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Enhanced Oxidation of Aniline Derivatives by Two Mutants of Cytochrome *c* Peroxidase at Tryptophan 51*

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Two hyperactive mutants of cytochrome *c* peroxidase (CCP), W51F and W51A, catalyze the enhanced oxidation of a number of substituted anilines. The reaction of CCP compound *E*S with mesidine is biphasic, while similar reactions using compound II give monophasic kinetics. These data, in addition to the ratio of the Fe^{4+}=O and free-radical species observed during steady-state turnover, indicate that reduction of the Trp-191 free radical of compound *E*S is more rapid than the reduction of the Fe^{4+}=O species. Transient kinetics were examined for the oxidation of eight mono-substituted anilines by CCP, W51F, and W51A. Each of the aniline derivatives were oxidized by the mutants at rates that exceeded that of the wild-type enzyme, and the rate constant for *m*-chloroaniline was 400-fold faster for W51F than for wild-type CCP. Variations in the rate constants for the different substrates follow a linear free-energy relationship using the Hammet substituent effect parameter σ^+, implicating electron transfer from the aniline ring in the transition state. For aniline oxidation, the free energy of activation is 3 kcal/mol lower for the mutants than for wild-type CCP, and this is due primarily to an increase in the activation entropy. These results indicate that the enhanced kinetics of W51F and W51A result from a generalized increase in enzyme reactivity characterized by an exo-entropic transition state such as dissociation of bound H_2O from the Fe^{4+}=O center.

A clear understanding of the differences in the reactivity of the heme peroxidases with various substrates and the various ways in which the enzyme structure may be used to modulate this reactivity is of considerable interest. An important step toward this understanding would be achieved by the development and characterization of variants of a given peroxidase that are capable of oxidizing non-native substrates. The transformation of substrate specificity will provide valuable information not only about the details of mechanism and substrate-enzyme interaction, but may also lead to the engineering of peroxidases with novel practical utility. Cytochrome *c* peroxidase (CCP)[1] (EC 1.11.1.5) is a 34-kDa yeast mitochondrial heme enzyme that catalyzes the oxidation of yeast cytochrome *c* (cyt *c*) by H_2O_2 (Yonetani, 1976; Poulos and Finzel, 1984). CCP functions by the normal peroxidatic cycle via two oxidized intermediates, *E*S and II, which can be approximated as:

$$\mathrm{CCP} + H_2O_2 \xrightarrow{k_1^{app}} ES + H_2O \qquad \text{(Eq. 1)}$$

$$ES + A \xrightarrow{k_2^{app}} \mathrm{II} + A^{+\cdot} \qquad \text{(Eq. 2)}$$

$$\mathrm{II} + A \xrightarrow{k_3^{app}} \mathrm{CCP} + A^{+\cdot} \qquad \text{(Eq. 3)}$$

where *A* is the reducing substrate.[2] The first oxidized intermediate for CCP has historically been referred to as the *E*S complex (Yonetani *et al.*, 1966). It is analogous to the compound I intermediate of other peroxidases in that it contains two oxidative equivalents: one is stored as an oxyferryl center (Fe^{4+}=O), and the other as a protein free radical. Unlike horseradish peroxidase (HRP) compound I, which carries the second oxidation equivalent as a porphyrin cation radical (Dolphin *et al.*, 1971), the radical associated with *E*S resides as a stable and reversibly oxidized form of Trp-191 (Sivaraja *et al.*, 1989). The *E*S complex is reduced by substrate in two 1-electron steps to generate the half-reduced species (compound II) before returning to the native ferric state. A similar mechanism is utilized by a number of heme peroxidases to carry out the oxidation of an enormous diversity of substrates, including indoles, phenols, aromatic amines (Saunders, 1973), lignin (Tien and Kirk, 1983), manganese (Pribnow *et al.*, 1989), and halide ions (Dawson, 1988). CCP is unusual in that its natural substrate is reduced cytochrome *c* (cyt c^{red}), although it has been shown to slowly oxidize the archetypal peroxidase substrates guaiacol and pyrogallol (Yonetani and Ray, 1965) (DePillis *et al.*, 1991) in addition to $Fe(CN)_6^{4-}$ (Jordi and Erman, 1974) and other substitutionally inert Fe^{2+} complexes (Purcell and Erman, 1976). A CCP mutant, W51A, has recently been shown to display a weak monooxygenase activity toward styrene (Miller *et al.*, 1992), thus displaying a

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[1] The abbreviations used are: CCP, cytochrome *c* peroxidase; CCP(MKT), the form of the enzyme which is expressed in and purified from *E. coli*, and contains Met-Lys-Thr (MKT) at the N terminus; CCP-Fe^{2+}, the reduced, ferrous form of CCP; compound II, the half-reduced state of *E*S, containing the oxyferryl heme only, and not the Trp-191 free radical; cyt *c*, cytochrome *c*; *E*S, the oxidized state of CCP generated by reaction with 1 equivalents of H_2O_2, containing both the oxyferryl center (Fe^{4+}=O) and the Trp-191 free-radical; HRP, horseradish peroxidase; W51A, the CCP mutant in which Trp-51 is replaced by Ala; W51F, the CCP mutant in which Trp-51 is replaced by Phe.

[2] The terms k_2 and k_3 are used to denote the true bimolecular rate constants for the elementary steps shown in Schemes 1 and 2. The terms k_2^{obs} and k_2^{obs} denote the observed pseudo-first order rate constants at a given substrate concentration, and k_2^{app} and k_3^{app} denote the experimentally derived apparent bimolecular rate constants.

considerable plasticity in its mechanism and substrate specificity.

It has been observed that mutations at Trp-51 of CCP significantly affect the coordination and functional properties of the enzyme (Goodin *et al.*, 1987; Wang *et al.*, 1990; Smulevich *et al.*, 1990; Goodin *et al.*, 1991). Trp-51 (distinct from the Trp-191 radical site) forms part of the distal active-site cavity near the open coordination position of the heme iron. The tryptophan side chain is in van der Waals contact with the heme and forms a hydrogen-bond with one of the water molecules which is displaced on binding H_2O_2 (Finzel *et al.*, 1984) (see Fig. 1). Other peroxidases such as lignin peroxidase (Edwards *et al.*, 1993) and horseradish peroxidase (Welinder, 1985, 1992) appear to have a Phe in this position in place of Trp-51. The CCP variants W51F and W51A have been shown to be more active than the wild-type enzyme, under steady-state conditions, in catalyzing the oxidation of cyt c^{red} (Goodin *et al.*, 1987, 1991). These studies have suggested that the enhanced kinetics of W51A and W51F result from an increased rate of electron transfer between the oxidized enzyme and cytochrome *c*, and have shown that the bimolecular rate constants for the reaction of the mutant enzymes with peroxide to form CCP *E*S were relatively unaffected by the mutations. The simplest interpretation of these data was that the mutations of Trp-51 serve to increase the intrinsic reactivity of the oxyferryl heme center of compound *E*S and/or compound II, although possible effects of these mutations on the mode of binding or interaction between the oxidized enzyme and cytochrome *c* could not be ruled out. A replacement of the analogous residue, F41V, of HRP has recently been reported (Smith *et al.*, 1992), and in contrast to those of CCP, this substitution was shown to cause an 8-fold reduction in the rate of reaction of the ferric enzyme with H_2O_2. However, in close correspondence to the hyperactivity observed for the CCP mutants, this HRP variant exhibited a small increase in the kinetics over the wild-type enzyme for the oxidation of *p*-aminobenzoic acid by HRP compounds I and II. These data were interpreted in terms of structural perturbations near the substrate binding site of HRP, consistent with the observed decrease in K_d for binding to another substrate, benzhydroxamic acid. Thus, the alteration of analogous distal residues of CCP (Goodin *et al.*, 1991) and HRP (Smith *et al.*, 1992) are highly complementary in that replacement of the naturally occurring residue at this position results in an increase in the rate of oxidation of substrate by the hypervalent porphyrin. For CCP, it was proposed that the hyperactivity is due to the enhanced reactivity of the oxyferryl heme with respect to electron transfer (Goodin *et al.*, 1991), while the HRP study (Smith *et al.*, 1992) concluded that altered substrate binding may be responsible for the altered kinetics.

If the enhanced reactivity of these mutants toward cytochrome *c* results from a generally more reactive enzyme intermediate, these mutants should also exhibit enhanced reactivity toward more classical peroxidase substrates, such as aniline. Indeed, we have found that the substitution of Trp-51 by Phe or Ala significantly enhances the reactivity of CCP compound II with respect to the oxidation of a number of aniline derivatives, and we will present and discuss a kinetic characterization, structure-activity relationships, and the thermodynamic activation parameters associated with aniline oxidation. The results demonstrate a generalized increase in the reactivity of these mutants with a wide variety of non-native substrates, and indicate that this effect is a consequence of an increase in the entropy of activation for the reduction of the ferryl iron center.

MATERIALS AND METHODS

Reagents and Chemicals—*p*- and *m*-chloroaniline were obtained from Kodak as practical grade. Aniline and all other aniline derivatives were purchased from Sigma as reagent-grade. Horse heart cytochrome *c* type VI and bovine hemin were also obtained from Sigma. Additional chemicals were reagent-grade and used as received. Solutions of H_2O_2 were standardized by titration with $KMnO_4$ (Kolthoff and Belcher, 1957).

CCP Expression and Purification—Expression and purification of CCP and mutants from *Escherichia coli* have been described previously (Goodin *et al.*, 1991). CCP concentrations were determined by using extinction coefficients of ϵ_{408} = 101.2, 121.5, and 107.1 $mM^{-1} cm^{-1}$ for CCP, W51F, and W51A (Goodin *et al.*, 1991).

Optical and EPR Spectra—UV/visible spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer interfaced to an AST/286 desktop computer. EPR spectra were collected at 9.51 GHz on a Bruker ESP300 spectrometer equipped with an Air Products LTR3 liquid helium cryostat.

Preparation of CCP-Fe^{2+} and Compound II—Reduced CCP (CCP-Fe^{2+}) was prepared by the electrolytic reduction of CCP mediated by methyl viologen (Goodin and McRee, 1993). A 3-ml solution of approximately 10 μM CCP buffered at pH 6.0 with 100 mM potassium phosphate and containing 1 mM methyl viologen was sealed in an airtight cuvette. The cell was continuously flushed with a stream of scrubbed nitrogen gas and thermostated at 15 °C. The cell was equipped with a gold foil working electrode, a platinum wire indicating electrode, and a silver/silver chloride reference electrode. The latter

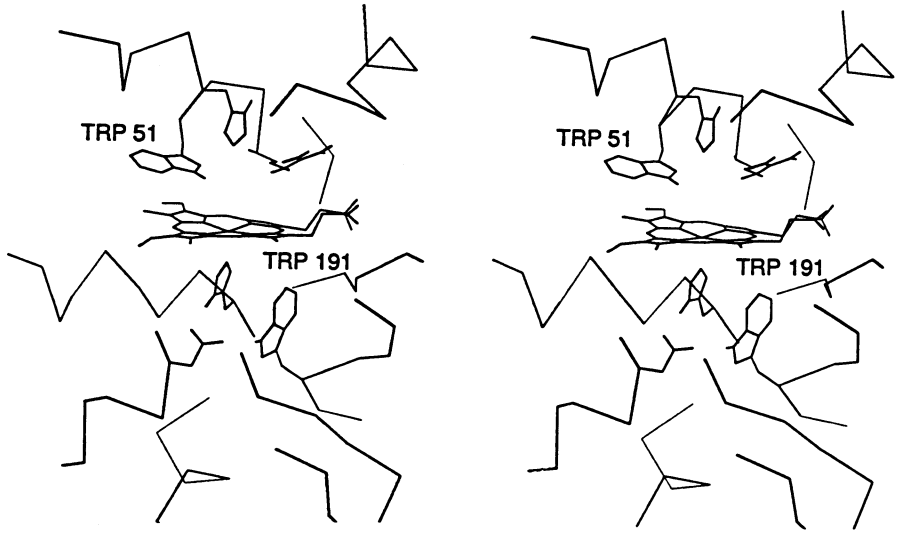


FIG. 1. **A stereo view of the distal heme face of CCP showing the relationship of Trp-51 with the active site.** The coordinates were obtained from the structure of CCP(MKT) (Goodin and McRee, 1993), and the figure was prepared with the program XFIT (McRee, 1992).

two electrodes were isolated from the solution by a double salt bridge. The reduction was carried out by reducing methyl viologen at the working electrode by applying 8 μA across the electrode. The reduced methyl viologen diffused from the gold electrode and reduced the ferric heme of CCP to Fe^{2+}. The reduction was monitored as an increase of absorbance at 438 nm. The reduction was considered complete when no further changes occurred at 438 nm, or an excess of reduced methyl viologen was formed as monitored at 394 nm.

Compound II, the form of the enzyme which contains the oxyferryl moiety but not the radical, was prepared by the stoichiometric addition of H_2O_2 to an anaerobic solution of the Fe^{2+} enzyme, a procedure shown previously to give the $Fe^{4+}{=}O$ peroxidase (Ho *et al.*, 1983).

Stopped-flow Kinetics—Transient kinetics were measured on a Hi-Tech Scientific SHU stopped-flow single-wavelength spectrophotometer interfaced to a Hewlett-Packard 9000 computer. The temperature of both the syringes and the sample cell was maintained by a variable temperature water bath to ±0.2 °C. All measurements were made in solutions buffered at pH 6.0 with 100 mM potassium phosphate. Typically, one syringe was filled with substrate and 2 μM H_2O_2 and the other contained 2.5 μM of the native enzyme or a mutant. Final concentrations of peroxide and enzyme in the reaction cell were 1 and 1.25 μM, respectively. The reaction was followed at 424 nm as the rapid increase of absorbance as peroxide reacted with the enzyme to form the oxidized species, and a much slower decrease as the oxyferryl group was reduced by substrate. Kinetic measurements were also made on pre-formed CCP-*E*S and CCP-$Fe^{4+}{=}O$. In these experiments, the *E*S complex was formed by the addition of 2 μM H_2O_2 to 2.5 μM CCP immediately before the stopped-flow experiment was carried out. CCP-$Fe^{4+}{=}O$ was produced as described above, and diluted 4-fold into ice-cold buffer to give a final concentration in the stopped-flow cell of 1.25 μM CCP-$Fe^{4+}{=}O$. The stopped-flow measurements were performed within 0.5 h after generating the CCP-$Fe^{4+}{=}O$ species.

RESULTS

Reaction of CCP(MKT) with Anilines—We have observed that CCP(MKT) will catalyze the oxidation of a range of aniline derivatives. Fig. 2 shows the observation of an isotropic EPR signal generated at 25 °C under steady-state conditions in a solution of 4-aminophenol and H_2O_2 which is dependent upon the presence of CCP. This signal is essentially identical to that of the 4-aminophenoxy radical produced by ascorbate peroxidase (Chen and Asada, 1990). As also observed for ascorbate peroxidase, the addition of ascorbate to the reaction results in the quenching of the 4-aminophenoxy radical and the appearance of the ascorbate radical. We have also observed a slow steady-state oxidation of 2,4,6-trimethylaniline (mesidine) by CCP and H_2O_2. Addition of 0.1 μM CCP(MKT) to a solution containing 100 μM H_2O_2 and 2 mM mesidine in 100 mM KP_i, pH 6.0, results in the appearance of an oxidation product as measured by an increasing absorbance at 350 nm. The initial slope of the absorbance *versus* time is linear in enzyme concentration and independent of H_2O_2 concentration, indicating that the oxidation is limited by the reaction of the enzyme with the reducing substrate. In addition the total change in absorbance over the course of the reaction is proportional to the quantity of H_2O_2 added. During the reaction, the absorbance at 424 nm, reflecting the oxidation state of the heme, is constant and equal to 100 ± 10% of that expected for the enzyme in the oxyferryl state. Under these steady-state turnover conditions, samples of the reaction were frozen and examined by EPR at 10 K for the appearance of the Trp-191 radical. In this case we observe 52 ± 13% of the radical signal expected for the *E*S state. These data indicate that the reaction is limited by the reducing substrate, and that k_3^{app} and k_2^{app} are of similar magnitude, assuming that k_3^{app} represents the reduction of the $Fe^{4+}{=}O$ center and k_2^{app} represents the reduction of the Trp-191 free radical.

Following these initial observations, we have examined the transient kinetics for the reaction of mesidine with the oxidized states of CCP(MKT). Previous studies of the reaction of CCP *E*S and HRP compound I with small molecule reductants have been performed by preparing CCP compound *E*S (Erman, 1975; Summers and Erman, 1988) or HRP compound I (Huang and Dunford, 1991; Perez and Dunford, 1990) before loading into one syringe and placing the reductant in the other syringe of a stopped-flow apparatus. These studies have shown that the reaction is typically biphasic, with $k_2^{app} \cong 3k_3^{app}$, but the faster rate constant (k_2^{app}) is often difficult to determine reliably (Purcell and Erman, 1976; Erman, 1975). For this reason, most studies of this reaction have concentrated on the value of k_3^{app} as determined by analysis of the latter part of the decay curve (Summers and Erman, 1988; Jordi and Erman, 1974).

Two methods were used to obtain values of k_3^{app} for the reaction of CCP(MKT) compound *E*S with mesidine. Fig. 3*A* shows the reaction of 1.2 μM CCP(MKT) *E*S with 0.6 mM mesidine at 25 °C. The absorbance at 424 nm was used for the measurement of the oxyferryl state as it represents the wavelength for the maximum difference in absorbance between the ferric state and either compound *E*S or compound II (Jordi and Erman, 1974). Fig. 3*A* also shows the results of a single exponential fit to the final 80% of the decay curve to give $k_3^{obs} = 0.337 \pm 0.003\ s^{-1}$. The fit indicates that the decay is biphasic in that an initial lag is observed in the reduction of the ferryl group. This behavior, which is also observed in the reaction of CCP(MKT) *E*S with ferrocyanide (Jordi and Erman, 1974) is to be expected from the proposed mechanism if it is assumed that the radical has no effect on the absorbance at 424 nm. In this case, the kinetics for the reaction of *E*S are described by,

$$\frac{A_{424}^{t} - A_{424}^{t=\infty}}{A_{424}^{t=0} - A_{424}^{t=\infty}} = \frac{1}{k_3^{obs} - k_2^{obs}} \left(k_3^{obs} e^{-k_2^{obs} t} - k_2^{obs} e^{-k_3^{obs} t}\right) \qquad \text{(Eq. 4)}$$

where A_{424}^{t} is the absorbance at 424 nm at time *t* and $k_n^{obs} = [A]k_n^{app}$. Equation 4 is a generalized model for the change in absorbance of a reaction which undergoes two sequential steps in which the reactant and intermediate species contribute equally to the absorbance. As shown in Fig. 3*B*, the data are adequately described by this model to give $k_3^{obs} = 0.345 \pm 0.004\ s^{-1}$, which compares well with the single exponential result. While two exponentials were observed for the oxidation of mesidine by the wild-type enzyme, this was not always the case. For the reaction of other substrates with wild-type enzyme, and particularly for the reactions of the mutants, it was difficult to determine reliable values of k_2^{app}. Thus, although the bi-exponential model most accurately reflects the proposed mechanism, single exponential fits to the final portion of the decay were used as the most general method for obtaining k_3^{app} in this work.

We wished to verify that the slower of the two observed kinetic phases, assigned to k_3^{app}, corresponds to the step in which the ferryl species is reduced. Thus, we have examined the reaction of mesidine with compound II of CCP(MKT), which contains the oxyferryl ($Fe^{4+}{=}O$) center without the Trp-191 free radical. CCP compound II can be prepared quantitatively by the reaction of CCP-Fe^{2+} with a stoichiometric amount of H_2O_2 (Ho *et al.*, 1983). CCP-Fe^{2+} was prepared by reductive titration of CCP-Fe^{3+} in an anaerobic electrochemical cell using methyl viologen as an electron transfer mediator (Goodin and McRee, 1993). The resultant species gave an optical spectrum with absorbance maxima at 438, 560, and 592(sh) nm, consistent with those observed in previous studies of CCP-Fe^{2+} (Ho *et al.*, 1983; Miller *et al.*, 1990). Compound II was generated by addition of a slight excess (1.1 equivalents) of H_2O_2 to a 10 μM solution of CCP-

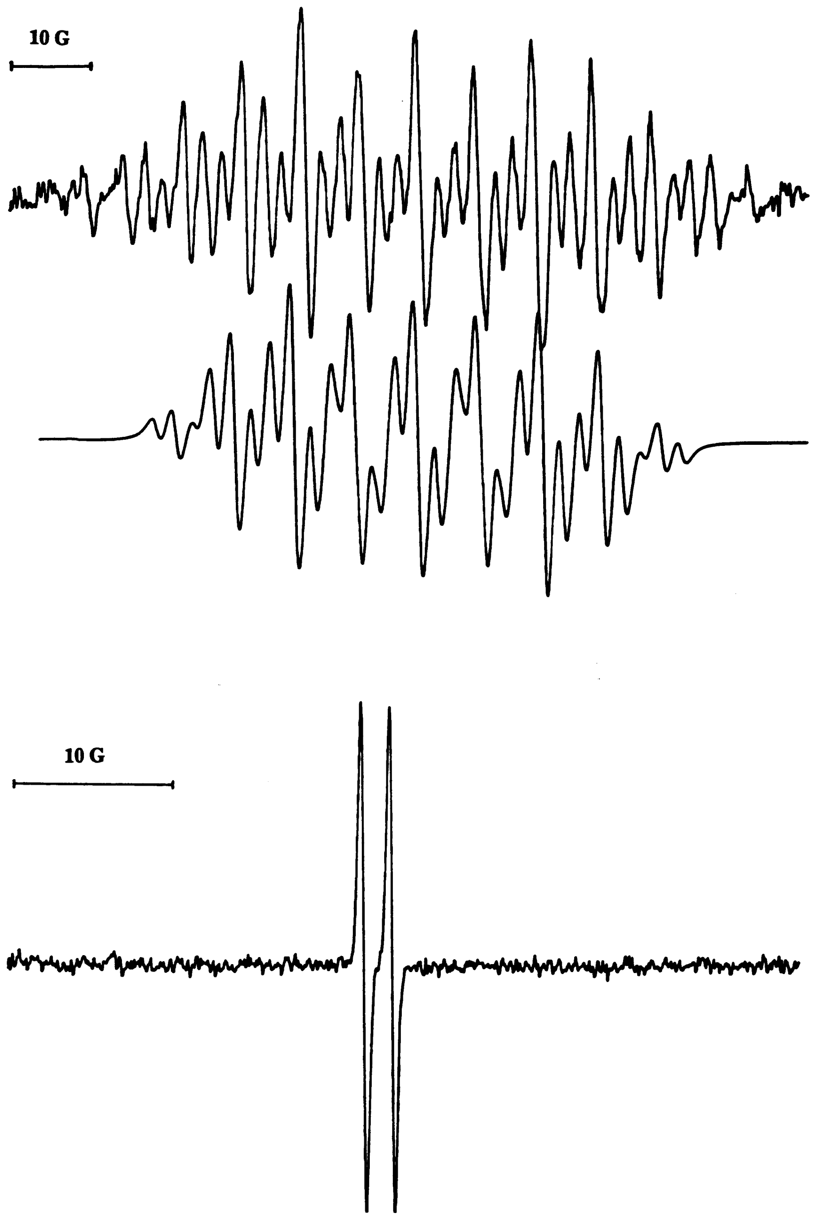


FIG. 2. **Observation of the 4-aminophenoxy radical produced by oxidation of 4-aminophenol by CCP(MKT) under steady-state conditions, and the trapping of this radical by ascorbate.** *Top*, the reaction mixture contained 10 mM 4-aminophenol, 10 mM H_2O_2, and 0.5 μM CCP(MKT) in 100 mM KP_i, pH 6. After mixing, the sample was immediately loaded into a Wilmad WG-812-Q flat cell, and EPR data were collected at room temperature. Spectrometer conditions: 9.75 GHz microwave frequency, 200 microwatt power, 100 KHz field modulation, 0.5 G modulation amplitude. *Middle*, a simulation of the radical using the parameters reported previously for this radical species (Chen and Asada, 1990). The simulation included isotropic hyperfine interactions with one nitrogen, a_N = 5.26 G, 4 equivalent protons with a_{H1} = 2.63 G, and 2 protons with a_{H2} = 0.88 G, and a Lorentzian line width of 0.5 G. *Bottom*, EPR spectra of the same reaction mixture as shown above, but with the addition of 10 mM ascorbate.

Fe^{2+} under anaerobic conditions. This species was characterized by absorbance maxima at 418, 530, and 560 nm, as reported for CCP-Fe^{4+}=O (Ho *et al.*, 1983). The rate constant for the decay of the Fe^{4+}=O enzyme in the absence of reductant was measured by the change of absorbance at 424 nm at 15 °C as 3.1 ± 0.1 × 10^{-4} s^{-1}, which compares well with the value of 3.0 × 10^{-4} s^{-1} determined earlier (Ho *et al.*, 1983). We measured the difference in the absorption spectra of *E*S and CCP as well as the difference between compound II and CCP. In both cases, the maximum difference in absorbance occurred at 424 nm ($\Delta\epsilon_{424}$ of approximately 35 mM^{-1} cm^{-1}) and an isosbestic point was observed at 414 nm, which compares well with measurements made of the optical difference spectra between CCP and *E*S (Balny *et al.*, 1987). In addition, the *E*S minus Fe^{4+}=O optical difference spectrum gave a weak band with λ_{max} = 580 nm which has been attributed to the Trp-191 free-radical center (Ho *et al.*, 1983). Consistent with earlier studies (Coulson *et al.*, 1971; Ho *et al.*, 1983), we observe that the Soret band intensity at 424 nm is very similar for *E*S and compound II, and differs by $\Delta\epsilon_{424} \leq 8$ mM^{-1} cm^{-1}. Thus, our kinetic measurements at 424 nm predominantly represent either of the two states (*E*S and compound II) containing the oxyferryl center rather than the presence of the Trp-191 free radical. In contrast to an earlier report (Ho *et al.*, 1983), we were not able to prepare a mixture of CCP-Fe^{2+} and CCP-Fe^{4+}=O. For example, when 0.5 equivalents of H_2O_2 were added to a solution of CCP-Fe^{2+}, visible spectra were measured which were characteristic of CCP-Fe^{3+}, having absorbance maxima at 408, 530, and 645 nm. These results would be expected for the facile reduction of CCP-Fe^{4+}=O by CCP-Fe^{2+}.

The reaction of CCP-Fe^{4+}=O with mesidine was measured under the same conditions as described for CCP *E*S above, and as shown in Fig. 3*C*, we observe that the kinetics are monophasic with a value of k_3^{obs} = 0.339 ± 0.005 s^{-1}. This corresponds well with the values obtained from the two methods of analysis of the biphasic reaction with compound *E*S. Thus, the value of k_3^{app} extracted from the biphasic kinetics of *E*S appears to correspond to the reduction of the oxyferryl Fe^{4+}=O species. The determinations of the apparent bimolecular rate constant, k_3^{app}, over a mesidine concentration range of 0.2 to 1 mM for the reaction of CCP(MKT) *E*S and for the reaction with compound II are shown in Fig. 4. Each of the above methods of obtaining k_3^{app}, 1) the reaction with *E*S and

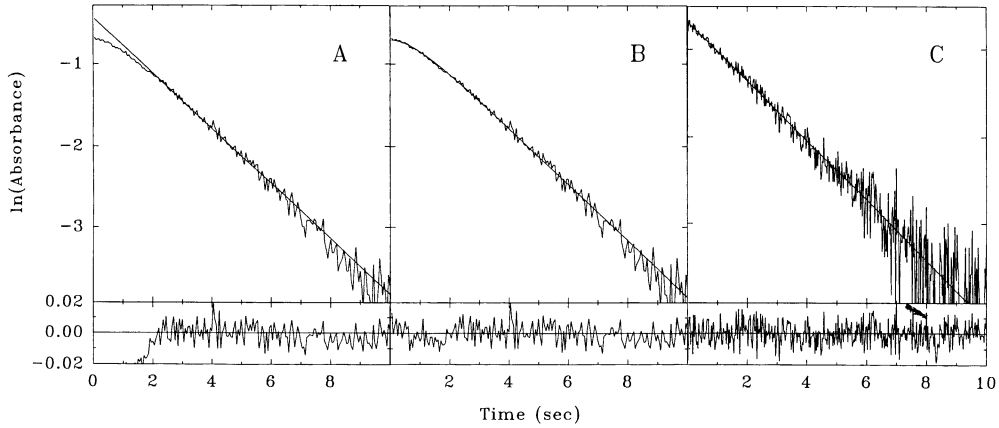


FIG. 3. **Stopped-flow kinetic traces for the reaction of CCP(MKT) compound *E*S and CCP (Fe^{4+}=O) with mesidine.** *A*, reaction of pre-formed CCP(MKT) compound *E*S with mesidine. The data were fit to a single exponential in which the data for the first 1.8 s were excluded from the fit. The fit gave k_3^{obs} = 0.337 ± 0.003 s^{-1}. *B*, same data as in *A*, analyzed by Equation 4 to give k_2^{obs} = 1.31 ± 0.05 s^{-1}, k_3^{obs} = 0.345 ± 0.004 s^{-1}. The data, representing A_{424}^{t}, were fit to Equation 4 by nonlinear least squares minimization using k_2, k_3, $A_{424}^{t=\infty}$, and ($A_{424}^{t=0} - A_{424}^{t=\infty}$) as adjustable parameters. *C*, CCP(MKT) (Fe^{4+}=O) fit to single exponential to give k_3^{obs} = 0.339 ± 0.005 s^{-1}. All reactions were performed at 25 °C in 100 mM KP_i, pH 6.0, and monitored by absorbance at 424 nm. The enzyme and mesidine concentrations were 1.2 μM and 0.6 mM, respectively, after mixing. In each case the fits were performed on linear absorbance, and the results were plotted on a logarithmic scale for visual evaluation.

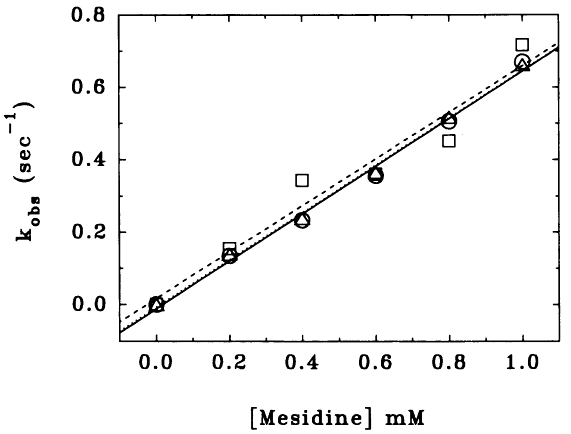


FIG. 4. **Pseudo-first order rate constants (k_3^{obs}) for the oxidation of mesidine by CCP(MKT) analyzed by three different methods.** ○, the reaction of pre-formed CCP(MKT) compound *E*S with mesidine and analyzed by single exponential fit to the final 80% of the decay (k_3^{app}= 653 ± 20 M^{-1} s^{-1}). △, reaction of CCP *E*S fit to a double exponential (k_3^{app} = 655 ± 27 M^{-1} s^{-1}). □, CCP(MKT) (Fe^{4+}=O) fit to a single exponential (k_3^{app} = 635 ± 73 M^{-1} s^{-1}). Conditions other than the concentration of mesidine are given in Fig. 3.

fit to Equation 4, 2) the reaction with *E*S using single exponential fits to the final part of the decay, and 3) the reaction with compound II, are compared in Fig. 4. The values of k_3^{app} obtained are 655 ± 27, 653 ± 20, and 635 ± 73 M^{-1} s^{-1}, respectively. The agreement of these values suggests that, under these conditions, any of these methods may be used fruitfully in the determination of k_3^{app}. In addition, k_2^{app} was determined for the reaction of CCP(MKT) *E*S with mesidine by fitting to Equation 4 to be 2720 ± 260 M^{-1} s^{-1}. The observed ratio of k_2^{app}/k_3^{app} = 4.2 is similar to that observed for the reaction of *E*S with ferrocyanide (Coulson *et al.*, 1971; Jordi and Erman, 1974).

Enhanced Oxidation of Non-native Substrates by W51F and W51A—Determination of the rate constants for the reaction of anilines with the oxidized states of the CCP mutants W51F and W51A was complicated by the fact that the oxyferryl states for these mutants are much less stable than that of CCP(MKT) (Goodin *et al.*, 1991). This precludes the reliable preparation of *E*S prior to loading the syringe. We have found that the reaction may be examined by placing a solution of the enzyme in one syringe, and a mixture of H_2O_2 and the reductant in the other syringe of the stopped-flow spectrophotometer. No reaction was observed between the anilines and H_2O_2 over the time of the experiment in the absence of CCP. In this way, the *E*S complex is formed in the stopped-flow cell in the presence of reductant. The formation of *E*S occurred so rapidly that it did not interfere with the analysis of the much slower kinetics for the reaction of the oxyferryl center with aniline substrates. To demonstrate this, we compared the rate of oxidation of several aniline derivatives by the *in situ* formed CCP(MKT) *E*S with that of preformed CCP(MKT) *E*S. The values obtained by either method compared well. For example, the second-order rate constant for the reduction of the oxyferryl group by *p*-anisidine was determined to be 1680 ± 40 M^{-1} s^{-1} by the first method and 1460 ± 250 M^{-1} s^{-1} when preformed CCP(MKT) *E*S was reduced.

We have found that the hyperactivity with respect to cytochrome *c* oxidation exhibited by the mutants W51F and W51A extends to the oxidation of all aniline derivatives that we have examined. The transient kinetics for the reaction of the *E*S complexes of CCP(MKT), W51F, and W51A with aniline and 4-aminophenol are presented in Fig. 5, which shows that the mutants oxidize these non-native substrates at significantly higher rates than the wild-type CCP(MKT). The values of k_3^{app} at 25 °C, pH 6.0, were determined for aniline and seven of its monosubstituted derivatives. For all substrates, the mutants W51F and W51A are significantly more reactive than CCP(MKT). Specifically, the least difference is seen in the reaction with p-anisidine where compound II of W51F is only 7-fold faster than that of the wild type. The greatest difference was observed with W51F *versus* WT in the oxidation of *m*-chloroaniline, where the mutant is over 400-fold faster, under identical conditions. This clearly demonstrates that a single mutation at W51 dramatically enhances the reactivity of compound II with a variety of reducing substrates in addition to cyt *c*.

The observed rate constants, k_3^{app}, for aniline oxidation by compound II of CCP and the mutants increase with the electron donating ability of the substituent. The Hammet plots of Fig. 6, *A* and *B*, quantifies this dependence by depicting free-energy relationships of the observed rate constants

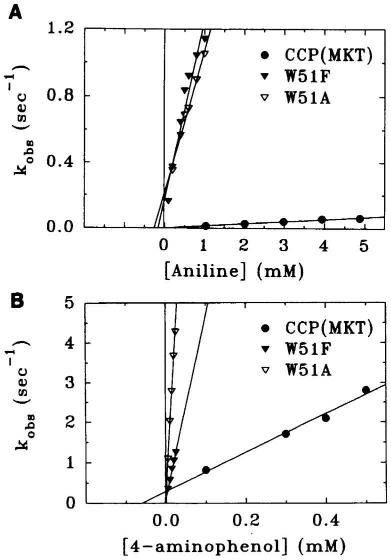


FIG. 5. **Plots of the pseudo-first order rate constant (k_3^{obs}) for aniline (*A*) and 4-aminophenol (*B*) oxidation by CCP(MKT), W51F, and W51A.** Reactions were carried out at 25 °C by flowing CCP(MKT) or mutant enzyme at approximately 2 μM against a solution containing both 1.2 μM H_2O_2 and various concentrations of substrate. Data were analyzed by single exponential fits to the final 80% of the decay curves. For aniline oxidation, k_3^{app} = 13.3 ± 0.1, 1500 ± 50, and 1010 ± 90 M^{-1} s^{-1} for CCP(MKT), W51F, and W51A, respectively, and for 4-aminophenol oxidation, k_3^{app} = 5200 ± 400, 49,000 ± 2000, and 170,000 ± 7000 M^{-1} s^{-1} for CCP(MKT), W51F, and W51A, respectively.

with the substituent parameters σ and σ^+. The data were fit to

$$\log(k_3^{app}) = \rho\sigma + C \qquad \text{(Eq. 5)}$$

where σ is the Hammet substituent parameter, C is a constant, and ρ is the observed slope which measures the sensitivity of the transition state to the electronic properties conferred by the substituent (March, 1985). An analogous equation for the Okamoto-Brown parameter (σ^+) applies. As shown in Fig 6, *A* and *B*, the data for each of the enzymes CCP(MKT), W51F, and W51A are somewhat better described by the σ^+ values than the σ values. The values obtained for ρ were −2.7, −1.5, and −2.0 for CCP(MKT), W51F, and W51A, respectively, when fit to σ^+, and −4.7, −2.5, and −3.3 when fit to σ.

Activation Parameters of Compound II Reduction by Aniline—We have examined the temperature dependence of for the reaction of CCP(MKT), W51F, and W51A with aniline, in order to extract activation parameters. Arrhenius plots are shown in Fig. 7 for aniline oxidation by these enzymes over a temperature range of 5–30 °C. The temperature dependence of each of the enzymes are very similar, and the most significant difference appears in the temperature-independent offset between CCP(MKT) and the two mutants. The activation energies, enthalpies, and entropies calculated from standard transition state theory for these reactions are presented in Table I. From these data it is seen that the activation free-energy for the reduction of the oxyferryl center of both W51F and W51A by aniline is reduced by approximately 3 kcal/mol with respect to that of CCP(MKT). However, there is very little difference in the activation enthalpies for these three enzymes, and it is apparent that the reduced activation energies for the W51F and W51A mutants arises from a significant increase in the entropy term. It is also significant that both W51F and W51A are characterized by a positive entropy of activation.

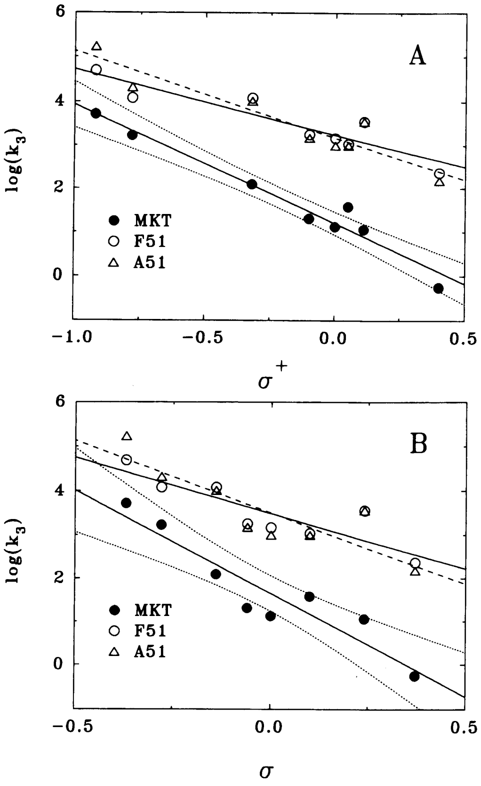


FIG. 6. **Linear free energy relationships for the oxidation of monosubstituted anilines by the Fe^{4+}=O center of CCP(MKT), W51F, and W51A.** *A*, Okamoto-Brown (σ^+) and *B*, Hammet (σ) substituent constants (March, 1985) for each aniline derivative were plotted against log(k_3^{app}) for CCP(MKT) (*closed circles, solid line*), W51F (*open circles, solid line*), and W51A (*open triangles, dashed line*). For each plot, the *curved dotted lines* give 95% confidence limits for the fit to the CCP(MKT) data. The observed slopes (ρ) for the σ^+ plots are −2.7, −1.5, and −2.0 for CCP, W51F, and W51A, respectively, and the corresponding correlation coefficients for the fits are 0.98, 0.92, and 0.94, respectively. For the σ plots, the slopes (ρ) are −4.7, −2.5, and −3.3 for CCP, W51F, and W51A, respectively, and the corresponding correlation coefficients for the fits are 0.94, 0.87, and 0.87, respectively. Each experiment was carried out at least three times at five concentrations of the aniline derivative. The individual values of k_{obs} were averaged before plotting against aniline concentration to obtain the second-order rate constant shown in the figure. The aniline substituent for each point, from *left* to *right* is *p*-OH, *p*-OMe, *p*-Me, *m*-Me, H, *m*-OMe, *p*-Cl, *m*-Cl.

DISCUSSION

Although it is not surprising that CCP will slowly oxidize anilines, it is significant that the mutants W51F and W51A oxidize these substrates with increased rates. This supports the proposal (Goodin *et al.*, 1991) that the hyperactivity of W51F and W51A with cyt *c* as substrate is in fact due to a general increase in the reactivity of the enzyme and not to other factors such as the interaction between the two proteins. In addition, analysis of the kinetics for a series of aniline derivatives allows the examination of free-energy relationships for the reactions, so that the origins of the increased reactivity may be examined in greater detail. These results are even more significant when consideration is given to the relationship that these mutants have to the amino acid sequence and substrate diversity of other peroxidases.

Oxidation of Anilines by CCP(MKT)—We observe that the kinetics for oxidation of anilines by CCP(MKT) is consistent with earlier reports of its reaction with inorganic substrates and with most studies which indicate that the Trp-191 radical center is reduced more rapidly than the Fe^{4+}=O center. The

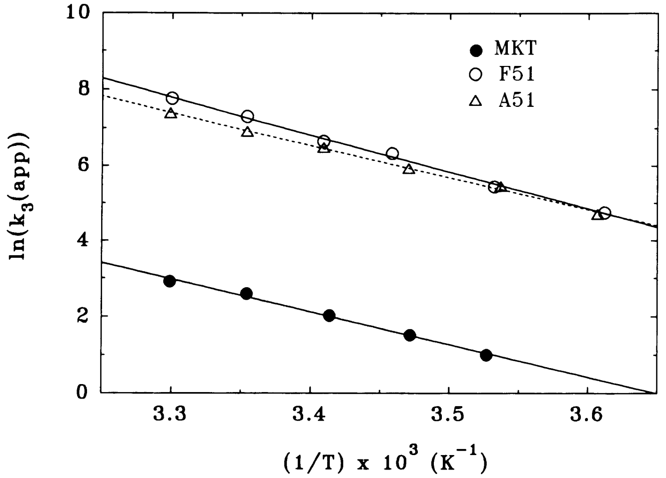


FIG. 7. **Arrhenius plot for the reaction of compound II with aniline for CCP(MKT) (*closed circles, solid line*), W51F (*open circles, solid line*), and W51A (*open triangles, dashed line*).** Thermodynamic parameters are given in Table I.

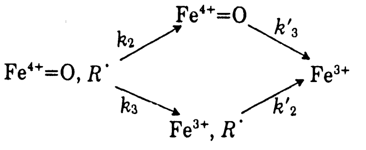
TABLE I

Thermodynamic parameters for the reaction of CCP(MKT), W51F, and W51A with aniline

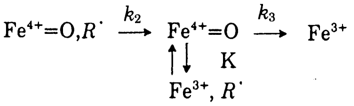
Enzyme	ΔE_a	ΔH_0*	ΔS_0*	ΔG_0*
	kcal/mol		*eu.*	*kcal/mol*
CCP(MKT)	17.0 ± 0.7	16.4 ± 0.7	1.6 ± 2.4	16.5 ± 3.1
W51F	19.6 ± 0.5	19.0 ± 0.5	19.7 ± 0.7	13.1 ± 1.0
W51A	17.0 ± 0.4	16.4 ± 0.4	10.2 ± 1.5	13.4 ± 0.8

kinetics for the loss of the ferryl center are biphasic, and the initial phase is characterized by a lag in the decay profile rather than a more rapidly decaying component. This is most easily explained by the mechanism in Equations 2 and 3 in which the conversion of compound *ES* to compound II is optically silent at 424 nm, as expected for the more rapid reduction of the radical species. The value of k_3^{app} obtained in the reaction of mesidine with CCP(MKT) *ES* corresponds well with the single exponential results for its reaction with the Fe^{4+}=O species of compound II. Previous studies of the reaction of ferrocyanide with CCP *ES* have given biphasic kinetics for the loss of the oxyferryl species (Jordi and Erman, 1974; Purcell and Erman, 1976), and these studies have also been interpreted in terms of the faster reduction of the free-radical species. However, with cytochrome *c* as the reducing substrate, there is no clear consensus in the literature on the assignment of these kinetic phases. In a stopped-flow study (Summers and Erman, 1988), two exponentially decaying phases in the absorbance were observed. These results were interpreted in terms of the more rapid reduction of the ferryl species and a slower reduction of the radical because the amplitude of the slower phase was somewhat smaller than for the faster phase. At variance with these interpretations, recent studies of the complex of a ruthenium-modified cytochrome *c* with CCP have shown that the intramolecular reduction of the Trp-191 free radical occurs much more rapidly than the reduction of the ferryl center (Geren *et al.*, 1991; Hahm *et al.*, 1993). In fact, the intramolecular rate constant derived for the reduction of the ferryl species in the fast phase of the stopped flow studies, 450 s^{-1} (Summers and Erman, 1988), is very similar to that observed for the reduction of the ferryl species of compound II, 450 s^{-1} (Hazzard *et al.*, 1987) or 250 s^{-1} (Geren *et al.*, 1991). Thus, our results with aniline are consistent with previous data on small molecule oxidation, and possibly with cytochrome *c* as well, which indicate that the reduction of the Trp-191 radical is more rapid than reduction of the ferryl heme.

It has not been clear for CCP whether the reaction proceeds with an ordered sequential or random sequential mechanism. For HRP, the two-step reduction of compound I appears to be ordered sequential, and the porphyrin radical of compound I is cleanly reduced by one equivalent of reducing agent to give compound II containing the oxyferryl iron (George, 1952; Dunford and Stillman, 1976; Job and Dunford, 1976). However, the half-reduced state of CCP *ES* produced by adding one equivalent of reducing agent to a sample of *ES* appears to be a mixture of Fe^{3+} and Fe^{4+} species. Two mechanisms have been proposed to account for this result (Coulson *et al.*, 1971),



SCHEME 1



SCHEME 2

in which the two sites are either reduced independently (Scheme 1), or in which the radical is reduced first, followed by intramolecular equilibration between the sites (Scheme 2). Scheme 2 is a special case of the ordered sequential mechanism in which the equilibrium between the half-reduced species was introduced to account for the mixed oxidation states upon reductive titration. However, the fact that a stable Fe^{4+}=O species can be quantitatively prepared by oxidation of CCP-Fe^{2+} (Ho *et al.*, 1983, 1984) indicates that the equilibrium shown in Scheme 2, if it occurs at all, may be slow or may greatly favor the Fe^{4+}=O state. The difficulty in distinguishing kinetically between the two mechanisms has been noted previously (Coulson *et al.*, 1971). Scheme 2 is expected to result in biphasic stopped flow kinetics with the apparent rate constants corresponding to k_2 and k_3. The independent site model (Scheme 1) can give three-phase kinetics with apparent rate constants of k_2, k_3, and $(k_2 + k_3)$ (Coulson *et al.*, 1971), assuming that $k_2 = k_2'$ and $k_3 = k_3'$. However, the small value of $\Delta\epsilon_{424}$ between the Fe^{4+}=O and *ES* states significantly reduces the amplitudes of the phases involving k_2, and in the limit where the Trp-191 radical contributes nothing to ϵ_{424}, only the k_3 phase will be observed (Coulson *et al.*, 1971). Thus, either mechanism is consistent with our observed biphasic kinetics for aniline oxidation. However, regardless of the actual mechanism in operation, a kinetic phase characterized by the bimolecular rate constant k_3 (reduction of the Fe^{4+}=O center) will be observed, and we have shown that this corresponds to the slower of the two observed phases for the oxidation of anilines by CCP(MKT).

The details of the mechanism may also depend on the nature of the substrate. For example, replacement of Trp-191 with Phe results in a severely hindered rate of electron transfer from cytochrome *c* to the Fe^{4+}=O center of CCP, but only a minor change in the rate of ferrocyanide reduction (Mauro *et al.*, 1988). This is consistent with the proposal that reduction of *ES* by cytochrome *c* requires electron transfer through the Trp-191 free radical, but that reduction by ferrocyanide is indiscriminate with respect to the two centers. Such differences in reaction mechanism may reflect the location or orientation of the sites of interaction with each type of substrate. For example, studies of CCP containing heme substituents have concluded that small substrates, such as guaiacol, are oxidized at a different site on CCP than that utilized by cytochrome *c* (DePillis *et al.*, 1991), and it was proposed that guaiacol may approach the heme edge by partially entering the H_2O_2 binding channel on the distal side of the heme. This does not necessarily mean, however, that reduction of CCP *E*S by one equivalent from a small molecule substrate must give a reduced heme and a free radical state. The Trp-191 free radical is close enough to the heme to suggest rapid equilibration of oxidizing equivalents, and a single electron introduced into CCP *E*S may rapidly localize to the site of lowest potential (Ho *et al.*, 1983; Coulson *et al.*, 1971).

The relatively slow kinetics observed in this study for the oxidation of mesidine by CCP allow steady-state conditions to be maintained at a high enough enzyme concentration that its optical and EPR properties may be examined during turnover. Under these conditions, we observe that 100% of the enzyme is in the ferryl state, yet it contained approximately 50% of the quantity of the Trp-191 free radical observed for *E*S. Assuming that $[H_2O_2]k_I \gg [\text{mesidine}]k_2^{app} \cong [\text{mesidine}]k_3^{app}$, then the steady-state concentration of the native Fe^{3+} enzyme may be neglected, and given that $k_2^{app}/k_3^{app} \cong 4$, the expected quantities of the $Fe^{4+}{=}O$ and radical species are 100 and 20%, respectively, for the sequential mechanism (Scheme 1) and 95 and 24% for the random mechanism (Scheme 2). Thus, the steady-state observations are consistent with either mechanism and are reasonably consistent with the k_2^{app}/k_3^{app} ratio obtained by stopped-flow.

Free Energy Relationships in the Enhanced Oxidation by Trp-51 Mutants—The fact that the mutants W51F and W51A oxidize both cytochrome *c* and a number of substituted aniline derivatives with rates that exceed that of the wild-type enzyme strongly implies that this phenomenon is due to an intrinsic change in the reactivity of the oxidized state of the mutants. The significant variation observed in the activities of these mutants with the various anilines might be due either to specific interactions of a given derivative with the enzyme, or simply due to the differences in the reactivity of the given substrate. Thus, we have examined these reactions in terms of the structure-activity relationships normally used to probe substituent effects in organic substitution reactions by correlating the observed second-order rate constants with Hammet's substituent parameter. We have found that these relationships provide a satisfactory description of our data for the oxidation of mono-substituted anilines by CCP(MKT), W51F, and W51A. This indicates that the transition state is sensitive to the donating properties of the substituent, and thus electron transfer from the substrate is rate-limiting. In addition, the absence of outliers from the correlation indicates that there is no specific interaction of a particular aniline with CCP in a manner that would either enhance or inhibit the expected structure-activity relationship. The fact that our data are better correlated with the σ^+ coefficients than the σ values indicates a transition state in which partial charge is developing directly on the aromatic ring. This is in contrast to similar correlations which have been made for the reaction of HRP compounds I and II with anilines and phenols (Huang and Dunford, 1990; Sakurada *et al.*, 1990), in which the data are better correlated with the σ values. These results were interpreted in terms of a transition state involving a coordinated electron and proton transfer from the amine group of the aniline to the ferryl heme center (Huang and Dunford, 1990). The quite different behavior of CCP and the distal cavity mutants of this study may indicate that the mechanism of substrate oxidation differs significantly in this respect for the two enzymes. The negative slopes (ρ) observed for these reactions indicate that electron donating substituents assist traversal of the transition state, as expected for electron transfer from the substrate. The similar magnitudes of ρ for CCP(MKT), W51F, and W51A demonstrate a similar sensitivity of these reactions to electronic effects of the substituents and thus indicate a similar mechanism for each enzyme.

Activation Parameters for the Oxidation of Anilines—Although it is possible that W51F and W51A induce hyperactivity with respect to a given substrate by favorably altering its association with the enzyme, it is unlikely that the same mutations would exert similar effects simultaneously for cytochrome *c* and each of a number of substituted anilines. A similar mutation (F41V) of HRP has been shown to result in a 2-fold reduction in K_d for benzhydroxamic acid binding, but the effect of this mutation on binding to *p*-aminobenzoic acid, in which higher than wild-type activity was observed, is unknown (Smith *et al.*, 1992). Thus, the increased rate constants observed for the distal heme cavity mutants of both CCP and HRP could be due to changes in substrate binding or to a generalized increase in ferryl reactivity of the nature proposed for CCP W51F and W51A (Goodin *et al.*, 1991). Activation parameters for the oxidation of aniline by the $Fe^{4+}{=}O$ state of CCP(MKT), W51F, and W51A were examined in order to obtain more information about the nature and cause of the hyperactivity exhibited by the mutants. The data show that both of the mutants have a free energy of activation that is approximately 3 kcal/mol lower than that of CCP(MKT), and that this is due to an increase in the activation entropy. The positive activation entropies for these mutants is at variance with the results for a number of substitution-inert iron complexes as reductants, in which $\Delta S_0{}^*$ values of −10 to −30 e.u. were observed (Purcell and Erman, 1976). The negative activation entropies are more typical of a transition state that is determined by the bimolecular encounter between enzyme and substrate. The positive $\Delta S_0{}^*$ values observed in this work for the oxidation of aniline by CCP(MKT) indicates an increase in entropy as the transition state is traversed, such as a unimolecular species dissociating into distinct units. Most importantly, the effect of the mutations at Trp-51 is to dramatically enhance this entropy increase as the transition state is traversed. While it is possible that the mutations alter the flexibility or solvation of the protein, no such changes have been noted in the reported structure of W51F (Wang *et al.*, 1990). We therefore propose that in the reaction of CCP compound II with anilines, the transition state is associated with reduction of the ferryl heme, in which the $Fe^{4+}{=}O$ oxygen is converted to Fe^{3+}-OH or subsequently released as H_2O. It is noted that the indole nitrogen of Trp-51 is within hydrogen-bonding distance to the $Fe^{4+}{=}O$ oxygen (2.9 Å), and it has been reported that the W51F mutation results in a shift in the frequency of the $Fe^{4+}{=}O$ bond (Wang *et al.*, 1990). We have previously proposed (Goodin *et al.*, 1991) that the increased reactivity of Trp-51 mutants may result from an intrinsic increase in the reactivity of the oxidized heme center, and this study fully supports this conclusion. We further suggested that this effect may have been due to a change in the energies of the heme molecular orbitals brought about by the altered hydrophobic packing of Trp-51 around the heme. Such an effect would be expected to manifest itself in changes in the activation enthalpies. However, this study indicates that, at least for the reaction of the $Fe^{4+}{=}O$ group with anilines as the reducing agent, the hyperactivity of the W51F and W51A mutants may be a direct result of the loss of the hydrogen bond between the Trp-51 indole proton and the ferryl oxygen, facilitating the release of the ferryl oxygen atom as H_2O. Thus, the role of Trp-51 in CCP may be that of stabilizing the enzyme in the ferryl state, through its hydrogen-bonding interaction with the ferryl oxygen.

It is possible that small molecule substrates such as aniline may have access to two different sites for reduction of the $Fe^{4+}=O$/Trp-191 center of CCP. Recent evidence has implicated the surface residues Ala-193 and Ala-194 near the Trp-191 radical center as the site for electron transfer from cytochrome *c* (Pelletier and Kraut, 1992), and this surface exposed site is certain to be accessible to small organic substrates. However, HRP appears to interact with a number of substrate analogs near the heme 8-methyl group, indicating access to heme (Morishima and Ogawa, 1979; Sakurada *et al.*, 1986; Veitch and Williams, 1990). This position would roughly correspond to the entrance to the peroxide access channel, assuming a similar general topology for CCP and HRP (Welinder 1992). CCP appears to oxidize small molecule substrates such as guaiacol and ferrocyanide by interacting at a site on CCP that is different than that utilized by cyt *c*. Reconstitution of apo-CCP with modified hemes containing substituents at the δ-meso position resulted in diminished reactivity with small molecule reductants but not cytochrome *c* (DePillis *et al.*, 1991). In addition, CCP W51A has been recently found to epoxidize styrene by incorporation of the oxyferryl oxygen, and to partially form the iron-phenyl complex upon reaction with phenyldiazene (Miller *et al.*, 1992). These results, more typical of the P_{450} enzymes, indicate that the W51A mutant may have a more open distal heme face than most peroxidases. One interpretation of the data is that the increased volume in the distal heme cavity expected for W51A is responsible for this reactivity. However, W51A and W51F exhibit a similar increase in reactivity, but would extend significantly different side chain volumes into the cavity. Therefore, the removal of the indole-ferryl interaction is more likely to be responsible for the altered reactivities than changes in the distal cavity volume. Without this stabilizing interaction to the oxyferryl oxygen, the heme may be more reactive with respect to phenyldiazene addition to the heme as well as increased electron transfer from cytochrome *c* and a number of artificial donors. While it is yet unresolved to what extent small molecules gain access to the distal heme face of CCP, it is clear that modification of the distal cavity may be utilized to alter the intrinsic reactivity of this enzyme to such substrates.

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