalent is accompanied by the appearance of covalently linked CCP(MI) dimers and trimers; and (4) the "lost" equivalent of dioxygen can be reduced by cyt c^{II} prior to the covalent cross-linking of the enzyme. These observations can be explained by a reaction sequence involving electron transfer from Trp 191 to dioxygen followed by heterolysis of the O–O bond, as represented below:

$$\text{CCP(MI)}^{II} + O_2 \rightarrow \text{CCP(MI)}^{II}\text{–}O_2 \quad (5)$$

$$\text{CCP(MI)}^{II}\text{–}O_2 + H^+ \rightleftarrows \text{CCP(MI)}^{III}(OOH^-)(trp^{\bullet}_{191}) + H^+ \quad (6)$$

$$\text{CCP(MI)}^{III}(OOH^{-1})(trp^{\bullet}_{191}) + H^+ \rightarrow \text{CCP(MI)}^{IV}{=}O(por^{\bullet+})(trp^{\bullet}_{191}) + H_2O \quad (7)$$

$$\text{CCP(MI)}^{IV}{=}O\ (por^{\bullet+})(trp^{\bullet}_{191}) \rightarrow \text{CCP(MI)}^{IV}{=}O(trp^{\bullet}_{191})(R^{\bullet}_{x}) \quad (8)$$

The initial reaction of CCP(MI)^{II} with dioxygen produces the Fe^{II}–O_2 complex (eq 5). The coordinated dioxygen is subsequently reduced to the level of the peroxide anion through the contribution of one electron from the ferrous iron and a second electron from the indole side chain of Trp 191 (eq 6). The reaction represented in eq 6 assumes that the coordinated peroxy ligand is protonated (from solvent or an internal proton donor) and that the indole nitrogen loses a proton following oxidation, on the basis of the apparent pK_a = 4.3 for the indolyl radical in solution (Posener et al., 1976).[2] Heterolytic cleavage of the O–O bond of the Fe^{III}(OOH$^-$) intermediate when Trp 191 is already oxidized would be expected to produce an oxyferryl iron center and a transient porphyrin π-cation radical (eq 7). The porphyrin radical formed by this reaction could be reduced by one or more endogenous donors to produce

[2] The protons required to balance eqs 6 and 7 may not be taken up from (and released to) bulk solvent. For example, the oxidation of CCP^{II} to CCP^{III} is accompanied by loss of a proton (Conroy et al., 1978), suggesting that the enzyme may supply a proton to the peroxy ligand during the electron transfer step. On the other hand, oxidation of Trp 191 does not cause a net loss of a proton from the enzyme (Conroy & Erman, 1978), suggesting that this proton may simply be transferred to the carboxylate side chain of Asp 235.

a transient intermediate with a protein-based radical at a second enzyme moiety (eq 8). The protein-based radical is short-lived, and the loss of this oxidizing equivalent apparently proceeds by several mechanisms, ultimately producing the heterogeneous enzyme population, designated compound I′, that is oxidized by two equivalents above the ferric state.

The initial reaction of $CCP(MI)^{II}$ with dioxygen is comparable to that observed for HRP^{II}. The bimolecular rate constants for the reaction of $CCP(MI)^{II}$ (3.8×10^4 M^{-1} s^{-1}) and HRP^{II} (5.7×10^4 M^{-1} s^{-1}) with dioxygen and the absorption spectra of the Fe^{II}–O_2 products are comparable under similar conditions (Wittenberg et al., 1967; Phelps et al., 1974). The stability of the Fe^{II}–O_2 complex is dramatically different between these two enzymes, however. The unimolecular rate constant for conversion of the Fe^{II}–O_2 complex to compound I′ is 4 s^{-1} at pH 6.0, 23 °C, while autoxidation of HRP^{II}–O_2 to the ferric enzyme proceeds with a unimolecular rate constant of approximately 3×10^{-4} s^{-1} under comparable conditions (Makino et al., 1976). Thus, the half-life of the Fe^{II}–O_2 complex formed by CCP(MI) is decreased by 12000-fold relative to the analogous complex formed by HRP.

The proposed reaction sequence predicts that the dramatic decrease in the lifetime of $CCP(MI)^{II}$–O_2 relative to that of HRP^{II}–O_2 reflects the rapid intramolecular electron transfer from Trp 191 to the coordinated dioxygen. The ability of one-electron donors to decrease the lifetime of Fe^{II}–O_2 complexes formed by heme proteins is well-known. The stability of Fe^{II}–O_2 complexes formed by heme proteins such as hemoglobin and HRP results from the unfavorable energetics of one-electron reduction of dioxygen and the slow rate of dissociative loss of superoxide from Fe^{II}–O_2 complexes (George, 1965; Wallace et al., 1974; Satoh & Shikama, 1981; Wallace et al., 1982). These factors make the autoxidation of Fe^{II}–O_2 complexes by one-electron reduction of dioxygen slow. The rate of heme protein autoxidation is increased significantly by one-electron donors, which link one-electron oxidation of the iron to the more favorable two-electron reduction of dioxygen to give an Fe^{III}–OOH complex. The peroxy ligand can dissociate to give peroxide and ferric heme (Wallace & Caughey, 1975; Kawanishi & Caughey, 1985) or react further by cleavage of the O–O bond (Tamura & Yamazaki, 1972).

The transfer of an electron from Trp 191 to the coordinated dioxygen will be promoted by both proximal and distal features of CCP(MI). The proximal environment of Trp 191 apparently decreases the redox potential of the indole side chain significantly relative to Trp in solution. Trp 191 is rapidly oxidized by the reaction of the ferric enzyme with peroxide, and the radical at Trp 191 is stable for several hours at room temperature, despite its location in van der Waals contact with the heme and in proximity to a number of Tyr residues (Yonetani et al., 1966, Erman & Yonetani, 1975; Finzel et al., 1984). Thus, the Trp 191 radical does not readily oxidize nearby Tyr side chains, as would be expected for these species in solution (Merenyi et al., 1988). The anomalous stability of the Trp 191 radical may result in part from the interaction between the Asp 235 carboxylate anion and the indole nitrogen of Trp 191, which will stabilize the developing positive charge on the indole ring and provide an acceptor for the proton released by Trp 191 oxidation. The dramatic increase in the lifetime of $CCP(MI)^{II}$–O_2 when Asp 235 is mutated to Asn provides evidence that the environment of the Trp 191 side chain exerts a strong influence on its ability to serve as an electron donor.

The distal heme environment of CCP(MI) promotes the transfer of electron density from the iron to coordinated ligands (Smulevich et al., 1988). This effect can be attributed to the presence of the positively charged guanidinium side chain of Arg 48 and the imidazole side chain of His 52, both of which can form hydrogen bonds with the bound ligand. These interactions should promote electron transfer from Trp 191 to dioxygen by stabilizing the increased negative charge on the dioxygen ligand. Mutations at His 52 and Arg 48 do not prevent the rapid further reaction of the Fe^{II}–O_2 complex, however, indicating that neither residue is required for electron transfer. The influence of these residues on the rate of conversion of $CCP(MI)^{II}$–O_2 to compound I′ remains to be evaluated.

Despite the features that promote the transfer of an electron from Trp 191 to dioxygen, it is unlikely that this reaction proceeds spontaneously. In fact, the Fe^{IV}=O form of CCP is more stable than the $Fe^{III}(trp^{\bullet}_{191})$ form (Ho et al., 1983; Summers & Erman, 1988), indicating that the equilibrium for the electron transfer from Trp 191 to the Fe^{II}–O_2 complex (eq 6) probably lies far to the left. The reaction can proceed to completion, however, if formation of the $Fe^{III}(OOH^-)$ intermediate is followed by rapid heterolytic cleavage of the O–O bond (eq 7). In this way, endothermic electron transfer from Trp 191 to dioxygen can be driven by the essentially irreversible cleavage of the O–O bond.

The proposed reaction sequence predicts that the heterolytic cleavage of the O–O bond occurs following oxidation of Trp 191. This reaction would be analogous to the reaction of peroxide with ferric CCP(MI,F191), a mutant that lacks a stable radical site. The porphyrin radical formed by the reaction of $CCP(MI,F191)^{III}$ with peroxide (Erman et al., 1989) is rapidly reduced by an endogenous donor; the rate constant for this reaction is 51 s^{-1} at 25 °C. A similar reaction would be expected for heterolysis of peroxide by $CCP(MI)^{III}$ when Trp 191 is already oxidized. Since the rate of reduction of the porphyrin radical in CCP(MI,F191) is approximately 10-fold faster than the rate of conversion of $CCP(MI)^{II}$–O_2 to compound I′, this intermediate would not be detected in the transient spectra.

The reduction of the porphyrin radical by an endogenous electron donor(s) would produce a protein-based radical at an unspecified amino acid chain(s). The oxidized side chain(s) would be capable of reaction to form covalent intermolecular cross-links or destruction by undetermined mechanisms. Tew and Ortiz de Montellano (1988) have provided precedent both for the formation of intramolecular dityrosine cross-linkages and for the destruction of Tyr residues as a result of the formation and endogenous reduction of a porphyrin radical following the reaction of metmyoglobin with peroxide. A similar mechanism would explain both the loss of 1 oxidizing equiv of dioxygen and the appearance of covalent CCP(MI) dimers during the conversion of $CCP(MI)^{II}$–O_2 to compound I′, where the formation of covalent CCP(MI) trimers may result from a free-radical polymerization reaction. It is interesting to note that when analyzed by SDS–PAGE, the products of the CCP(MI,F191) + peroxide reaction in the presence and absence of cyt c^{II} are identical to those observed during the reaction of $CCP(MI)^{II}$ with dioxygen (M. Miller, unpublished observation). Thus, the rapid loss of 1 oxidizing equiv from the enzyme and formation of covalent intermolecular cross-links accompany the formation of a porphyrin radical in CCP(MI) and its reduction by an endogenous donor (such as the phenolic side chain of a Tyr residue).

The proposed reaction sequence predicts that the conversion of $CCP(MI)^{II}$–O_2 to compound I′ will proceed via one or more intermediates that retain all of the oxidizing equivalents of

dioxygen in an activated form. The observation that 3 mol of cyt c^{II} are oxidized during the reaction of $CCP(MI)^{II}$ with dioxygen and its subsequent reduction to CCP^{III} is consistent with this prediction. In view of the suggestion that cyt c^{II} can reduce an intermediate of the CCP^{III} + peroxide reaction prior to formation of the compound I radical (Summers & Erman, 1988), it is possible that cyt c^{II} reduces the $CCP(MI)^{III}(OOH^-)(trp^{\bullet}_{191})$ intermediate (eq 6) or one of the subsequent biradical intermediates represented in eqs 7 and 8. The failure of cyt c^{II} to react with the Fe^{II}–O_2 complex formed by the Trp 191 → Phe mutant argues against a mechanism involving direct reduction of the Fe^{II}–O_2 complex, however.[3]

The mechanism(s) of autoxidation of mutants of CCP(MI) and other heme peroxidases that lack an electron donor equivalent to Trp 191 is not clear at this time. Although $CCP(MI,F191)^{II}$–O_2 and HRP^{II}–O_2 spontaneously return to the ferric state without detectable intermediates, the failure of CN^- to increase the rate of autoxidation of these enzymes argues against a mechanism that involves simple dissociative loss of superoxide (Wittenberg et al., 1967). It seems likely that the autoxidation of these enzymes proceeds by a mechanism similar to the one presented in eqs 5–8, where one-electron reduction of the Fe^{II}–O_2 complex by an endogenous electron donor is followed by O–O bond cleavage and conversion of the enzyme to a higher oxidation state. The much more rapid conversion of $CCP(MI)^{II}$–O_2 to compound I′ in CCP(MI) relative to other heme peroxidases would be explained by the much slower rate of the initial reduction of the coordinated dioxygen. The failure of oxidized enzyme intermediates to accumulate requires that the initial conversion of the enzyme to compound I is slow relative to the rate of conversion of the enzyme to the ferric state. This is consistent with the finding that compound I and compound II of HRP react rapidly with HRP^{II}–O_2 to produce 3 and 2 mol of the ferric enzyme, respectively (Tamura & Yamazaki, 1972). The changes in the absorption spectra of the ferric CCP(MI,N235) and CCP(MI,F191) enzymes that result from autoxidation (Figure 3) may reflect modifications of the enzyme that occur during transient oxidation above the ferric state.

Relevance for Catalysis by CCP. The observations reported here may have relevance to the catalytic mechanism of CCP(MI). Kinetic evidence (Hazzard et al., 1987; Summers & Erman, 1988) suggests that cyt c^{II} initially reduces the oxyferryl center of compound I, producing a Fe^{III} intermediate that retains the compound I radical (compound II_R; eq 9). According to this mechanism, the subsequent reduction of the compound I radical would require reformation of the oxyferryl center through an intramolecular electron transfer step (eqs 10–11). The transfer of the oxidizing equivalent from Trp 191 to the iron (eq 10) is too slow to account for the steady-state catalytic rate of CCP, indicating that the compound I radical is not reduced by cyt c^{II} under steady-state conditions (Summers & Erman, 1988). These authors suggested that cyt c^{II} must reduce a hypothetical intermediate of the CCP^{III} + HOOH reaction that precedes compound I under steady-state conditions.

$$\text{compound I} + \text{cyt } c^{II} + 2H^+ \rightarrow CCP^{III}(trp^{\bullet}_{191}) + \text{cyt } c^{III} + H_2O \quad (9)$$

$$CCP^{III}(trp^{\bullet}_{191}) + H_2O \rightleftarrows CCP^{IV}{=}O + 2H^+ \quad (10)$$

$$CCP^{IV}{=}O + \text{cyt } c^{II} + 2H^+ \rightarrow CCP^{III} + \text{cyt } c^{III} + H_2O \quad (11)$$

An alternative possibility can now be suggested. Reaction of the ferric iron center of compound II_R with a second molecule of peroxide (eq 12) would produce an intermediate

$$CCP^{III}(trp^{\bullet}_{191}) + HOOH \rightarrow CCP^{III}(OOH^-)(trp^{\bullet}_{191}) + H^+ \quad (12)$$

that is essentially identical to that proposed in eq 6 above. If the rate of reaction of the ferric enzyme with peroxide is not decreased by the oxidation of Trp 191, the rate of reaction of compound II_R with peroxide (eq 12) under the steady-state conditions reported by Kim et al. (1990) would be 1500 times the reported rate for the conversion of compound II_R to compound II_F (Summers & Erman, 1988). The formation of a $CCP^{III}(OOH^-)(trp^{\bullet}_{191})$ intermediate therefore seems likely under these conditions. The present results show that once formed, an intermediate such as that suggested in eq 12 (or its subsequent reaction products; eqs 7 and 8) can be quantitatively reduced to the ferric enzyme by cyt c^{II}.

On this basis, it is reasonable to suggest that under steady-state conditions the enzyme alternates between the $Fe^{III}(OOH^-)(trp^{\bullet}_{191})$ and compound II_R oxidation states. One-electron reduction by cyt c^{II} of an intermediate resulting from the reaction of compound II_R with peroxide (eq 12) would produce compound I, and a second molecule of cyt c^{II} would reduce the oxyferryl center of compound I, thus re-forming the $Fe^{III}(trp^{\bullet}_{191})$ (i.e., compound II_R) intermediate. The nature of the intermediate reduced initially by cyt c^{II} would depend on the rate of reaction of the $Fe^{III}(OOH^-)(trp^{\bullet}_{191})$ intermediate to form biradical intermediates (e.g., eqs 7 and 8) relative to the rate of electron transfer from cyt c^{II}. It is interesting to note, however, that the formation of biradical intermediates provides a means for transferring both oxidizing equivalents of peroxide to sites other than the (unreactive) Trp 191 radical. It is tempting to speculate that under steady-state conditions the electron transfer between cyt c^{II} and CCP(MI) results in part from the formation of Tyr radicals at the molecular surface of CCP, and their subsequent reduction by cyt c^{II}.

It is also possible to imagine a physiological role for the rapid conversion of the CCP^{II}–O_2 complex to compound I′. Although CCP^{II} is not present under normal physiological conditions, a complex identical to CCP^{II}–O_2 could be formed by the reaction of CCP^{III} with superoxide (see eq 5, above) produced in the intermembrane space of yeast mitochondria as a consequence of normal aerobic metabolism (Loschen et al., 1974; Beyer, 1990). Several other ferric heme peroxidases are known to react with superoxide to form a complex identical to the Fe^{II}–O_2 complex (Yamazaki & Piette, 1963; Odajima & Yamazaki, 1972; Wariishi & Gold; 1990). The formation of such a complex would severely inhibit the peroxidase activity of CCP, since the mutant studies indicate that the Fe^{II}–O_2 complex does not react rapidly with cyt c^{II}, and thermal dissociation of dioxygen from the Fe^{II}–O_2 complex is very slow. The pathway for facile electron transfer from the radical site to coordinated dioxygen decreases the half-life of the Fe^{II}–O_2 complex by approximately 40 000-fold relative to the mutants that lack this feature, and allows CCP to catalyze the reduction of superoxide by cyt c^{II} without damage to the enzyme. The compound I radical site may therefore represent an adaptation that permits the ferric enzyme to function in the presence of superoxide.

[3] Although previous studies have shown that the $Fe^{IV}{=}O$ form of the Trp 191 → Phe mutant is reduced very slowly by cyt c^{II} (Mauro et al., 1988), more recent results in this laboratory indicate that when the ferric enzyme is mixed with peroxide in the presence of cyt c^{II}, rapid, stoichiometric oxidation of cyt c^{II} occurs. Thus, the failure of cyt c^{II} to reduce the Fe^{II}–O_2 complex formed by this mutant enzyme does not result from a general defect that prevents electron transfer from cyt c^{II} to coordinated ligands.

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