Tryptophan-191 → Phenylalanine, a Proximal-Side Mutation in Yeast Cytochrome *c* Peroxidase That Strongly Affects the Kinetics of Ferrocytochrome *c* Oxidation†

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ABSTRACT: On the basis of X-ray structural information, it was previously proposed that tryptophan-191 of yeast cytochrome *c* peroxidase (CCP) may be important in determining the spectroscopic and catalytic properties of the enzyme [Edwards, S. L., Xuong, Ng. H., Hamlin, R. C., & Kraut, J. (1987) *Biochemistry 26*, 1503–1511]. By use of site-directed mutagenesis and an *Escherichia coli* expression system, a mutant phenylalanine-191 (F191) CCP was prepared in order to examine the effects of altering the H-bonding and π–π interactions that occur between Trp-191 and the iron-coordinated proximal His-175 in the parent enzyme. The F191 mutant enzyme exhibits a dramatic decrease ($\sim$3000-fold at pH 7) in V_0/e for catalysis of peroxide-dependent ferrocytochrome *c* oxidation, while V_0/e for oxidation of ferrocyanide is decreased only 4.6-fold compared to that of the parent. The Fe^{3+}/Fe^{2+} $E_{m,7}$ and the stability of the oxyferryl center in the H_2O_2-oxidized mutant enzyme are relatively unaffected by the mutation, but the species responsible for a radical-like signal centered at $g = 2.00$ has been destabilized $\sim$100-fold with respect to spontaneous decay. Steady-state kinetic assays as well as transient-state laser flash photolysis experiments utilizing flavin semiquinones as reductants indicate that the mutant CCP forms a complex with cytochrome *c* but the oxyferryl center in the oxidized enzyme is no longer able to be rapidly reduced by ferrocytochrome *c*. The most likely reasons for this kinetic behavior are either that new steric constraints exist in the mutant which impede relaxation of the iron center to the resting ferric state or that the indole ring of Trp-191 is important in a specific interprotein electron-transfer pathway that exists between the heme centers of CCP and cytochrome *c*.

Understanding the means by which the protein portion controls the properties of the bound heme in hemoproteins is a longstanding goal of much biochemical research. Recent work has focused on heme-containing peroxidases, which catalyze the peroxide-dependent oxidation of a variety of substrates by mechanisms which involve cycling between Fe^{3+} (ferric) and Fe^{4+}=O (oxyferryl) states of the heme prosthetic group (Hewson & Hager, 1979). Among these enzymes, yeast cytochrome *c* peroxidase (CCP)[1] (EC 1.11.1.5) and its specific macromolecular redox partner, cytochrome *c*, have been studied as a model system for the intermolecular electron-transfer process in proteins (Poulos & Kraut, 1980a,b; Waldemeyer et al., 1982; Ho et al., 1986; Cheung et al., 1986; Hazzard et al., 1987). It has been proposed that the placement of and interactions among amino acid side chains on the heme proximal side are important factors in the control of the formation, stabilization, and eventual breakdown of intermediates in the CCP enzymic mechanism (Fujita et al., 1983; Poulos & Kraut, 1980a,b; Finzel et al., 1984). More specifically, it is believed that stabilization of the oxyferryl heme in compounds I and II[2] of CCP may be accomplished by charge delocalization onto the proximal His-175 and that the redox properties of the heme are further modulated by hydrogen bonding and more extensive charge delocalization among adjacent proximal amino acid residues (Poulos & Finzel, 1984; Chance et al., 1987).

An intriguing aspect of the chemistry of CCP compound I is the existence of an unusually stable radical that is believed to reside within the protein portion of the H_2O_2-oxidized enzyme (Yonetani et al., 1966a; Hoffman et al., 1979; Hori & Yonetani, 1985). While previous mutagenesis studies aimed at elucidating the site or sites of the unpaired electron in compound I have demonstrated the delicate nature of the exact heme-cleft structure responsible for the details seen in EPR spectra (Fishel et al., 1987; Goodin et al., 1987), they have failed to unambiguously define the protein features responsible for the principal paramagnetic spectral characteristics of preparations of the oxidized enzyme, namely, two overlapping signals in the EPR spectrum, consistent with at least two radical centers having different symmetry and temperature-dependent relaxation properties (Hori & Yonetani, 1987;

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; CCP(MI), *E. coli* produced cytochrome *c* peroxidase; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate; ENDOR, electron nuclear double resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; modified TB medium, 10.8 g of tryptone, 21.6 g of yeast extract, 3.6 g of glycerol, 10 g of NaCl, 2.3 g of KH_2PO_4, and 12.5 g of K_2HPO_4 per liter; cytc, cytochrome *c*; $E_{m,7}$, midpoint potential measured at pH 7; HRP, horseradish peroxidase; metMb, ferric sperm whale myoglobin; DEAE-cellulose, (diethylaminoethyl)cellulose; LFH•, lumiflavin semiquinone; 5-DRf, oxidized 5-deazariboflavin; 5-DRfH•, semiquinone of 5-deazariboflavin; R•, radical site in H_2O_2-oxidized CCP; NO, nitric oxide.

[2] Compound I is the two-equivalent oxidation product of ferric CCP; compound II is the one-equivalent reduction product of compound I.

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Goodin et al., 1987). With these results in mind and on the basis of the model studies of Fujita et al. (1983) as well as the refined X-ray structures of both resting (Fe^{3+})CCP (Finzel et al., 1984) and compound I (Edwards et al., 1987) we believed that proximal Trp-191 may be important in the formation and stabilization of both the radical and oxyferryl heme centers of compound I. The position of the indole ring of Trp-191, which lies parallel to and in van der Waals contact with the imidazole of proximal His-175, suggests the likelihood of π–π interactions by which the redox properties of the heme might be modulated by relatively small protein movements. Furthermore, we previously proposed (Edwards et al., 1987) that the indole ring of Trp-191 may function in the oxidized CCP intermediate as part of an extended π–orbital system on the heme proximal side which may stabilize the radical site(s) of compound I, and it is possible that the complex magnetic behavior exhibited by compound I may arise as a result of such a delocalized stabilization apparatus. Finally, the indole nitrogen of Trp-191 participates in a hydrogen-bonded network, which includes the side chains of proximal His-175 and Asp-235, an assembly which may also contribute to the stabilization of the oxyferryl states of the enzyme.

We have recently developed a system for the mutation and expression of yeast CCP in *Escherichia coli* (Fishel et al., 1987) and are using deliberately engineered mutant enzymes constructed with this system to investigate various aspects of the CCP enzymic mechanism. In this paper we describe the initial characterization of a mutant CCP in which Trp-191 has been replaced with a phenylalanine. Observations of the mutant enzyme included optical spectra, EPR at 89 K, properties of the H_2O_2-oxidized enzyme, steady- and transient-state kinetics, and redox potential measurements. The results of these studies show that this Trp-191 → Phe mutant exhibits a profoundly impaired ability to catalyze the peroxide-dependent steady-state oxidation of ferrocytochrome *c*. Furthermore, although the mutant enzyme retains the ability to stabilize the oxyferryl center upon reaction with peroxide, the radical species in the oxidized mutant enzyme has been strongly destabilized as a result of the mutation. Finally, the rate at which the oxyferryl center can accept electrons from reduced cytochrome *c* has been greatly decreased as a result of the mutation at position 191. These results lead us to conclude that Trp-191 is absolutely required for the efficient function of physiologically significant redox processes involving the heme iron of CCP and for the stability of the protein-based radical in compound I.

Experimental Procedures

Materials. Materials for site-specific mutagenesis were procured from the sources described by Fishel et al. (1987). A synthetic 25-mer DNA fragment was prepared on an Applied Biosystems 380A DNA synthesizer at the Agouron Institute, La Jolla, CA. The sequence of the mutagenic 25-mer DNA primer was based on the sequence for the gene published by Kaput et al. (1982). The nucleotide sequence was 5′-GTTAGCGGCTCCGAATGGCCCTTCG-3′, where the indicated bases allowed the original TGG(Trp) codon to be changed to TTC(Phe) in the mutant DNA sequence as a result of the mutagenesis protocol.

Horse heart cytochrome *c* used in steady-state assays of peroxidase activity was Sigma type VI and was used as received. Horse heart cytochrome *c* used for laser flash experiments was further purified by ion-exchange chromatography on CM-cellulose. Bakers' yeast CCP was isolated as described previously (Hazzard et al., 1987). Sperm whale myoglobin (type II) was purchased from Sigma and assumed to be 100% metmyoglobin as received; horseradish peroxidase (HRP) was purchased from Boehringer and was used without further purification.

H_2O_2 was diluted from either 3% (for laser flash experiments) or 30% stock solutions obtained from Mallinkrodt. Potassium ferrocyanide, $K_4Fe(CN)_6$ was of reagent grade from Mallinkrodt. 5-Deazariboflavin was the generous gift of Drs. William McIntire and Thomas Singer. The source of lumiflavin has been previously described (Simondsen & Tollin, 1983).

Methods. Oligonucleotide-directed site-specific mutagenesis was carried out by the methods described by Fishel et al. (1987), with a modification in mutant selection protocol suggested by Kunkel (1985). Selection of the mutant was also aided by digestion of candidate DNA with the restriction endonuclease *Nco*I. Mutation of the CCP gene resulting in a change of the Trp-191 codon (TGG) to the Phe codon (TTC) resulted in the loss of an *Nco*I restriction site, an event which was easily observed by analyzing the restricted DNA from mutant candidates on an acrylamide gel. Following plaque purification, the mutant gene in its phage vector [M13mp8F191CCP(MI)] was completely sequenced using ^{35}S dideoxy methodology (Sanger et al., 1977; Biggin et al., 1983) in order to verify that the proper mutation had been obtained and to demonstrate that the remainder of the CCP gene remained unchanged. The mutated DNA was moved into the pUC8 plasmid for expression of the protein in *E. coli* as described by Fishel et al. (1987).

The expression system previously described (Fishel et al., 1987) was used to produce both the parent cytochrome *c* peroxidase and the Trp-191 → Phe mutant protein used in these studies. The parent peroxidase expressed in *E. coli* is designated CCP(MI), where MI represents the amino acids Met-Ile appended onto the N-terminus of the mature peroxidase amino acid sequence. This construction was used to allow facile DNA manipulation as well as to provide an obligatory translation initiation signal.

Cell growth conditions, addition of bovine hemin, and purification procedures were performed for CCP(MI) and the phenylalanine-substituted mutant [CCP(MI,F191)] as previously described (Fishel et al., 1987), with the exception that cells transformed with pUC8CCP(MI,F191) were grown in a richer (modified-TB) medium supplemented with 300 μg/mL ampicillin in order to obtain increased cell density and higher mutant protein production. All protein used in these experiments was crystallized two times by dialysis of 0.5–1 mM protein solutions dissolved in 30 mM potassium phosphate (pH 6.0 at 4 °C) against distilled water at 4 °C. Protein was stored at cryogenic temperatures as crystal suspensions in water. The twice-crystallized proteins were judged to be homogeneous by SDS–polyacrylamide gel electrophoresis.

UV/visible spectra were recorded at 23 °C on a Perkin-Elmer Lambda 3B spectrophotometer which was computer controlled with software supplied by Softways, Inc., Riverside, CA. Buffers for spectra had a constant ionic strength of 0.10 M, except where otherwise indicated. Compositions of buffers were as follows: pH 5, sodium acetate; pH 6 and 7, potassium phosphate; pH 8.4, sodium glycylglycine.

The 30% hydrogen peroxide stocks were periodically standardized with $KMnO_4$. All peroxide solutions were prepared in cold 10 mM potassium phosphate, pH 7.0, containing 1 mM EDTA.

The extinction coefficient for CCP(MI,F191) (0.1 M potassium phosphate, pH 6.0, 23 °C) was determined by the basic pyridine hemochromogen method (Paul et al., 1953). A

value of 109 ± 2 mM^{-1} cm^{-1} at 408 nm was determined by assuming 1 mol of heme/mol of mutant protein. Concentrations of CCP(MI) were calculated with ϵ = 102 mM^{-1} cm^{-1} at 408 nm (Fishel et al., 1987). Concentrations of bakers' yeast CCP were calculated with ϵ = 93 mM^{-1} cm^{-1} at 408 nm. Ferrocytochrome *c* concentrations were calculated with ϵ = 27.6 mM^{-1} cm^{-1} at 550 nm and an $\epsilon_{red} - \epsilon_{ox}$ of 19.6 mM^{-1} cm^{-1}. Concentrations of HRP and sperm whale metMb were determined using ϵ = 108 mM^{-1} cm^{-1} at 403 nm for HRP and 157 mM^{-1} cm^{-1} at 409 nm for metMb.

Steady-state assays of CCP activity were performed with $Na_2S_2O_4$-reduced horse heart cytochrome *c* that had been separated from excess reducing agent by chromatography on Sephadex G-25. A ternary buffer system of constant ionic strength μ = 50 mM in the pH range 4–8 as described by Ellis and Morrison (1982) and consisting of acetic acid/MES/Tris was used to assay the pH dependence of peroxide-dependent steady-state ferrocytochrome *c* oxidation by CCP(MI), CCP(MI,F191), sperm whale metMb, and HRP. Adjustments of pH in this buffer system were made with $HClO_4$ or NaOH. Steady-state peroxide-dependent oxidation of ferrocytochrome *c* was assayed in the presence of initial pseudo-first-order concentrations of reduced cytochrome *c* and H_2O_2 of 45 and 160 μM, respectively. The values for V_0/e reported were calculated by use of the initial background-corrected change in absorption at 550 nm that occurred upon oxidation of ferrocytochrome *c*. Molar protein concentrations in the 1-mL assay cuvette in steady-state measurement of peroxide-dependent oxidation of ferrocytochrome *c* were as follows: CCP(MI), 8.7×10^{-11}; CCP(MI,F191), 2.6×10^{-7}; sperm whale metMb, 1.2×10^{-6}; HRP, 4.8×10^{-7}.

Steady-state apparent K_m values for horse heart cytochrome *c* were determined for CCP(MI) and CCP(MI,F191) at pH 6 and 23 °C in the μ = 50 mM ternary acetic acid/MES/Tris buffer system previously described. In these assays, the initial concentration of H_2O_2 was held constant at 160 μM while the initial concentration of ferrocytochrome *c* was varied from 3 to 75 μM, and apparent K_m values were derived from standard double-reciprocal plots of $1/V_0$ versus $1/[cytc^{2+}]$.

Steady-state assays of peroxide-dependent oxidation of potassium ferrocyanide, $K_4Fe(CN)_6$, were conducted in the ternary buffer system previously described, with 8 mM ferrocyanide and 500 μM H_2O_2 initially present in the assay cuvette; these conditions were selected to provide pseudo-first-order kinetics on the basis of previous published work by Jordi and Erman (1974b). V_0/e values were calculated by use of the initial change in absorption at 420 nm by assuming an extinction coefficient of 1.0 mM^{-1} cm^{-1} for ferricyanide. No effort was made at this time to more rigorously elucidate steady-state kinetic parameters. Molar protein concentrations in the 1-mL assay cuvette were as follows: CCP(MI), 5.5×10^{-9}; CCP(MI,F191), 3.4×10^{-8}; HRP, 1.2×10^{-9}.

The procedures for the laser flash experiments have been described previously (Simondsen & Tollin, 1983; Hazzard et al., 1987). Reaction kinetics were measured at low ionic strength (μ = 8 mM) in a 3 mM phosphate buffer containing 500 μM EDTA and 70 μM flavin. For all experiments involving the 1:1 complexes, cytochrome *c* and CCP were maintained at equimolar concentrations. For reduction of cytochrome *c* in the 1:1 complex with ferric CCP, lumiflavin was used as a reductant. Under these conditions, lumiflavin semiquinone does not reduce ferric CCP (Hazzard et al., 1987). For the reduction of the H_2O_2-oxidized CCP species in the complex with cytochrome *c*, ferric CCP in the presence of the cytochrome *c* was titrated with 5 mM H_2O_2 immediately prior to all experiments. It has been shown previously (Hazzard et al., 1987) that 5-deazariboflavin does not reduce free bakers' yeast CCP compound I on a time scale comparable to the lifetime of the flavin semiquinone. In the presence of ferric cytochrome *c*, one-electron reduction of the peroxidase does occur; however, the mechanism involves direct flavin reduction of the cytochrome *c*, followed by a first-order intracomplex reduction of compound I (see Results). 5-Deazariboflavin semiquinone was used as the reductant due to its extremely high rate of reaction with cytochrome *c* (Meyer et al., 1983). Thus, the first-order intracomplex electron transfer, rather than the second-order reduction of the complexed cytochrome *c*, is rate limiting. Reduction of the peroxide-oxidized CCP species was monitored at 550 nm (Hazzard et al., 1987). Pseudo-first-order rate constants (k_{obsd}) were derived from linear plots of log ΔS versus time, where ΔS was the measured signal intensity for the averaged value of a minimum of four transient decay curves. Second-order rate constants were determined from the slopes of linear plots of k_{obsd} vs concentration.

Reduction potentials of CCP(MI) and CCP(MI,F191) were measured by the method of mixtures with photochemical titration with riboflavin/EDTA as the mediator/reductant system. Solutions contained 10 mM EDTA, 20 mM potassium phosphate, 33 μM riboflavin, and 30 μM protein at pH 7.0 (23 °C). The solution, minus protein, was purged of oxygen by bubbling for 1 h with deoxygenated argon gas. After introduction of the protein, photochemical reduction was accomplished by direct illumination with a fluorescent lamp for increasing increments of time. Reduction of the heme/riboflavin system was monitored at 560 and 480 nm for the protein-bound heme (Fe^{3+}/Fe^{2+}) and riboflavin, respectively. Redox potentials were calculated by use of a potential of −205 mV for riboflavin (Draper & Ingraham, 1975).

The UV/visible spectra of peroxide-oxidized CCP(MI) and CCP(MI,F191) at pH 6 (0.1 M potassium phosphate, 23 °C) were recorded immediately after addition of H_2O_2 to either protein contained in a cuvette at 10 μM protein concentration.

Spectrophotometric observation of the spontaneous decay of peroxide-oxidized CCP(MI,F191) enzyme in dilute solutions was accomplished by adding a 1:1 stoichiometric amount of H_2O_2 to the protein at 10 μM in 0.1 M potassium phosphate, pH 6.0, 23 °C. Visible spectra were recorded immediately (120 nm/min scan speed) and at timed intervals for 20 h after the addition of peroxide. The half-life for spontaneous decay was calculated by measuring the decrease in absorbance at 424 nm over time (Erman & Yonetani, 1975).

EPR spectra (89 K) of the resting and peroxide-oxidized forms of CCP(MI,F191) were obtained on an IBM ER 200D EPR spectrometer as described by Fishel et al. (1987). The pH dependence of the resting-state EPR spectra was investigated in 0.1 M buffer at pH 6 and 7 (phosphate) and in sodium glycylglycine at pH 8.4 and approximately 0.75 mM protein concentration. The EPR spectrum of peroxide-oxidized CCP(MI,F191) was obtained on a sample that was prepared by mixing and rapidly freezing a solution of ice-cold enzyme with ice-cold H_2O_2 at pH 6.

Time-dependent changes in the EPR spectrum of peroxide-oxidized CCP(MI,F191) were studied by mixing the protein dissolved at 0.75 mM in 0.1 M potassium phosphate, pH 6, with a 10% stoichiometric excess of H_2O_2 at room temperature. At various measured times, aliquots were removed into quartz EPR tubes and rapidly frozen in liquid nitrogen. In this experiment, the elapsed time between mixing the protein/peroxide solutions and freezing the first-removed

aliquot was 60 s. A parallel experiment was conducted in which aliquots of the oxidized protein were removed, and the visible absorption spectrum was recorded after appropriate dilution into 0.1 M potassium phosphate pH 6.0 (23 °C). This allowed an estimate of the effect of the high protein concentration used in the EPR experiments on the rate of decay of the oxyferryl iron visible spectrum.

Determination of equilibrium constants for dissociation of cyanide from CCP(MI) and CCP(MI,F191) was performed at 23 °C in 0.1 M potassium phosphate, pH 6, according to the titrimetric method described by Yonetani and Ray (1965). K_D values were calculated from Scatchard plots as described by Erman (1974).

Visible spectra of CCP(MI) and CCP(MI,F191) at liquid nitrogen temperature were recorded on an Aminco DW-2 spectrophotometer equipped with the manufacturer's low-temperature attachment. Protein solutions were injected into lucite cells (2-mm path length) which had been precooled in liquid nitrogen to ensure rapid freezing. Buffers used for the low-temperature pH profile were the same as those used to collect ambient temperature spectra, except that 0.25 M sucrose was present in each case to aid in devitrification. The exact temperature of the frozen cell was not monitored; however, an effort was made to position the cell in the N_2 vapor in the same way each run.

Results

Expression of Yeast CCP and the Trp-191 → Phe Mutant in E. coli. Cytochrome *c* peroxidase expressed in the *E. coli* system we have developed (Fishel et al., 1987) is referred to as CCP(MI). The CCP gene was derived from *Saccharomyces cerevisae* by Goltz et al. (1982). Construction of the parent CCP(MI) expression vector involved replacement of the codons specifying amino acid residues −2 and −1 of the CCP leader sequence (Kaput et al., 1982) with codons specifying the amino acids Met-Ile [hence CCP(MI)] in order to create a *Bcl*I restriction site and the required Met initiation codon. The new *Bcl*I restriction site allowed substitution of the CCP(MI) gene into the *Bcl*I site of the plasmid pUC8DHFR in order to take advantage of its highly efficient mutant promoter (Fishel et al., 1987).

The CCP(MI) parent enzyme as well as the mutant enzyme described in these experiments has two additional sequence variations as compared with bakers' yeast CCP (Takio et al., 1980; Kaput et al., 1982): Gly-152 instead of Asp and Ile-53 instead of Thr. The combined effects of these two differences in primary sequence as well as the two amino acids Met-Ile appended onto the protein N-terminus have resulted in no functionally significant difference in properties of the CCP(MI) enzyme compared to those of bakers' yeast CCP which would be expected to influence the interpretation of the present work.

The CCP(MI) mutant with Trp-191 changed to Phe is designated CCP(MI,F191). The apoprotein of the latter was isolated from *E. coli* by the method previously described for CCP(MI) (Fishel et al., 1987). The mutant apoprotein was able to accept bovine hemin at pH 7, and after removal of unbound excess heme by chromatography on DEAE-cellulose, the mutant holoenzyme was readily crystallized by slow dialysis against distilled water. As with CCP(MI), freezing and storage at −80 °C of the crystalline mutant enzyme suspended in water had no observed adverse effect on its properties, as judged by comparison with freshly prepared mutant holoenzyme that had not been frozen.

Optical Spectroscopy of Resting CCP(MI,F191) at Room Temperature. UV/visible absorption spectra were obtained

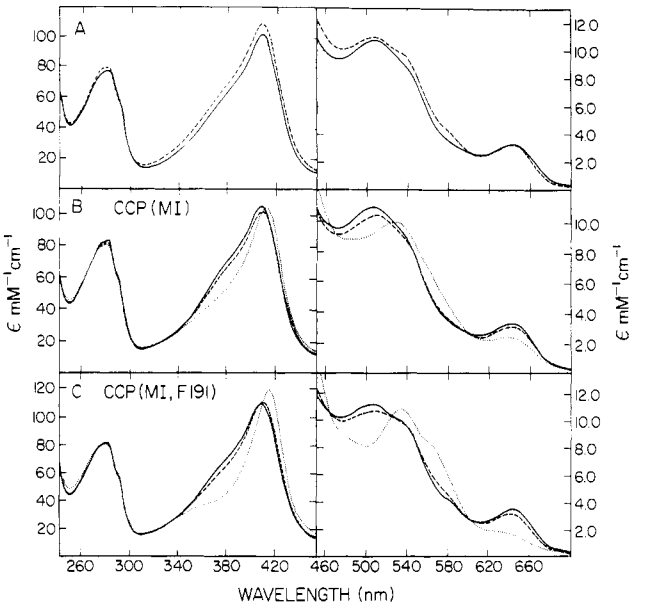

FIGURE 1: UV/visible spectra of ferric CCP(MI) and CCP(MI,F191). Panel A: CCP(MI) (solid) and CCP(MI,F191) (dashed) at pH 6. Panel B: CCP(MI) at pH 5 (solid), pH 7 (dashed), and pH 8.4 (dotted). Panel C: CCP(MI,F191) at pH 5 (solid), pH 7 (dashed), and pH 8.4 (dotted).

for CCP(MI,F191) and compared with the spectra of CCP(MI) in order to assess the effect of the mutation on the spin-state equilibrium of the ferric heme of the enzyme. Figure 1A compares the optical spectra of CCP(MI) and CCP(MI,F191) in 0.1 M potassium phosphate at pH 6, conditions under which both proteins are stable in dilute solution for several hours. The spectra are similar and indicate the presence of largely high-spin ferric iron as judged by comparison with the published spectrum of (aquo)metMb (Eaton & Hochstrasser, 1968). However, the mutant enzyme has a somewhat higher extinction at the Soret maximum (ϵ_{408nm} = 109 mM^{-1} cm^{-1}) than CCP(MI) (ϵ_{408nm} = 102 mM^{-1} cm^{-1}) (Fishel et al., 1987), and the bands centered at approximately 540 and 580 nm are slightly more pronounced compared to these features in the CCP(MI) visible spectrum. These differences were reproducibly present for CCP(MI,F191) from several independent preparations and are most probably due to the presence of a small amount of 6-coordinated heme, as we have recently demonstrated using the resonance Raman technique (Smulevich et al., 1988). The 408/280 ratio for CCP(MI,F191) is normally ~1.4, an increase from the 1.3 value seen for pure CCP(MI); this result is undoubtedly due to a decrease in absorption at 280 nm resulting from the replacement of one of the seven tryptophan residues in CCP(MI) with phenylalanine.

The room-temperature UV/visible spectrum of bakers' yeast CCP exhibits only small changes over the pH range 5–7; conversion to a low-spin alkaline form occurs when the pH is further elevated (Yonetani et al., 1966b). The *E. coli* derived CCP(MI) parent enzyme used in the present experiments also exhibits this behavior, as shown in Figure 1B, in which the altered spectrum observed for the alkaline form at pH 8.4 is readily observable. The CCP(MI,F191) mutant enzyme (Figure 1C) behaves in a very similar manner as the pH is raised. At pH 8.4, the spectrum of the mutant enzyme indicates that conversion to a low-spin form whose spectrum clearly resembles that of the alkaline form of CCP(MI) has occurred; however, the mutant spectrum has more well-defined bands centered at 540 and 580 nm as well as a larger decrease in the intensities of the bands centered at 505 and 640 nm

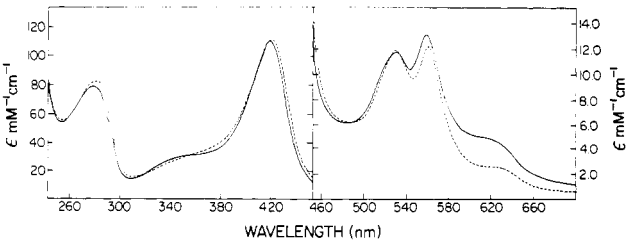

FIGURE 2: UV/visible spectra of H_2O_2-oxidized CCP(MI) and CCP(MI,F191) at pH 6. Protein concentrations were 10 μM; a 50% molar excess of H_2O_2 was added to the ferric form in either case. CCP(MI) (solid); CCP(MI,F191) (dashed).

relative to these features in the CCP(MI) alkaline spectrum.

It is evident that the mutation at position 191 has affected the coordination chemistry of the ferric heme to some extent so that interaction with a sixth ligand, which is probably a water molecule with variable degrees of hydroxide character depending on pH, has been enhanced. The pK of conversion to a low-spin alkaline form is lowered somewhat in CCP(MI,F191), judging by the spectrum at pH 8.4 which shows a greater conversion toward a low-spin form.

Optical Spectroscopy of H_2O_2-Oxidized CCP(MI,F191). When a 5 μM solution of CCP(MI,F191) at pH 6 was treated with increasing amounts of H_2O_2, it was found that 1 mol of H_2O_2/mol of mutant enzyme was required to achieve the maximum change in absorbance measured at 424 nm (data not shown). Beyond the 1:1 ratio of added H_2O_2, no significant change in optical density was observed. Thus, the initial reaction that occurs when the mutant enzyme reacts with H_2O_2 appears to be a two-electron redox event, analogous to the reaction that occurs with the parent CCP(MI) enzyme and H_2O_2.

The compound that is observed immediately after mixing the mutant enzyme with approximately equimolar H_2O_2 has an optical absorption spectrum which is closely similar to, but not identical with, the spectrum of CCP(MI) compound I (Figure 2). Both the Soret band and the band centered at approximately 560 nm are red-shifted by about 2 nm in the mutant. The prominent shoulder that occurs between 580 and 640 nm in compound I of CCP(MI) is about 50% as intense in the spectrum of the H_2O_2-oxidized mutant enzyme; the diminished intensity of this feature probably accounts for the lowered intensity of the adjacent and overlapping 560-nm band in the oxidized mutant enzyme.

Considering the rapid room-temperature decay of the g = 2.00 signal seen in the 89 K EPR spectrum of the H_2O_2-oxidized mutant enzyme (see below), we assume that the absorption spectrum shown in Figure 2 is essentially that of the Fe(IV) protein lacking the protein-based radical that exists in compound I of CCP(MI) (Fishel et al., 1987). Both the visible absorption spectrum of the HRP compound II (Adar, 1978) and the derived Fe(IV) spectrum of bakers' yeast CCP (Coulson et al., 1971) represent peroxidase enzymes in the oxyferryl state without an associated radical; in both spectra the 580–640-nm shoulder, which is a major feature of the spectrum of compound I of CCP, is much diminished. Since the same shoulder is also similarly diminished in the spectrum of H_2O_2-oxidized CCP(MI,F191), we infer that electronic features associated with the oxidized radical site are correlated with the enhanced light absorption in this region that occurs in compound I of CCP(MI) and bakers' yeast CCP.

Spontaneous Decay of the Oxyferryl (Fe^{4+}=O) Center in H_2O_2-Oxidized CCP(MI,F191). An interesting feature of the chemistry of cytochrome c peroxidase is the ability of the enzyme to stabilize a reactive oxyferryl heme center within the protein interior; a reported value for the $t_{1/2}$ of the oxyferryl spontaneous decay of bakers' yeast CCP is 6.6 ± 1.4 h [k = (2.9 ± 0.6) × 10^{-5} s^{-1}] (Erman & Yonetani, 1975). In order to examine the effect of the Trp-191 → Phe mutation on the stability of the oxyferryl center in the H_2O_2-oxidized enzyme, the spontaneous decay of the visible spectrum was measured. This decay obeyed first-order kinetics for at least 2.5 half-lives, having a $t_{1/2}$ of 4.9 ± 0.5 h [k = (3.9 ± 0.4) × 10^{-5} s^{-1}]. At a 75-fold higher protein concentration, the decay again obeyed first-order kinetics; however, $t_{1/2}$ was 48 ± 4 min [k = (2.4 ± 0.2) × 10^{-4} s^{-1}] under these conditions. Thus, the first-order rate constant increased approximately 6-fold with a 75-fold increase in protein concentration. Since the decay process obeys first-order kinetics at either concentration of mutant enzyme but occurs with a shorter half-life at the higher concentration, it seems likely that spontaneous decay of the oxyferryl center occurs via at least two parallel processes. At the low enzyme concentration first-order intraprotein decay likely dominates, while at the higher protein concentration the oxidized mutant enzyme may form aggregates which facilitate reduction of the oxyferryl species via a first-order process whose rate is enhanced as a result of intermolecular interactions. The decay of the oxyferryl center in the aggregated protein would be expected to exhibit first-order kinetics if aggregate formation is not rate limiting. In support of this aggregation hypothesis, it is noted that Spangler and Erman (1986) have recently reported the formation of significant amounts of covalently linked aggregates in bakers' yeast CCP as a result of the decay of the oxyferryl species.

The published results of analogous experiments (Erman & Yonetani, 1975), in which the spontaneous decay rates of the oxyferryl centers of 10 and 300 μM compound I of bakers' yeast CCP were compared, showed that the first-order decay constant at the higher concentration increased by a factor of about 1.5; although the present experiments compared 10 and 750 μM CCP(MI,F191) mutant enzyme concentrations, the concentration dependence of the oxyferryl decay rate appears to be somewhat greater in this case. Regardless of the detailed reasons for the observed decay kinetics, it is concluded that CCP(MI,F191) is able to effectively stabilize the oxyferryl heme, although the propensity for reduction of the Fe^{4+}=O center by intermolecular mechanisms, possibly involving aggregates at high protein concentration, appears to be enhanced for the mutant enzyme.

89 K EPR Spectroscopy of Ferric CCP(MI,F191). EPR spectra of CCP(MI) and CCP(MI,F191) were obtained at 89 K in order to ascertain the effects of the mutation on the magnetic properties of the ferric heme at cryogenic temperature. It is well-known that ferric CCP undergoes significant spin-state changes when cooled below room temperature, and when observed frozen at 89 K, the EPR and visible absorption spectra are strongly dependent on pH as well (Yonetani et al., 1966; Yonetani, 1971; Hori & Yonetani, 1985). The pH dependence is manifested as a steady high- to low-spin trend between pH 5 and pH 9, with evidence of loss of heme orientation and/or protein denaturation occurring above pH 9 (Hori & Yonetani, 1985). The enzyme apparently exists in a pH-dependent equilibrium with pentacoordinated high-spin heme favored at low pH and hexacoordinated low-spin heme favored at high pH (Yonetani & Anni, 1987); the spin transition that occurs with increasing pH is likely due to coordination of hydroxide ion to the ferric iron.

When 89 K EPR spectra for the CCP(MI) parent enzyme were obtained at pH 6, 7, and 8.4, the expected shift toward low-spin iron with increasing pH was observed, as judged by

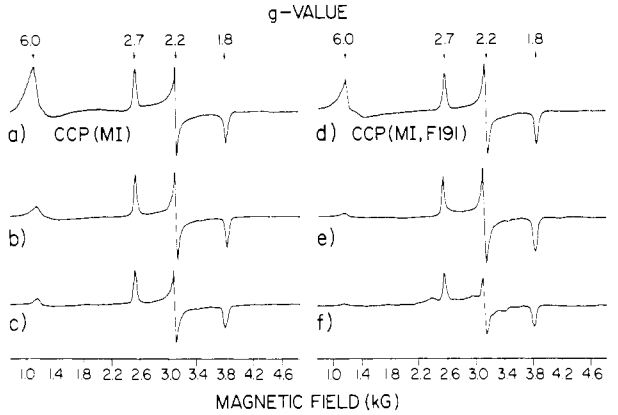

FIGURE 3: First-derivative EPR spectra of ferric CCP(MI) and CCP(MI,F191) measured at 89 K. CCP(MI) at (a) pH 6, (b) pH 7, and (c) pH 8.4. CCP(MI,F191) at (d) pH 6, (e) pH 7, and (f) pH 8.4. Enzyme concentrations were 0.68 mM for CCP(MI) and 0.89 mM for CCP(MI,F191); the traces were scaled accordingly. Spectrometer conditions: 9.4 GHz, 54-mW (GdB) microwave power, and 8-G modulation amplitude. Receiver gain varied from 4.0 to 8.0 ($\times 10^4$).

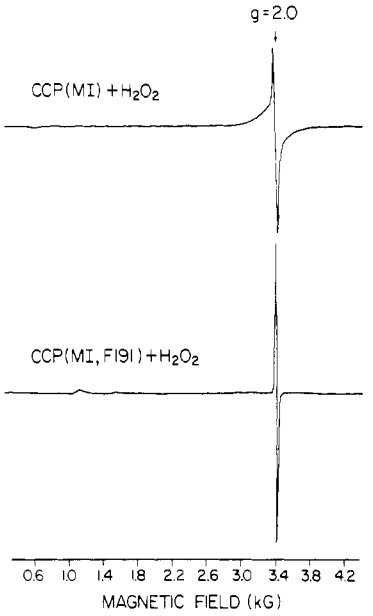

FIGURE 4: Comparison of first-derivative line shapes of $g = 2.00$ EPR signals of H_2O_2-oxidized CCP(MI) and CCP(MI,F191) at 89 K. CCP(MI) at 0.74 mM final enzyme concentration with 10% molar excess H_2O_2. CCP(MI,F191) of 0.73 mM final enzyme concentration with 50% molar excess of H_2O_2. Rapidly mixing with 10% excess H_2O_2 gives essentially identical results (unpublished observations). Spectrometer conditions: 9.4 GHz, 54-mW (GdB) microwave power, and 8-G modulation amplitude. Receiver gain was 7.96×10^4 for CCP(MI) and 3.17×10^4 for CCP(MI,F191).

the decreasing amplitude of the broad $g_\perp = 7$–4 high-spin signal relative to the rhombic low-spin signals at $g = 2.7$, 2.2, and 1.8 (Figure 3a–c). The broad signal in the region $g_\perp = 7$–4 has been demonstrated to be due to a rhombically distorted axially symmetric pentacoordinated high-spin ferric heme (Anni & Yonetani, 1987). When the EPR spectra of CCP(MI,F191) were obtained at pH 6, 7, and 8.4, a high- to low-spin trend was again observed (Figure 3d–f). A significant difference, however, between the spectra for the mutant and the parent enzymes was the shifted pH dependence of the disappearance of spin intensity of the $g_\perp = 7$–4 signal. Thus, at pH 6, the spectra for both proteins are essentially identical, but at pH 7 the relative intensity of this signal in the mutant spectrum is much less than for the CCP(MI) parent (compare spectra b and e of Figure 3). In fact, the spectrum of the mutant at pH 7 is much like that of the parent CCP(MI) at pH 8.4, indicating that the CCP(MI,F191) mutant enzyme undergoes some transition(s) that result(s) in the disappearance of the broad $g_\perp = 7$–4 high-spin signal at a pH of 1–1.5 units lower than occurs for the parent. Additional evidence for a shifted pK for some transition(s) which affect(s) the iron spin equilibrium may be seen by examining the EPR spectrum of the mutant at pH 8.4 (Figure 3f). The intensities of the low-spin rhombic signals decrease considerably at pH 8.4 for the mutant protein, in a manner which is very similar to the behavior exhibited by bakers' yeast CCP above pH 9.5 (Hori & Yonetani, 1985). This phenomenon has been attributed to loss of a unique heme binding geometry at pH 9.5; it is therefore possible that the pH at which changes in heme binding occur has been lowered for CCP(MI,F191). Visible absorption spectra recorded at 89 K (not shown) for CCP(MI) and CCP(MI,F191) confirmed the results of the 89 K EPR studies; the pH-dependent spin transition for the mutant occurred at least 1 pH unit lower for the mutant enzyme, as judged by comparison to frozen spectra of CCP(MI)

The reasons for the pH dependence of the spin equilibrium observed in CCP at low temperatures, in contrast to its relative pH independence at room temperature, are not well understood. However, it is likely that freezing-induced protein structural changes slightly alter the relative positions of the heme and iron so that the interaction with a sixth ligand becomes more favorable at low temperature. If this is the case, then we can conclude that the proximal Trp-191 → Phe mutation allows the heme iron to more effectively interact with a sixth ligand, probably by allowing the iron to move toward the heme distal side. Thus, the lowered pK for the high- to low-spin transition in the mutant may be due to a more favorable interaction of the heme iron with a coordinated water molecule, leading to a lowered pK for formation and binding of hydroxide ion as the sixth ligand.

89 K EPR Spectroscopy of H_2O_2-Oxidized CCP(MI,F191). Upon reaction with a stoichiometric quantity of H_2O_2, CCP(MI) forms a two-equivalent oxidized compound I species with an 89 K EPR spectrum which is identical with that of compound I of bakers' yeast CCP and which is characterized by a single, isotropic, radical-like signal centered at $g = 2.004$ (Fishel et al., 1987) (Figure 4). Previous EPR studies performed in this laboratory on the distal-side CCP mutant, Trp-51 → Phe, (Fishel et al., 1987) and work by Goodin et al. (1987) on the proximal-side mutant, Met-172 → Ser, have demonstrated the sensitivity of the exact shape and line width of the radical-like signal to these structural perturbations in the heme binding cleft. Additionally, the previous work revealed a much-reduced stability of the oxidized site responsible for the radical signal in the Phe-51 mutant. In order to test the hypothesis that Trp-191 plays a role in stabilizing and determining the magnetic characteristics of the radical site in CCP, we obtained the 89 K EPR spectra of a sample of CCP(MI,F191) that was frozen immediately after addition of H_2O_2 and also of samples of the H_2O_2-oxidized mutant enzyme that were allowed to stand at room temperature for measured time periods after reaction with peroxide.

When a 0.73 mM solution of ice-cold CCP(MI,F191) was mixed with a 50% molar excess of H_2O_2 at pH 6 and rapidly frozen, the 89 K EPR spectrum showed nearly complete conversion to a new species with an isotropic signal centered at $g = 2.00$ (Figure 4). It is notable that the "wings" flanking the $g = 2.00$ signal, which are characteristic of compound I of bakers' yeast CCP (Yonetani et al., 1966) and CCP(MI) (Figure 4), are essentially missing in the spectrum of the

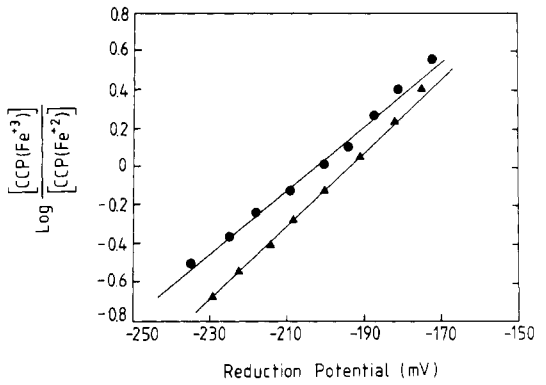

FIGURE 5: Plots of log ([CCP(Fe^{3+})]/[CCP(Fe^{2+})]) versus redox potential for the photochemical titration of CCP(MI) and CCP(MI,F191). CCP(MI): (▲) $E_{m,7}$ = −194 mV. CCP(MI,F191): (●) $E_{m,7}$ = −202 mV. Experimental details are given under Methods.

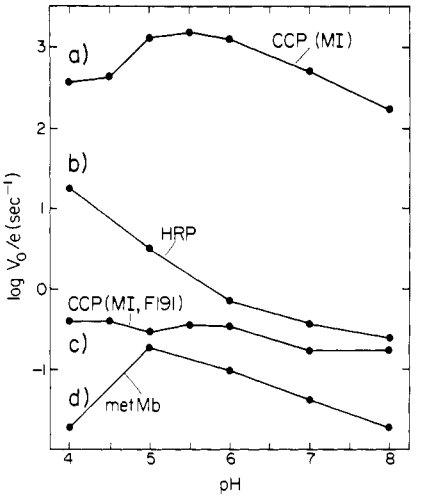

FIGURE 6: Steady-state peroxidase activities of CCP(MI) and CCP(MI,F191) with horse heart ferrocytochrome *c* as reducing substrate. Samples of HRP and metMb were assayed under identical conditions for comparative purposes. V_0/e = initial rate of reaction (mol L^{-1} s^{-1}) divided by the enzyme concentration (mol L^{-1}). Experimental details are given under Methods.

H_2O_2-oxidized CCP(MI,F191) mutant. Diminished wings have been observed for the radical signals of the H_2O_2-oxidized Phe-51 and Ser-172 CCP mutants previously studied (Fishel et al., 1987; Goodin et al., 1986). Thus, the Phe-191 mutant is the third modified CCP for which the detailed characteristics of the 89 K EPR spectrum of the H_2O_2-oxidized protein have been affected.

An experiment which monitored the decay at room temperature of the chemical species that gives rise to the radical signal centered at g = 2.00 in the 89 K EPR spectrum of H_2O_2-oxidized CCP(MI,F191) revealed that this center decayed very rapidly (data not shown). Our preliminary estimate of $t_{1/2}$ for the disappearance of the narrow isotropic EPR signal is ~1 min. This result stands in contrast to the room-temperature stability of the radical center of compound I of bakers' yeast CCP, which decays with a $t_{1/2}$ of ~2.5 h (Erman & Yonetani, 1975). Although the rapid decay of the radial center in CCP(MI,F191) made estimation of its exact decay rate impossible, it is evident that the lifetime of this species is less than one percent of the lifetime of the stabilized radical site in bakers' yeast CCP. This rapid decay rate for the *proximal* Trp-191 → Phe mutant is similar to the decay rate obtained for the *distal* Trp-51 → Phe mutant previously described (Fishel et al., 1987). The tryptophan residues positioned on *both* sides of the heme therefore appear to be necessary to achieve the stability of the radical observed in the parent protein. We conclude that the CCP(MI,F191) mutant enzyme is able to react with H_2O_2 to give a radical species but is unable to effectively stabilize that species within the protein structure. Whether or not this radical site in the H_2O_2-oxidized mutant is identical with that of compound I of CCP is not known; this determination will require more extensive EPR investigations (see Discussion).

Reduction Potential of CCP(MI,F191). The midpoint potential ($E_{m,7}$) of the Fe^{3+}/Fe^{2+} couple for different proteins containing the iron protoporphyrin IX prosthetic group varies over a wide range, e.g., HRP (−260 mV) (Yamada et al., 1975), CCP (−190 mV) (Conroy et al., 1978), and hemoglobin (+160 mV) (Abraham & Taylor, 1975). This range of values undoubtedly reflects the sensitivity of this thermodynamic parameter to specific heme–protein interactions; therefore, we measured the $E_{m,7}$ for the CCP(MI,F191) mutant protein, as a further means to investigate the functional effects of any structural perturbations that had occurred as a result of the mutation. Although the Fe^{3+}/Fe^{2+} couple is not believed to be involved in any enzymically relevant reaction of CCP, we expected that significant changes in this reduction potential might reflect similar changes in the potential for the enzymically relevant Fe^{4+}/Fe^{3+} couple.

Figure 5 shows a plot of log ([Fe^{3+}]/[Fe^{2+}]) versus potential for the reducing phototitrations carried out at pH 7. The $E_{m,7}$ value for CCP(MI) was calculated to be −194 mV (n = 1.1), a value which is in excellent agreement with the −190-mV midpoint potential previously reported for bakers' yeast CCP (Conroy et al., 1978). Under identical conditions, CCP(MI,F191) was found to have an $E_{m,7}$ of −202 mV (n = 1.0). We note that the present titrations were performed in the reductive direction only, but isosbestic points were maintained in the visible spectra for greater than 90% of the reduction process in either case, indicating that clean conversion to a single reduced species occurs (data not shown).

On the basis of these measurements, which reflect the relative stabilities of the Fe^{3+}/Fe^{2+} states of the heme embedded in the CCP protein matrix, we conclude that only a small effect on the redox potential of the enzyme has occurred as a result of the present mutation.

Steady-State and Transient Kinetics of CCP and CCP(MI,F191). The results of steady-state assays measuring peroxide-dependent oxidation of ferrocytochrome *c* revealed a dramatic decrease in activity for CCP(MI,F191) relative to that of CCP(MI). At pH 5.5, near the pH optimum for CCP(MI) activity, the mutant is 0.02% as active as the parent enzyme (Figure 6, curves a and c). This greatly depressed catalytic oxidation of cytochrome *c* was observed from pH 4 to pH 8 (Figure 6, curve c). Furthermore, the shape of the pH vs CCP activity curve for the mutant is clearly altered. The pH profile for CCP(MI) shown in Figure 6 is nearly identical with that obtained for bakers' yeast CCP (Yonetani & Ray, 1966), with the added feature that no buffer-related discontinuities occur since the assays were performed in a ternary buffer system at constant ionic strength (μ = 50 mM) (see Experimental Procedures).

The extent of diminution of catalytic activity resulting from the mutation was made apparent by comparing the activity of CCP(MI,F191) to the cytochrome *c* peroxidase activity of commercially obtained sperm whale metmyoglobin (metMb) and horseradish peroxidase (HRP) across the pH 4–8 range. The background-corrected log (V_0/e) versus pH profiles for these proteins in the catalysis of peroxide-dependent oxidation of horse heart ferrocytochrome *c* are shown in Figure 6, curves b and d. Placed within this context, the extent of impairment

Table I: Second-Order Rate Constants for Reduction of Ferric Horse Cytochrome *c* in 1:1 Complexes with Various Ferric CCP Species

CCP species	k ($\times 10^{-7}$ M^{-1} s^{-1})[a]
bakers' yeast	1.9
CCP(MI)	3.0
CCP(MI,F191)	2.2
{free cytc(Fe^{3+})}[b]	7.4

[a] Values obtained from plots of K_{obsd} vs concentration. See Methods. [b] Data from Hazzard et al. (1987).

of activity for the mutant CCP is especially striking, since HRP, which presumably has no significant degree of specificity for ferrocytochrome *c* as a reducing substrate, is a superior catalyst at all pH values compared to CCP(MI,F191). However, the mutant enzyme *does* turnover substrates at all pH values at a rate which is clearly discernible from the background uncatalyzed rate; furthermore, this remaining activity is higher than that found for metMb under the same conditions.

The apparent K_m for horse cytochrome *c* was determined for both the CCP(MI) and the mutant enzyme at μ = 50 mM and pH 6, in order to examine effects of the mutation on cytochrome *c* binding. The residual activity displayed by CCP(MI,F191) clearly showed saturation kinetics, and the apparent K_m for horse cytochrome *c*, derived from double-reciprocal plots, was 12.8 ± 1.5 μM, whereas for CCP(MI) a value of 10.2 ± 1.5 μM was obtained. These results suggest that the steady-state binding of horse ferrocytochrome *c* by the mutant CCP is not seriously perturbed as a result of the Trp → Phe mutation.

In order to examine in more detail the nature of the complex formed between cytc and the ferric species of CCP(MI) or CCP(MI,F191), we have measured the effect of complexation at low ionic strength (8 mM) on the kinetics of reduction of horse cytc(III) by photogenerated lumiflavin semiquinone (LfH•). By this technique, we have previously been able to determine the relative steric accessibilities of the heme groups of several cytochromes *c* within electrostatically stabilized 1:1 complexes with bakers' yeast CCP (Hazzard et al., 1987, 1988). Second-order rate constants for the reduction of free and peroxidase-complexed cytochrome *c* are given in Table I. In the complex with ferric CCP(MI), a 60% decrease in the reduction rate constant was observed, which is similar to the decrease obtained in the presence of ferric bakers' yeast CCP. In the case of complexation with CCP(MI,F191), a 70% decrease was observed. As the percent decrease in the rate constant is determined mainly by the relative steric accessibility of the cytochrome *c* heme (Tollin et al., 1987), we conclude that there is only a slight difference between the relative cytochrome *c* heme accessibilities in complexes with the three different forms of CCP. Thus, the Trp → Phe mutation apparently has little or no effect on the degree of exposure of the cytochrome *c* heme group within the complex with CCP, which is consistent with the conclusion drawn above from the steady-state data.[3]

In order to further explore the markedly diminished catalytic capability of CCP(MI,F191), relative to that of bakers' yeast CCP or CCP(MI), direct measurements were made of one-electron transfer from cytochrome *c*(II) to the H_2O_2-oxidized intermediates of the mutant and CCP(MI) parent enzymes. It has been shown previously (Hazzard et al., 1987) that in

[3] The data are not interpreted as showing the exact geometric orientations of the proteins within any complexes formed but only the relative cytochrome *c* heme accessibility.

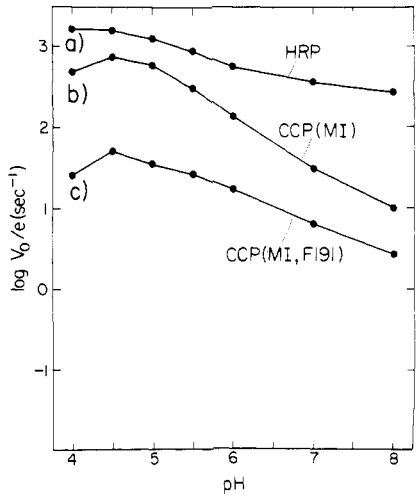

FIGURE 7: Steady-state peroxidase activities of CCP(MI) and CCP(MI,F191) with potassium ferrocyanide [$K_4Fe(CN)_6$] as reducing substrate. A sample of HRP was assayed under identical conditions for comparative purposes. Experimental details are given under Methods.

the absence of cytochrome *c* baker's yeast CCP compound I is not appreciably reduced on a rapid time scale (~50 ms) by free flavin semiquinones. However, in the presence of cytochrome *c*(III) at low ionic strength, rapid one-electron reduction of compound I occurs by the following mechanism:

$$5\text{-DRfH}^{\bullet} + \text{cytc}(Fe^{3+})\text{–CCP}(Fe^{4+},R^{\bullet}) \rightarrow 5\text{-DRf} + \text{cytc}(Fe^{2+})\text{–CCP}(Fe^{4+},R^{\bullet})$$

$$\text{cytc}(Fe^{2+})\text{–CCP}(Fe^{4+},R^{\bullet}) \rightarrow \text{cytc}(Fe^{3+})\text{–CCP}(Fe^{3+},R^{\bullet})$$

where 5-DRfH• and 5-DRf stand for the semiquinone and oxidized forms of 5-deazariboflavin, respectively. Thus, the reduction of CCP compound I occurs by first-order intracomplex electron transfer from cytochrome *c*(II).

When we measured the intracomplex electron-transfer rate constants for reduction of the oxyferryl center in compound I of bakers' yeast and CCP(MI) by horse ferrocytochrome *c* at low ionic strength, very similar values were obtained for bakers' yeast and *E. coli* derived CCP(MI) peroxidase species, 750 and 850 s^{-1}, respectively. This result suggests that the relative orientations of and/or distance between the protein-bound heme groups in the two complexes are very similar. In marked contrast, in the presence of cytochrome *c* no reduction of the peroxide-oxidized species of CCP(MI,F191) could be detected on time scales as long as 1 s, even though the reduction of complexed ferric cytochrome *c* had rapidly occurred. Therefore, one important factor in the extremely slow turnover of cytochrome *c* observed in the steady-state assays is apparently the inability of the mutant peroxidase to accept reducing equivalents from ferrous cytochrome *c* on a rapid time scale.

H_2O_2 Oxidation of Ferrocyanide Catalyzed by CCP(MI,F191). Ferrocyanide ion $Fe(CN)_6^{4-}$ has previously been used as a model iron-containing small-molecule reductant in investigations of both steady-state and transient kinetics of bakers' yeast CCP (Jordi & Erman, 1974a,b). Although the steady-state catalysis by CCP of the oxidation of ferrocyanide by H_2O_2 is characterized by saturation kinetics, the apparent K_m varies from 1 to 10 mM depending upon pH and buffer conditions; the binding of ferrocyanide is thus 100–1000 times weaker than that for ferrocytochrome *c*. To observe the effects of the Trp-191 → Phe mutation on the steady-state kinetics with the relatively nonspecific ferrocyanide substrate, assays were carried out over the pH range 4–8. Figure 7 displays

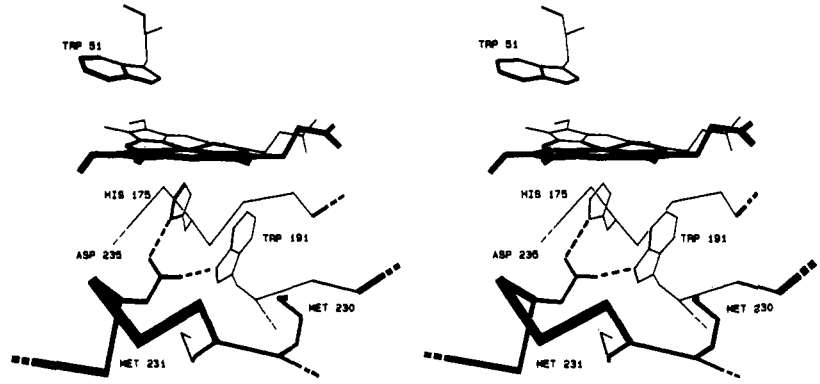

FIGURE 8: Stereoscopic view of some important amino acid residues on the heme proximal side. Distal Trp-51 is also shown. Hydrogen bonds between the Asp-235 carboxyl and His-175 and Trp-191 are shown by dotted lines.

the log (V_0/e) versus pH for assays of the reaction catalyzed by CCP(MI,F191), CCP(MI), and HRP. The results of assays using CCP(MI,F191) show that the mutant has reduced activity compared to CCP(MI) across the entire pH range studied. The CCP(MI) parent enzyme exhibits a profile closely similar to the one published by Jordi and Erman (1974b) for bakers' yeast CCP; the shapes of the pH versus V_0/e profiles for oxidation of ferrocyanide are quite similar for CCP(MI) and CCP(MI,F191) (Figure 7b,c), with 4.5 being the optimum pH for either enzyme.

Comparison of the log (V_0/e) versus pH profiles for assays conducted with either ferrocytochrome *c* or ferrocyanide as the reducing substrate reveals that the initial rate of H_2O_2-dependent ferrocyanide oxidation has been much less affected by the Trp-191 → Phe substitution. Thus, the ratio of V_0/e for ferrocyanide oxidation by CCP(MI,F191) to V_0/e for CCP(MI) varies as follows: pH 4, 5.1%; pH 5, 6.0%; pH 6, 12.5%; pH 7, 21.7%; pH 8, 26.2%. In contrast, the initial rate of ferrocytochrome *c* oxidation catalyzed by CCP(MI,F191) compared to CCP(MI) varies as follows: pH 4, 0.11%; pH 5, 0.022%; pH 6, 0.028%; pH 7, 0.033%; pH 8, 0.09%. Depending on the pH, the mutation has therefore had a 50–650-fold greater negative effect on V_0/e for ferrocytochrome *c* oxidation compared to ferrocyanide oxidation. The magnitude of the effect increases with increasing pH, with the maximal 650-fold differential occurring at pH 7.

Cyanide Equilibrium Dissociation Constant for CCP(MI) and CCP(MI,F191). On the basis of the analysis of optical and EPR spectra obtained in this study, as well as on resonance Raman results (unpublished observations), we believed that the ferric iron in CCP(MI,F191) is able to more effectively interact with distal ligands than the ferric iron of CCP(MI). On the basis of previous work by Yonetani and Ray (1965), we reasoned that determination of K_D for binding between cyanide and CCP(MI,F191) would allow a quantitative measure of changes in the binding properties of the heme iron in the mutant protein relative to those of bakers' yeast CCP or CCP(MI).

The titrimetric K_D value for cyanide dissociation from CCP(MI) was determined to be 2.5 ± 0.3 μM, in excellent agreement with the value of 3.0 ± 0.2 μM for bakers' yeast CCP (Yonetani & Ray, 1965). For CCP(MI,F191), the K_D for cyanide dissociation was found to be 0.8 ± 0.3 μM, or about 3-fold smaller than the value found for CCP(MI). The cyanide complex for the mutant is, therefore, about 0.6 kcal/mol more stable than the parent CCP(MI) cyanide complex at pH 6. On the basis of these equilibrium measurements, we conclude that the interaction of the heme ferric iron of CCP(MI,F191) with the strong-field cyanide ligand is increased slightly with respect to that of CCP(MI).

In summary, the Trp-191 → Phe mutation has resulted in a modified enzyme with the following characteristics, compared to those of CCP(MI). (1) The apoenzyme readily incorporates ferric heme, but a lowered pK is seen for some structural transition(s), which may ultimately lead to changes in heme binding. (2) The mutant forms a compound I like species with a stable oxyferryl center upon reaction with H_2O_2, but the stability of the site responsible for the radical signal centered at $g = 2.00$ is reduced ~100-fold. (3) The steady-state catalysis of oxidation of ferrocytochrome *c* is decreased ~1000–5000-fold depending on pH, while the oxidation rate of the nonspecific ferrocyanide substrate is only decreased ~4–20-fold. (4) The rate of reduction of the H_2O_2-oxidized mutant enzyme by bound ferrocytochrome *c* is decreased by at least 1000-fold, as determined by direct transient kinetic studies using the preformed macromolecular complex. (5) The Fe^{3+}/Fe^{2+} midpoint potential is only slightly affected by the mutation.

DISCUSSION

The positions of the amino acid residues on the heme proximal side that we consider important in evaluating the properties of the CCP(MI,F191) mutant are shown in Figure 8. It is seen that Asp-235 bridges iron-coordinated His-175 and Trp-191 by means of hydrogen bonds and that the indole ring of Trp-191 and the imidazole ring of His-175 are parallel and in van der Waals contact. Trp-191 is thus poised to indirectly interact with the heme iron mediated by π–π interactions with His-175. It may also affect the protonation state of His-175 through hydrogen-bonding interactions with bridging Asp-235. The feasibility of charge transfer between the heme iron and proximal His-175 has been demonstrated by the model studies of Fujita et al. (1983), and charge delocalization onto Trp-191 via π-orbital contact with His-175 would appear to be chemically reasonable. Furthermore, it has been proposed that the enhancement of electron density in the imidazole ring of His-175 that results from its H-bonding interaction with Asp-235 may aid in the formation of oxyferryl intermediates in the CCP enzymic mechanism (Finzel et al., 1984; Chance et al., 1986; Hashimoto et al., 1986); chemical tuning of the heme iron may thus be accomplished by an arrangement of electronic and hydrogen-bonded interactions on the proximal side of the heme. The important H-bonding triad would be His-175–Asp-235–Trp-191, with additional π-orbital stacking interactions also possible.

The refined 2.5-Å low-temperature X-ray structure of compound I of bakers' yeast CCP suggested that a number of small adjustments in the region of Trp-191 may occur in response to oxidation by H_2O_2 (Edwards et al., 1987). On the basis of these results, combined with computer graphics

modeling and chemical reasoning, we previously proposed that the protein-based radical in compound I may be delocalized over Trp-191 and the thioether sulfur atoms of methionine-230 and -231, which bracket the indole nitrogen of the tryptophan (Figure 8). Trp-191 was proposed to play a central role in this delocalization apparatus, since the sulfur atoms of Met-230 and -231 are too far apart (~6 Å) to stabilize a cation radical by direct S–S orbital overlap.

Prompted by these structural observations and speculation about their importance to the chemistry of CCP, we have replaced Trp-191 with phenylalanine by site-directed mutagenesis in order to obtain a variant enzyme with minimal structural perturbations but with altered hydrogen-bonding and π-orbital interactions at position 191. Additionally, we reasoned that the phenyl ring of phenylalanine would be likely to disrupt any radical stabilization apparatus that has the indole ring of Trp-191 as its centerpiece.

The most significant result of the present study is the approximately 1000–5000-fold decrease in the rate of catalysis of H_2O_2-dependent oxidation of ferrocytochrome *c* for the CCP(MI,F191) mutant compared to its CCP(MI) parent. Since it was observed that the mutant reacts rapidly with H_2O_2 (unpublished stopped-flow observations), it was surmised that the low overall steady-state activity may be due to a decreased ability of the mutant enzyme to complete the catalytic cycle by accepting reducing equivalents from ferrocytochrome *c*. This was indeed found to be the case, as was demonstrated by the results of laser flash experiments in which the rate of reduction of the oxyferryl intermediate of the F191 mutant in complex with cytochrome *c* was at least 1000-fold slower than the measured reduction rate in the compound I CCP(MI)–cytc complex. While the precise reasons for the diminution in the ability of the H_2O_2-oxidized mutant enzyme to be efficiently reduced by ferrocytochrome *c* remain unclear, some of the more obvious possibilities were explored in the present studies.

The observation that the $E_{m,7}$ for the Fe^{3+}/Fe^{2+} couple of the mutant enzyme is not significantly different from the $E_{m,7}$ for CCP(MI) demonstrates that the protein structural factors that govern the reduction potential of the *ferric* heme have not been seriously altered by the mutation. The compound II/Fe^{3+} redox potential for bakers' yeast CCP has been estimated to be approximately +1.1 V (Purcell & Erman, 1976), and similar values for both the compound I/compound II and compound II/Fe^{3+} couples have been measured for HRP (Hayashi & Yamazaki, 1979). If it is assumed that the redox behavior for the high oxidation state intermediates is similar for CCP and HRP and furthermore that, by extrapolation, there exists only about an 8–10-mV difference between the CCP(MI) and CCP(MI,F191) reduction potentials for the more highly oxidized couples, then a reasonable conclusion is that the present kinetic results cannot be explained on the basis of changes in reduction potential for the mutant enzyme. From the established similarities between HRP and CCP, the assumption of +1000 mV for the E_0' for the CCP(MI) compound I/compound II and compound II/Fe^{3+} redox couples is probably justified, but the second assumption, that the small change in the Fe^{3+}/Fe^{2+} $E_{m,7}$ for the mutant accurately reflects the effect of the mutation on the higher oxidation states, is more tenuous. For example, in the case of HRP, isozyme A has a Fe^{3+}/Fe^{2+} E_0' that is ~60 mV *more positive* than that of isozyme C (Yamada et al., 1975); but E_0' for the compound II/ferric couple of isozyme A is ~80 mV *more negative* than that of isozyme C (Hayashi & Yamazaki, 1979). However, on the basis of the ~3000-fold decrease in V_0/e observed for CCP(MI,F191) at pH 7, we estimate that the reduction potential of either the compound I/compound II or the compound II/Fe^{3+} couple (or both) would have to be lowered by at least 200 mV to account for the kinetic differences observed.[4] While we consider this to be unlikely, direct measurement of the reduction potentials for the oxyferryl centers of CCP(MI) and CCP(MI,F191) would be of value in order to allow more definitive conclusions to be made about the effects of the mutation on the redox thermodynamics of the enzyme.

The rapid rate of reaction of compound I with ferrocytochrome *c* has been attributed to the formation of a specific and tightly bound macromolecular complex which enables electron transfer to occur 10^3–10^4 times more rapidly than the reaction of compound I with metal complexes having redox potentials and self-exchange rates comparable to those of cytochrome *c* (Yandell & Yonetani, 1983). The present kinetic results show essentially no change in the apparent K_m for ferrocytochrome *c* or in the ability of the mutant enzyme to inhibit reduction of ferricytochrome *c* by free flavin semiquinones. These results indicate that the mutant enzyme and cytochrome *c* interact in solution in a manner analogous to the interaction of CCP(MI) and cytochrome *c*. It seems likely, therefore, that the Trp → Phe substitution at position 191 in the protein interior has no significant effect on the surface interactions required for cytochrome *c* binding. We have recently obtained further support for this conclusion, since there are no large structural perturbations in the crystallized mutant enzyme (J. Wang, unpublished X-ray crystallographic observations). Thus, the large difference in electron-accepting properties of CCP(MI) and CCP(MI,F191) probably cannot simply be attributed to altered binding of cytochrome *c* due to gross structural changes in the mutant enzyme.

If the reduction potential of the H_2O_2-oxidized F191 mutant enzyme and its ability to form an intermolecular complex or a closely related family of complexes (Northrup et al., 1987) with cytochrome *c* are essentially unchanged, then the rate of intermolecular electron transfer must be affected by other, less obvious, factors. It is likely that the Trp-191 → Phe mutation has altered the chemistry of the enzyme by disrupting the protein-based radical site or by altering the electron-accepting properties of the oxyferryl heme center in the mutant enzyme–cytochrome *c* complex, or both. A third possibility is that the indole of Trp-191 is crucial in some specific electron-transfer pathway which links the iron redox centers in the macromolecular electron-transfer complex.

If the ability to form a specific radical species is important for the catalytic performance of CCP (and this has not been proven), then disruption of the formation of this species by the present Trp → Phe mutation would be expected to seriously affect the kinetics of the catalyzed reaction. That rapidly prepared and frozen peroxide-oxidized CCP(MI,F191) had an 89 K EPR spectrum in which the narrow isotropic signal centered at $g = 2.00$ was clearly the dominant feature we interpret as evidence that the site responsible for this signal in unperturbed parent CCP(MI) is not Trp-191. However, a cautionary note applies to this conclusion, since in the heterolytic cleavage of H_2O_2 by CCP a proposed transient intermediate is formally a Fe(V) heme (Ho et al., 1983) which would likely be capable of rather nonspecifically oxidizing a

[4] This approximation, which assumes that no changes in intracomplex heme–heme distance or in reorganization energy for reduction of the mutant enzyme occur as a result of changes in protein structure, was made as follows:

$$\Delta EMF = (RT/nF) \ln 3000 = 205 \text{ mV}$$

$$n = 1 \quad T = 298 \text{ K}$$

variety of nearby amino acid side chains. For example, even metMb, which is an extremely weak peroxidase, forms a transient $g = 2.00$ radical species upon reaction with H_2O_2 (King & Winfield, 1963).

The species responsible for the narrow isotropic $g = 2.00$ radical signal in the 89 K EPR spectrum of H_2O_2-oxidized CCP(MI,F191) is at least 100-fold less stable with respect to spontaneous decay than the radical species that exists in compound I of the parent enzyme. However, *stability* of a radical species does not appear to be critical for enzyme activity, since the distal Trp-51 → Phe mutant we previously studied had a similarly unstable radical species but showed 2–4-fold *increased* overall steady-state peroxidase activity (Fishel et al., 1987). It is more likely, therefore, that determination of the exact chemical identity, rather than the stability, of any radical species that exist in CCP compound I and H_2O_2-oxidized mutants will lead to an understanding of the role of such species in the enzymic mechanism. It will very likely be necessary to use ENDOR spectroscopy, which is capable of examining more closely the chemical environment of an unpaired electron, to investigate the nature of the species responsible for the narrow isotropic signal in the H_2O_2-oxidized mutant enzyme (Hoffman et al., 1979, 1981). We are currently conducting additional magnetic spectroscopic studies in order to be able to draw more meaningful conclusions about the effects the Trp-191 → Phe mutation has had on the radical site or sites in CCP.

The broad wings which flank the narrow isotropic $g = 2.00$ radical signal in the 89 K EPR spectrum of preparations of compound I of CCP have been attributed to a superimposed signal due to the presence of an axially symmetric radical species which accounts for approximately 90% of the integrated spin intensity seen in the spectrum (Wittenberg et al., 1968; Hoffman et al., 1979; Hori & Yonetani, 1985). Below 25 K, the axial signal narrows and dominates the first-derivative spectrum, although the narrow isotropic signal still exists but is obscured in the powder EPR spectrum by the resolved axial features. Hori and Yonetani (1985) have thus proposed that two radical species exist in preparations of compound I of CCP.

The observation of narrowed wings flanking the $g = 2.00$ radical signal in the 89 K EPR spectrum of H_2O_2-oxidized CCP(MI,F191) indicates that the magnetic center responsible for the broad wings may have been altered or eliminated as a result of the mutation. However, Goodin and co-workers (Goodin et al., 1987) have shown the necessity of a thorough examination of the temperature dependence of the EPR spectrum of any H_2O_2-oxidized CCP mutant before final conclusions are drawn about the effects of the mutation. Thus, the *proximal* Met-172 → Ser (Goodin et al., 1986, 1987) and the *distal* Trp-51 → Phe mutants (Fishel et al., 1987) also had narrowed wings in the 89 K EPR spectrum of the H_2O_2-oxidized enzymes. However, at 10 K the spectrum of either H_2O_2-oxidized mutant enzyme showed the presence of the dominant axially symmetric radical species. From this it was concluded that neither mutation had abolished either of the radical sites in preparations of oxidized CCP; rather, the effect of these mutations appears to have been to alter the temperature dependence of the magnetic properties of the enzyme. Possible mechanisms for this behavior have been elaborated by Goodin et al. (1987). Whether the CCP(MI,F191) mutant will also exhibit the dominant axial EPR signal at 10 K must await the results of low-temperature EPR investigations.

It is intriguing that three separate mutations in CCP at quite different positions with respect to the heme have each had similar effects on the wing signals flanking the narrow $g = 2.00$ signal in the EPR spectrum of the H_2O_2-oxidized enzyme observed at 89 K. A constant feature of these mutants is the replacement of side chains very near to or in electronic contact with the heme by less easily oxidized ones (Met → Ser; Trp → Phe). Indeed, the strategy used to design these mutants has been to remove residues which might allow stabilization of a radical cation aided by nucleophilic interactions or by π-delocalization. The similarity of the observed changes in the 89 K EPR spectra of the oxidized mutant enzymes that result from these diverse mutations may be due to the delocalized π-orbital environment in the heme crevice provided by Met-172, Trp-51, His-175, and Trp-191, since an alteration at one site might be expected to affect the entire electronic system. Furthermore, the observation that a Trp → Phe mutation at *distal* position 51 or *proximal* position 191 destabilizes the radical species formed in the H_2O_2-oxidized mutant enzyme suggests that the stability of the radical site in CCP is also due to delocalization effects which occur in the relatively π-orbital-rich environment that exists in the CCP heme cavity, compared to other heme proteins such as the globins (Poulos & Finzel, 1984).

Since the stability to spontaneous decay of the oxyferryl center is relatively unaffected in the peroxide-oxidized mutant enzyme, it appears that the presence of an indole ring at position 191 is not crucial in determining this property in CCP. CCP(MI,F191) is, therefore, the first yeast peroxidase mutant described which has widely different stabilities for the oxyferryl iron and radical sites. We note that in our previous work the distal Trp-51 → Phe mutant displayed rapid spontaneous decay of both oxidized sites (Fishel et al., 1987).

It has recently been demonstrated experimentally that the oxyferryl heme in bakers' yeast CCP can be rapidly reduced by ferrocytochrome *c* without the radical species being present (Hazzard et al., 1987); however, this does not necessarily mean that the protein structural features responsible for formation and stabilization of the protein radical species are without influence on the reduction properties of the oxyferryl center. The intact structure of this site may be necessary to allow required conformational adjustments or some more exotic electron-gating phenomena leading to reduction of the oxyferryl heme in the compound I and II intermediates of CCP, regardless of the exact electronic disposition of the radical site or sites.

If the rate of reduction of the oxyferryl heme in CCP(MI,F191) by complexed ferrocytochrome *c* is largely independent of the oxidation state of any protein-based radical site, then the effect of the mutation has been to dramatically decrease the intrinsic ability of the Fe(IV) iron to be efficiently reduced by ferrocytochrome *c*. We propose that the two most likely reasons for impaired electron-accepting capability in oxidized CCP(MI,F191) are either (1) that altered steric constraints affecting the energy barrier for relaxation of the oxyferryl to the ferric state exist in the mutant or (2) that complete interruption or much-decreased conductivity of a specific intermolecular redox pathway which has Trp-191 as an important element has occurred as a result of the Trp-191 → Phe substitution.

The imposition of new steric constraints affecting the dynamics of protein movements and ultimately leading to a decreased rate of electron transfer from ferrocytochrome *c* may have occurred as a result of the proximal-side Trp-191 → Phe mutation. According to Marcus theory (Marcus & Sutin, 1985), an important factor which may influence the rate of outer-sphere fixed-distance electron transfer in proteins is the

energy required for reorganization of redox-active metal–ligand centers and their protein environments as they approach the transition-state geometry required for electron transfer. Evidence for the importance of this phenomenon has emerged from experiments with ruthenium-modified myoglobins, in which the energy of reorganization of the heme environment involved in the loss of an iron-coordinated water ligand when $MbFe^{III}$ is reduced to $MbFe^{II}$ is believed to be important in the slow rate of reduction of the ferric heme center by fixed outer-sphere ruthenium(II) electron donors (Crutchley et al., 1985). Furthermore, there is experimental evidence which indicates that changes in oxidation state in bakers' yeast CCP may take place with significant reorganization energy (Cheung et al., 1986). These workers have attributed the energy dependence of electron transfer in various nonphysiological CCP–cytochrome *c* complexes to a significant reorganization energy. A substantial reorganization energy could apply in the enzymically relevant CCP oxyferryl → ferric transition as well.

It is possible that structural adjustments in the CCP molecule that are required for efficient promotion of electron transfer may be induced upon binding of cytochrome *c*. In the F191 mutant, binding of cytochrome *c* may not be able to induce the adjustments that are necessary to lower the energy barrier between the oxyferryl and ferric states, leading to the observed dramatic kinetic results. Since any structural changes leading to efficient electron transfer that may occur as a result of formation of a CCP–cytochrome *c* complex would be expected to depend on the multiple and precise interactions that are possible between the macromolecular redox pair, it would not be possible for the simple ferrocyanide reductant to effect an enhancement of electron transfer by the same mechanism employed by ferrocytochrome *c*. The relatively small difference in V_0/e between parent CCP(MI) and CCP(MI,F191) observed with ferrocyanide as the reducing substrate (4.6-fold at pH 7) compared to the corresponding ~3000-fold difference in V_0/e for ferrocytochrome *c* could well be due largely to a modest difference in reduction potential that may exist between the oxyferryl centers of the mutant and parent enzymes. The very similar shapes of the V_0/e versus pH profiles for CCP(MI) and CCP(MI,F191) with ferrocyanide as the reducing substrate further suggest that oxidation of ferrocyanide proceeds by essentially the same mechanism in either case. In contrast, the divergent profiles observed with ferrocytochrome *c* as reductant indicate that the residual activity displayed by the mutant may be due to a minor or new and essentially different catalytic pathway operating in the F191 mutant. However, it is obvious that a more detailed kinetic analysis of the effects of the present mutation on the individual steps of the overall peroxidase mechanism using either reducing substrate will be necessary before more definitive conclusions can be made.

Evidence that structural changes which can affect the properties of the heme iron centers occur upon formation of a CCP–cytochrome *c* complex has recently been presented, on the basis of altered luminescence properties of the Zn-substituted complexed proteins (Koloczek et al., 1987) and alterations of the chemical shifts of the protons corresponding to the heme 3- and 8-positions and Phe-82 in cytochrome *c* that occur as a result of complex formation (Moench et al., 1988). Furthermore, on the basis of the stability of an isolated Fe(IV) intermediate of bakers' yeast CCP, Ho et al. (1983) have asserted that conformational equilibria are important in the CCP mechanism, although these experiments did not directly investigate CCP–cytochrome *c* interactions.

How could the transition-state energy required for the reorganization of the heme iron center in the Fe(IV) → Fe(III) reduction process be affected by the exact position or identity of the amino acid at proximal position 191? Results of resonance Raman studies on both Fe(II) and Fe(III) CCP(MI,F191) (Smulevich et al., 1988), the altered pH dependence of the electronic absorption and EPR spectra, and the 3-fold tighter binding of cyanide observed for the mutant all indicate that the heme iron in the mutant is less restricted with respect to movement perpendicular to the heme plane than in the parent enzyme. From comparison of the X-ray structures of the ferric and compound I forms of bakers' yeast CCP (Edwards et al., 1987), it is apparent that the iron must "snap back" at least 0.2 Å toward the proximal side upon reduction to the ferric form. The proximal-ligand-dependent tension which may be necessary to allow rapid iron relaxation into the resting-state position may not be present in the mutant due to the loss of the Asp-235–Trp-191 anchoring hydrogen bond that exists in the parent enzyme. Furthermore, small adjustments of the CCP proximal-side structure induced by the binding of cytochrome *c*, and mediated by Trp-191–Asp-235 interactions, may be responsible for transiently enhancing the tension applied to the oxyferryl heme through coordinated His-175 in the parent enzyme, possibly leading to a lowered transition-state energy for the reduction process. The mutant protein, which lacks the required proximal-side interactions, could not utilize the same mechanism.

The intriguing possibility also exists that Trp-191 may be an important link in an electron-transfer pathway which is disrupted by the phenylalanine substitution in CCP(MI,F191). Such a role for tryptophan has recently been asserted on the basis of anomalously high electron-transfer rates in ruthenium-modified myoglobin in which a fortuitously positioned indole ring was proposed to be acting as an electron-transfer enhancer in the "line of flight" between redox centers (Mayo et al., 1986; Kuki & Wolynes, 1987). If such a mechanism for the enhancement of electron transfer is possible, then it would not be surprising if evolutionary adaptation has occurred in which natural protein redox systems are capable of utilizing the same or some similar mechanism. In the present case, the specificity of intermolecular electron transfer between CCP and cytochrome *c* would be due to attainment of a properly aligned complex or set of complexes which would allow Trp-191 to be in a position to enhance electron transfer, possibly in concert with other important intervening residues. We note that, in order for the indole ring of Trp-191 to be in the line of flight between the heme centers in a CCP–cytc complex, the previously proposed model (Poulos & Kraut, 1980a,b) would have to be modified by alteration of the relative positions of the two macromolecular redox partners. The difference in the effect of the F191 mutation of V_0/e for ferrocyanide versus ferrocytochrome *c* would be explained on the basis of an electron-transfer mechanism in which Trp-191 acts as an electron-tunneling enhancer, since it is unlikely that ferrocyanide would bind at the same site on CCP and interact in the same way as ferrocytochrome *c*.

We note that there is evidence from X-ray structural studies which indicates that the position of the indole ring of Trp-191 is sensitive to the coordination state of the heme iron center. In the recently obtained refined structure of the NO complex of ferric bakers' yeast CCP, the indole ring of Trp-191 moves 0.25 Å away from the imidazole ring of His-175 (S. Edwards, personal communication). This movement appears to occur in response to delocalized electron density entering the Fe–His-175–Trp 191 system upon binding of the electron-donating

NO ligand (Yonetani et al., 1972). Additionally, the peptide segment 189–194 is positioned very near the proposed binding site for cytochrome *c* (Poulos & Kraut, 1980a,b), suggesting a possible structural basis for induction of specific protein movements, which may ultimately affect the heme center, upon binding of cytochrome *c*. We will report the results of X-ray structural studies on CCP(MI,F191) in a separate paper.

The greatly altered catalytic capability of cytochrome *c* peroxidase which results from the replacement of a single, non-iron-coordinated, amino acid on the heme proximal side attests to the precise structural requirements at this position which are necessary for efficient enzyme function. It is likely that the eventual elucidation of the detailed reasons for the characteristics exhibited by CCP(MI,F191) will result in an enhancement of our knowledge of peroxidase chemistry as well as of the mechanisms of long-range interprotein electron transfer.

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REFERENCES

Abraham, E. C. & Taylor, J. F. (1975) *J. Biol. Chem. 250*, 3929–3935.

Adar, F. (1978) in *The Porphyrins* (Dolphin, D., Ed.) Vol. III, pp 167–209, Academic New York.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Chance, M., Powers, L., Poulos, T., & Chance, B. (1986) *Biochemistry 25*, 1266–1270.

Cheung, E., Taylor, K., Kornblatt, J. A., English, A. M., McLendon, G., & Miller, J. R. (1986) *Proc. Natl. Acad. Sci. USA 83*, 1330–1333.

Conroy, C. W., Tyma, P., Daum, P., & Erman, J. E. (1978) *Biochim. Biophys. Acta 537*, 62–69.

Coulson, A. F. W., Erman, J. E., & Yonetani, T. (1971) *J. Biol. Chem. 246*, 917–924.

Crutchley, R. J., Ellis, W. R., & Gray, H. B. (1985) *J. Am. Chem. Soc. 107*, 5002–5004.

Draper, R. D., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 805–808.

Edwards, S. L., Xuong, Ng. H., Hamlin, R. C., & Kraut, J. (1987) *Biochemistry 26*, 1503–1511.

Ellis, J. K., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Erman, J. E. (1974) *Biochemistry 13*, 39–44.

Erman, J. E., & Yonetani, T. (1975) *Biochim. Biophys. Acta 393*, 350–357.

Finzel, B. C., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027–13036.

Fishel, L. A., Villafranca, J. E., Mauro, J. M., & Kraut, J. (1987) *Biochemistry 26*, 351–360.

Fujita, I., Hanson, L. K., Walker, F. A., & Fajer, J. (1983) *J. Am. Chem. Soc. 105*, 3296–3300.

Goltz, S., Kaput, J., & Blobel, G. (1982) *J. Biol. Chem. 257*, 11186–11190.

Goodin, D. B., Mauk, A. G., & Smith, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1295–1299.

Goodin, D. B., Mauk, A. G., & Smith, M. (1987) *J. Biol. Chem. 262*, 7719–7724.

Hashimoto, S., Teraoka, J., Inubushi, T., Yonetani, T., & Kitagawa, T. (1986) *J. Biol. Chem. 261*, 11110–11118.

Hayashi, Y., & Yamazaki, T. (1979) *J. Biol. Chem. 254*, 9101–9106.

Hazzard, J. T., Poulos, T. L., & Tollin, G. (1987) *Biochemistry 26*, 2836–2848.

Hazzard, J. T., McLendon, G., Cusanovich, M. A., Das, G., Sherman, F., & Tollin, G. (1988) *Biochemistry 27*, 4445–4451.

Hewson, W. D., & Hager, L. P. (1979) in *The Porphyrins* (Dolphin, D., Ed.) Vol. VII, pp 295–332, Academic, New York.

Ho, P. S., Hoffman, B. M., Kang, C. H., & Margoliash, E. (1983) *J. Biol. Chem. 258*, 4356–4363.

Ho, P. S., Sutoris, C., Liang, N., Margoliash, E., & Hoffmann, B. M. (1985) *J. Am. Chem. Soc. 107*, 1070–1071.

Hoffman, B. M., Roberts, J. E., Brown, T. G., Kang, C. H., & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6132–6136.

Hoffman, B. M., Roberts, J. E., Kang, C. H., & Margoliash, E. (1981) *J. Biol. Chem. 256*, 6556–6564.

Hori, H., & Yonetani, T. (1985) *J. Biol. Chem. 260*, 349–355.

Jordi, H. C., & Erman, J. E. (1974a) *Biochemistry 13*, 3734–3741.

Jordi, H. C., & Erman, J. E. (1974b) *Biochemistry 13*, 3741–3745.

Kaput, J., Goltz, S., & Blobel, G. (1982) *J. Biol. Chem. 257*, 15054–15058.

King, N. K., & Winfield, M. E. (1963) *J. Biol. Chem. 238*, 1520–1528.

Koloczek, H., Horie, T., Yonetani, T., Anni, H., & Vanderkooi, J. M. (1987) *Biochemistry 26*, 3142–3148.

Kuki, A., & Wolynes, P. G. (1987) *Science (Washington, D.C.) 236*, 1647–1652.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.

Mayo, S. L., Ellis, W. R., Crutchley, R. J., & Gray, H. B. (1986) *Science (Washington, D.C.) 233*, 948–952.

Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhattacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740–6744.

Moench, S. J., Shih, T. M., Erman, J. E., & Satterlee, J. D. (1988) *Biophys. J. 53*, p 27A.

Northrup, S. H., Boles, J. O., & Reynolds, J. C. L. (1987) *J. Phys. Chem. 91*, 5991–5998.

Paul, K. G., Theorell, H., & Åkeson, Å. (1953) *Acta Chem. Scand. 7*, 1284–1287.

Poulos, T. L., & Kraut, J. (1980a) *J. Biol. Chem. 255*, 10322–10330.

Poulos, T. L., & Kraut, J. (1980b) *J. Biol. Chem. 255*, 8199–8205.

Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115–171.

Purcell, W. L., & Erman, J. E. (1976) *J. Am. Chem. Soc. 98*, 7033–7037.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., & Rose, B. A. (1980) *J. Mol. Biol. 143*, 161–178.

Simondsen, R. P., & Tollin, G. (1983) *Biochemistry 22*, 3008–3016.

Smith, D. W., & Williams, R. J. P. (1968) *Biochem. J. 110*, 297–301.

Smulevich, G., Mauro, J. M., Fishel, L. A., English, A. M., Kraut, J., & Spiro, T. G. (1988) *Biochemistry 27*, 5477–5485.

Spangler, B. D., & Erman, J. E. (1986) *Biochim. Biophys. Acta 872*, 155–157.

Takio, K., Titani, K., Ericsson, L. H., & Yonetani, T. (1980) *Arch. Biochem. Biophys. 203*, 615-629.
Tollin, G., Hanson, L. K., Caffrey, M., Meyer, T. E., & Cusanovich, M. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3693-3697.
Waldmeyer, B., Bechtold, R., Bosshard, H. R., & Poulos, T. L. (1982) *J. Biol. Chem. 257*, 6073-6076.
Wittenberg, B. A., Kampa, L., Wittenberg, J. B., Blumberg, W. E., & Peisach, J. (1968) *J. Biol. Chem. 243*, 1863-1870.
Yamada, H., Makino, R., & Yamazaki, I. (1975) *Arch. Biochem. Biophys. 169*, 344-353.
Yandell, J. K., & Yonetani, T. (1983) *Biochim. Biophys. Acta 748*, 263-270.
Yonetani, T. (1971) in *Probes of Structure and Function of Macromolecules and Membranes II* (Chance, B., Yonetani, T., & Mildvan, A., Eds.) pp 545-552, Academic, New York.
Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 4503-4508.
Yonetani, T., & Ray, G. S. (1966) *J. Biol. Chem. 241*, 700-706.
Yonetani, T., & Anni, H. (1987) *J. Biol. Chem. 262*, 9547-9554.
Yonetani, T., Schleyer, H., & Ehrenberg, A. (1966a) *J. Biol. Chem. 241*, 3240-3243.
Yonetani, T., Wilson, D. F., & Seamonds, B. (1966b) *J. Biol. Chem. 241*, 5347-5352.
Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., & Reed, G. H. (1972) *J. Biol. Chem. 247*, 2447-2455.

A Novel Sodium Channel Inhibitor from *Conus geographus*: Purification, Structure, and Pharmacological Properties†

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ABSTRACT: A novel toxin, tentatively named conotoxin GS (CGS), has been isolated from a marine snail, *Conus geographus*. CGS was found to exist as a single polypeptide chain, consisting of 34 amino acid residues, cross-linked by three disulfide bonds. Its amino acid sequence was shown to be Ala-Cys-Ser-Gly-Arg-Gly-Ser-Arg-Cys-Hyp-Hyp-Gln-Cys-Cys-Met-Gly-Leu-Arg-Cys-Gly-Arg-Gly-Asn-Pro-Gln-Lys-Cys-Ile-Gly-Ala-His-Gla-Asp-Val. In competition experiments, CGS inhibited the bindings of [^{3}H]Lys-tetrodotoxin ([^{3}H]Lys-TTX) and [^{3}H]propionylconotoxin GIIIA to *Electrophorus electricus* electroplax membranes, with K_i values of 34 nM and 24 nM, respectively. The toxin inhibited the binding of [^{3}H]Lys-TTX (1 nM) to rat skeletal muscle homogenates with an IC_{50} value of 880 nM but showed very little effect on this binding to the rat brain P_2 fraction at 10 μM. These binding studies indicate that CGS belongs to the same group of Na channel inhibitors as TTX, STX (saxitoxin), and μ-conotoxins. Although CGS, like the μ-conotoxins, is a pharmacological probe for distinguishing between neuronal and muscle Na channel subtypes, the homology in the sequences of CGS and μ-conotoxins is very limited.

The voltage-dependent Na channel is the target of a variety of neurotoxins (Narahashi, 1974; Catterall, 1980, 1986). These toxins are useful in investigations on the structural and functional domains of the Na channel at the molecular level (Catterall, 1985). Studies have shown that different isoforms or subtypes of Na channels are present in various tissues and have indicated that these toxins are also valuable in studying heterogeneity of Na channels (Barchi, 1987). The class of neurotoxins that includes tetrodotoxin (TTX)[1] and saxitoxin (STX) is water-soluble heterocyclic guanidinium compounds which inhibit ion transport through Na channels by binding highly specifically to these channels. Two classes of Na channels differing in sensitivities to TTX have been described as TTX-sensitive and TTX-insensitive Na channels, and there are reports of the coexistence of these two types of Na channels in primary cultures of rat myoblasts and myotubes (Gonoi et al., 1985; Weiss & Horn, 1986) and in denervated muscles of adult rats (Pappone, 1980).

The venom of the marine snail *Conus geographus* contains a number of peptide toxins with various biological activities (Olivera et al., 1985). For example, ω-conotoxins inhibit Ca channels at the presynaptic terminus, α-conotoxins block nicotinic acetylcholine receptors at the postsynaptic membranes, and μ-conotoxins inhibit Na channels in the muscle.

μ-Conotoxins are basic peptides, each composed of 22 amino acid residues (Sato et al., 1983; Cruz et al., 1985). These peptide toxins can distinguish not only between neuronal and

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[1] Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; [^{3}H]Lys-TTX, [^{3}H]Lys-tetrodotoxin; [^{3}H]Pr-CGIIIA, [^{3}H]propionylconotoxin GIIIA; CGS, conotoxin GS; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; Gla, 4-carboxyglutamic acid; DTT, dithiothreitol; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; CamCys, carbamoylcysteine; Hyp, 4-hydroxyproline; CGIIIB, conotoxin GIIIB.

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