Compound I Radical in Site-Directed Mutants of Cytochrome *c* Peroxidase As Probed by Electron Paramagnetic Resonance and Electron–Nuclear Double Resonance†

Laurence A. Fishel,‡ Martin F. Farnum, J. Matthew Mauro,§ Mark A. Miller, and Joseph Kraut

Department of Chemistry, University of California, San Diego, La Jolla, California 92093

Yajun Liu, Xiao-ling Tan, and Charles P. Scholes*

Departments of Physics and Chemistry, Center for Biochemistry and Biophysics, State University of New York at Albany, Albany, New York 12222

Received July 26, 1990; Revised Manuscript Received November 8, 1990

ABSTRACT: The reaction of ferric cytochrome *c* peroxidase (CcP) from *Saccharomyces cerevisiae* with peroxide produces compound I, characterized by both an oxyferryl iron center and a protein-based free radical. The electron paramagnetic resonance (EPR) signal of the CcP compound I radical can be resolved into a broad majority component which accounts for approximately 90% of the spin intensity and a narrow minority component which accounts for approximately 10% of the integrated spin intensity [Hori, H., & Yonetani, T. (1985) *J. Biol. Chem. 260*, 3549–3555]. It was shown previously that the broad component of the compound I radical signal is eliminated by mutation of Trp-191 to Phe [Scholes, C. P., Liu, Y., Fishel, L. F., Farnum, M. F., Mauro, J. M., & Kraut, J. (1989) *Isr. J. Chem. 29*, 85–92]. The present work probed the effect of mutations in the vicinity of this residue by EPR and electron–nuclear double resonance (ENDOR). These mutations were obtained from a plasmid-encoded form of *S. cerevisiae* expressed in *Escherichia coli* [Fishel, L. A., Villafranca, J. E., Mauro, J. M., & Kraut, J. (1987) *Biochemistry 26*, 351–360]. The EPR line shape and ENDOR signals of the compound I radical were perturbed only by mutations that alter Trp-191 or residues in its immediate vicinity: namely, Met-230 and Met-231, which have sulfur atoms within 4 Å of the indole ring, and Asp-235, which forms a hydrogen bond with the indole nitrogen of Trp-191. Mutations of other potential oxidizable sites (tryptophan, tyrosine, methionine, and cysteine) did not alter the EPR line shapes of the compound I radical, although the integrated spin intensities were weaker in some of these mutants. Mutations at Met-230 and/or -231 perturbed the EPR line shapes of the compound I radical signal but did not eliminate it. ENDOR of these two methionine mutants showed alteration to the hyperfine couplings of several strongly coupled protons, which are characteristic of the majority compound I radical electronic structure, and a change in weaker hyperfine couplings, which suggests a different orientation of the radical with respect to its surroundings in the presence of these methionine mutations. Besides the Trp-191 → Phe mutation, only the Asp-235 → Asn mutation eliminated the broad component of the compound I signal. Loss of the broad compound I EPR signal coincides with both the loss of the Asp → Trp-191 hydrogen-bonding interaction and alteration of the position of the indole ring of Trp-191. These results argue against the involvement of a Trp other than Trp-191 in the formation of the compound I radical. In the Trp-191 → Phe and Asp-235 → Asn mutants, the narrow component of the EPR radical signal was detected, despite the loss of the broad component. The line shape of the narrow signals in these mutants, as well as the line shape of the narrow minority radical of CcP compound I with no site-directed mutations, resembled that observed for the Tyr radical of photosystem II of green plants. The presence of a narrow EPR signal in Phe-191 and Asn-235 mutants may indicate that replacement of Trp-191 or perturbation to its interaction with Asp-235 favors oxidation of tyrosine over tryptophan. The radical signal of the Asn-235 mutant differed from that of the Phe-191 mutant in EPR line shape, spin relaxation time, and solvent exposure. This, together with small differences in the narrow radical EPR line shapes among several other mutants, suggests that the narrow radical signal could arise from any of several sites. Such a conclusion is further supported by the failure to date of any single-site mutation to eliminate the narrow radical signal.

Cytochrome *c* peroxidase (CcP)[1] catalyzes the conversion of H_2O_2 to water, and in so doing, it oxidizes two molecules of ferrocytochrome *c*. When H_2O_2 reacts with CcP in the absence of a reducing substrate, the enzyme loses 2 reducing equiv, and compound I (also called compound ES) is formed. In compound I, the abstraction of the first equivalent oxidizes the heme iron from the ferric to oxyferryl state (Lang et al.,

† This work was supported in part by NIH Grant GM-35103 (C.P.S.), NSF Grant DMB 88-15718 (J.K.), NSF Grant BBS 8711617 (C.P.S.), NIH NRSA Postdoctoral Fellowships GM 12262-02 (M.F.F.) and GM 10292-02 (J.M.M.), and a postdoctoral fellowship from Hemoglobin and Blood Training Grant 5 T32 AM 072333-11 (M.A.M.). Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

* Author to whom correspondence should be addressed.

‡ Current address: Department of Chemistry, Michigan State University, East Lansing, MI 48824-1322.

§ Current address: Center for Advanced Research in Biotechnology, 9600 Gudelsky Dr., Rockville, MD 20850.

[1] Abbreviations: CcP, cytochrome *c* peroxidase; CcP (MI), bakers' yeast cytochrome *c* peroxidase expressed in *E. coli*; EPR, electron paramagnetic resonance; ENDOR, electron–nuclear double resonance; T_1, spin–lattice relaxation time; ptp, peak to peak; G, gauss (1 G = 10^{-4} T); RF, radio frequency; μW, microwatt; i.d., inside diameter; o.d., outside diameter; Trp, tryptophan; Tyr, tyrosine; Met, methionine; Cys, cysteine; Leu, leucine; Ile, isoleucine; Gly, glycine; Phe, phenylalanine; Asp, aspartic acid; Asn, asparagine; DPPH, 2,2′-diphenyl-1-picrylhydrazyl radical.

0006-2960/91/0430-1986$02.50/0

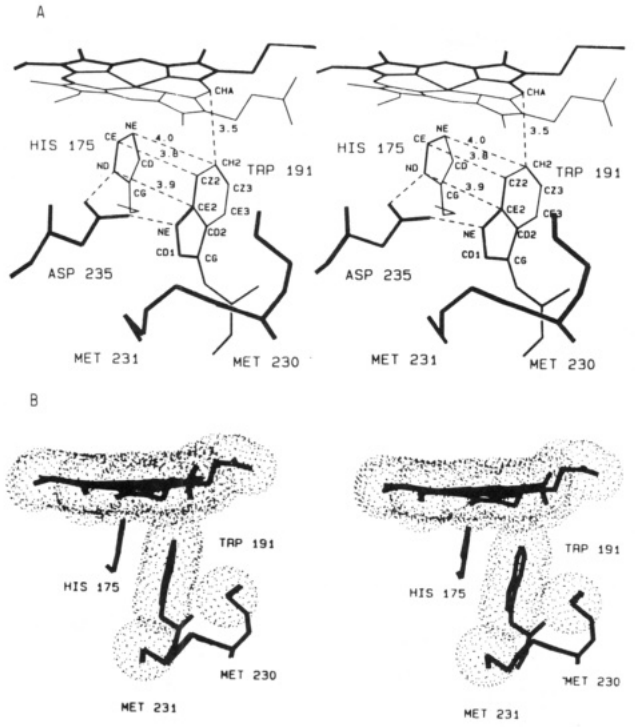

FIGURE 1: (Plate A) The environs of the Trp-191 and nearby His-175 and heme with nomenclature and distances. See Table II. (Plate B) View showing van der Waals contact of Trp-191, the porphyrin, and the sulfurs of Met-230 and Met-231. Data from Wang et al. (1990).

1976), while abstraction of the second equivalent creates a protein-based radical. Yonetani et al. (1966) initially suggested that the side chain of an aromatic amino acid provides the site for the protein-based radical.

EPR Characteristics of Compound I Radical. The reported g values of $g_{\parallel} = 2.038 \pm 0.002$ and $g_{\perp} = 2.005 \pm 0.002$ (Hori & Yonetani, 1985; Hoffman et al., 1981; Scholes et al., 1989) are unusual for a free radical. The EPR signal broadens to hundreds of gauss as the temperature is increased from 4.2 to 77 K. The relaxation time of the radical is remarkably short for a free radical at liquid helium temperatures; T_1 ranges from 15 ms at 2.1 K to 35 μs at 4.2 K (Scholes et al., 1989). Although the temperature-dependent properties of the compound I radical are unusual for a free radical, similar properties have been observed for ubisemiquinone radicals *near* paramagnetic Fe^{2+} in the bacterial photosynthetic reaction center (Butler et al., 1984). [Both Fe(IV) here and the Fe(II) in photosynthetic reaction centers are integer spin systems with significant zero-field splittings.] The unusual temperature-dependent properties of the compound I radical may therefore result from an electron–electron spin interaction of the radical with the heme iron.

Properties and Site of Compound I Radical. Initial site-directed mutagenesis experiments ruled out Trp-51 and Met-172 as the radical sites (Fishel et al., 1987; Goodin et al., 1986). Recently, several studies have provided circumstantial evidence that Trp-191 is the site of the radical in compound I. Single-crystal diffraction studies revealed small changes in electron density in the vicinity of Trp-191 when the ferric enzyme (Finzel et al., 1984) was converted to compound I (Edwards et al., 1987). The structure of CcP near Trp-191 (Figure 1) shows that a radical at Trp-191 would be sufficiently close to the heme iron and to the porphyrin ring for substantial magnetic spin–spin interaction.

Site-directed mutagenesis of Trp-191 to Phe gave a mutant protein from which the steady-state activity of the enzyme was diminished 3000-fold (Mauro et al., 1988). Although reaction of this Trp-191 → Phe mutant with peroxide in the absence of a reducing substrate formed a product with an absorption spectrum characteristic of the oxyferryl center of compound I (Mauro et al., 1988), EPR–ENDOR studies on it have shown that the characteristic compound I radical signal is absent in this mutant[2] (Scholes et al., 1989). Furthermore, reaction kinetics of the Trp-191 → Phe mutant with peroxide resulted in a transiently formed porphyrin cation radical, clearly establishing a role for Trp-191 in the formation of the typical compound I radical (Erman et al., 1989). Recent ENDOR measurements on CcP grown on perdeuterated tryptophan have provided further evidence that the compound I radical is located at a tryptophan residue (Sivaraja et al., 1989), rather than on methionine as suggested previously (Hoffman et al., 1979, 1981).

Although Trp-191 is strongly implicated as the radical site in compound I, the circumstantial evidence for this assignment remains equivocal since there are other tryptophans in CcP. The present work was undertaken to further elucidate the nature of the compound I radical and its interaction with the immediate environment of the putative radical site. We have focused on the EPR and ENDOR characterization of compound I in CcP mutants, and the most telling results were at residues in the immediate vicinity of the putative Trp-191 radical site. Of particular interest has been the mutation of Asp-235 → Asn, from which loss of the hydrogen-bonding interaction between Asp-235 and the indole NH of Trp-191 has recently been shown to result in a substantial reorientation of the side chain of Trp-191 (Wang et al., 1990). The mutation was therefore expected to alter the properties of a compound I radical at Trp-191. Met-230 and -231 have comprised a second region of interest near Trp-191, since the sulfur atoms in the side chains of these residues lie close to the indole ring of Trp-191. Moreover, oxidation of Met-230 and -231 to the sulfoxide has been shown to produce an enzyme form that does not show detectable reaction with H_2O_2 (Kim & Erman, 1988).

The Trp-191 environs contain a number of conspicuous aromatic tryptophans and tyrosines (for example, Trp-211, Trp-223, Tyr-187, Tyr-229, and Tyr-236) which might serve to channel charge into and out of a radical site (Edwards et al., 1987). Amino acid analysis of the products of CcP compound I decay *in solution* has shown degradation of tryptophan and tyrosine (Coulson & Yonetani, 1972; Spangler & Erman, 1986), as well as cysteine and methionine, and intramolecular (tyrosine) cross-linking (Spangler & Erman, 1986). Protein structural perturbations detected *in the crystal* structure of CcP that had been converted to compound I and allowed to decay before analysis were at Tyr-23, Tyr-42, Tyr-236, and Cys-128, as well as at Met-230 and Met-231 (Anderson, 1986). Therefore, a survey by EPR of site-directed mutants at these potentially interesting additional sites was undertaken to investigate their involvement in compound I formation.

Narrow Radical Signals. In the course of this and our previous study (Scholes et al., 1989), we have noted additional radicals in peroxide-oxidized CcP which had considerable intensity but which were not the majority or primary compound I signal. At liquid helium temperature, where the majority compound I EPR signal is relatively narrow, these features were obscured under the large majority compound

[2] We are aware that site-directed mutagenesis cannot unambiguously determine a location for the compound I radical. If a mutation were to cause nonappearance of the radical, such nonappearance could occur because of alteration of either the radical species that exists in the wild-type intermediate or a protein feature involved in its formation.

I signal. However, at liquid nitrogen temperature, the compound I EPR signal is substantially broadened; these features remain narrow and are easily detected. Such features have been observed as minority species from single crystals and frozen solutions of bakers' yeast CcP (Hori & Yonetani, 1985). When Trp-191 was mutated to Phe in CcP(MI), the same narrow radical signal that was previously found as the minority radical in unmutated CcP was now easily observed at all temperatures since no typical broad, temperature-dependent compound I signal was present. This radical signal had an appearance similar to that of a tyrosine radical observed in photosystem II (Prince, 1988; Debus et al., 1988; Barry & Babcock, 1987, 1988; Beck & Brudvig, 1987). Besides the tyrosine radicals of photosystem II that are associated with the highly oxidizing multimanganese center of that system, tyrosine radicals have also been identified as components of several proteins whose function is redox chemistry. Notably, a tyrosine radical is associated with a binuclear iron center in ribonucleotide reductase (Bender et al., 1989), with a ferryl heme in prostaglandin H synthase (Kulmacz et al., 1990), and with a copper in galactose oxidase (Whittaker & Whittaker, 1988, 1990). The overall electronic structure of these tyrosine radicals is thought to be similar, but the EPR line shapes of them can differ primarily because their methylene proton hyperfine couplings differ (Barry & Babcock, 1988). This difference in methylene proton couplings is due to the variation in conformation of the β-methylene group with respect to the tyrosine phenol head group (Barry et al., 1990).

It is possible that the narrow radical signals associated with compound I are not simply artifacts but may be important for CcP function, and furthermore, they may provide general insight into the behavior of potentially oxidizable protein groups like tyrosine near highly oxidizing metal centers. Hence, they merited study.

Experimental Procedures

Site-Directed Mutagenesis. Synthetic oligonucleotides were obtained from the UCSD Peptide Synthesis Facility. Most of the oligonucleotides used were about 23-mers, except for the one used for a double-tyrosine mutant (Tyr-36,Tyr-42 → Phe,Phe) which was a 33-mer. All mutagenesis reactions were carried out with the original M13mp8ccp(MI) template described in Fishel et al. (1987). The Asp-235 → Asn mutant was prepared (by J.M.M.) according to the method of Kunkel (1985). The Met-230,Met-231 → Leu,Leu mutant was prepared (by J.M.M.) as described previously (Fishel et al., 1987; Mauro et al., 1988). All materials and methods for other mutants (prepared by L.A.F.) were as described in Fishel et al. (1987) with the following additions or modifications. An Amersham oligonucleotide-directed in vitro mutagenesis kit was used. DNA sequence analyses using [^{35}S]dATPS were performed as described in Fishel (1987), or with a Sequenase kit (U.S. Biochemicals). All mutants were sequenced entirely following mutagenesis, including the mutant DHFR promoter region, to confirm that only the desired mutation had been introduced and that no other mutations had occurred elsewhere in the CCP(MI) gene. With the exception of the Asn-235 and Leu-230,Leu-231 mutants, final transfer of each mutant CcP gene from M13mp8ccp (mutant) into pUC8 for protein expression in *Escherichia coli* was accomplished with the aid of GeneClean (Bio101, La Jolla, CA) to isolate DNA from agarose gels.

Production of Mutant CcP Proteins in E. coli. Cell culture was as described in Fishel et al. (1987) with the following modifications: *E. coli* SK383 carrying the pUC8CCP (mutant) plasmids were cultured in modified TB broth (Tartof & Hobbs, 1987) containing the following ingredients: 0.1% D-glucose or glycerol, 10 g/L NaCl, and ampicillin (200–300 μg/mL). Yields of CcP mutant proteins were increased by culturing the cells at 39 °C for 40–48 h, but the Phe-191 and Asn-235 mutants were produced by 37 °C growth for 32 h.

Table I

amino acid	gene (5′ → 3′)	mutant gene (5′ → 3′)
36, 42 (Tyr, Tyr)	TAT, TAT	TTC, TTC (Phe, Phe)
39 (Tyr)	TAT	TTC (Phe)
119 (Met)	ATG	ATT (Ile)
128 (Cys)	TGT	GCT (Ala)
163 (Met)	ATG	ATT (Ile)
211 (Trp)	TGG	TTC (Phe)
223 (Trp)	TGG	TTT (Phe)
229 (Tyr)	TAC	TTC (Phe)
230 (Met)	ATG	ATC (Ile)
230 (Met)	ATG	TAC (Tyr)
231 (Met)	ATG	TTG (Leu)
230, 231 (Met, Met)	ATG, ATG	TTG, TTG (Leu, Leu)
236 (Tyr)	TAT	TTT (Phe)
191 (Trp)[a]	TGG	TTC (Phe)
235 (Asp)[b]	GAT	AAT (Asn)

[a] Mauro et al., 1988. [b] Smulevich et al., 1988.

Purification of Mutant CcP Proteins. Purification procedures used on CcP(MI), Trp-191 → Phe, and Asp-235 → Asn were essentially as described elsewhere (Fishel et al., 1987; Mauro et al., 1988). These mutants have been used for X-ray crystallography and were obtained following a complete 2× crystallization procedure. However, scaled-down, more rapid procedures were used to quickly isolate many other mutant proteins to screen them by EPR spectroscopy. After column chromatography (Sephadex G-75 and DEAE-cellulose), diafiltration, and concentration (Amicon PM-10 membrane, 62 mm), which left the protein in 0.1 M potassium phosphate, pH 6.0, each sample was ultracentrifuged for 30 min (40 000 rpm, Beckman Ti-60 rotor, 5 °C). Aliquots of the supernatant were then frozen in liquid nitrogen for later use. For the Met-230,Met-231 → Leu,Leu mutant, heme was added to the crude lysate before G-75 chromatography. Extinction coefficients and concentrations were determined as described elsewhere (Fishel et al., 1987; Mauro et al., 1988; Smulevich et al., 1988). Except for Trp-191 → Phe, Asp-235 → Asn, and CcP(MI), the samples surveyed were not purified to homogeneity by crystallization before EPR analysis. The four separate mutants at Met-230 and/or -231, which we consider most interesting because of their proximity to Trp-191, gave consistent spectroscopic results for samples prepared with different freezing times. The critical Met-230,Met-231 → Leu,Leu mutant gave consistent EPR and ENDOR results from two separate preparations of enzyme done over a 2-year period and with different freezing times.

CcP Mutants. A list of the CcP mutants prepared for the present experiments is given in Table I.

Preparation of CcP Samples for EPR–ENDOR Analyses. Standard 3-mm i.d./4-mm o.d. quartz EPR tubes from Wilmad Glass Co. were used for EPR analyses. Larger quartz (Heraeus-Amersil CFQ quartz) tubes (7-mm i.d./9-mm o.d.) were used for EPR–ENDOR.

Deuterated samples were prepared by resuspending crystals in 99.9% D_2O, centrifuging the enzymes, dissolving them in pD 6.0 deuterated buffer (prepared from dry, protonated salts), and letting them stand overnight at 4 °C. Samples were prepared by the reaction of CcP with a 1.1 to 1.5 molar excess of H_2O_2, all in a 0.1 M potassium phosphate, pH 6.0. When this preparation procedure was done by manual pipetting, the

elapsed time between mixing and immersion in liquid nitrogen was about 1 min. This manual method was used for some unmutated CcP(MI) samples and for some samples of the Asp-235 → Asn mutant. (To check for instability of the Asp-235 → Asn mutants, a series of these samples was reacted with H_2O_2 and allowed to remain incubating on ice for periods of up to 1 h before freezing in liquid nitrogen.) Asp-235 → Asn samples were also prepared by the rapid mixing/freeze–quench technique.

Rapid-Mixing/Freeze–Quench Technique. In the 30–60 s needed to hand mix and freeze, some samples, including Trp-191 → Phe and the mutations at Met-230 and/or Met-231, did not give maximum yield apparently because of instability of their compound I signal. Thus a rapid-mixing/freeze–quench system was developed, and it was subsequently used routinely on all new mutant CcP samples to increase the likelihood that most or all of compound I would be captured.

A Durrum D110 stopped-flow instrument was adapted (by M.F.F. and L.A.F.) for freeze–quench flow by replacing the ceramic drive syringes with Teflon plungers having silicone O-rings for sealing purposes. The flow cell of the stopped-flow unit was removed, and a special holder for the mixing (constructed of polycarbonate plastic) was placed on the outlet of the valve block. Minimum-bore Teflon tubing was used to direct the flow of mixed components to the bottom of EPR or ENDOR tubes. Protein and peroxide solutions, syringes, and the mixing block were maintained at 4 °C. The smaller EPR tubes were immersed in an isopentane freezing mixture cooled by liquid nitrogen (Bray, 1964) for precooling and lifted out of the bath just prior to introduction of CcP/H_2O_2 into them. After sample injection the tubes were rapidly frozen by immersion in freezing isopentane. We found that using a hypodermic needle to perforate the Teflon tubing along its bottom (distal) 3–5 cm prior to insertion into each empty EPR tube enabled us to fill the smaller EPR tubes directly and more rapidly with the reacted mixture. Such a procedure left no air gap at the bottom of the tube and diminished the chance of shattering the EPR tubes upon freezing. The Teflon tubing which carried the reacted mixture into the 3-mm i.d. EPR tubes was left frozen in place. Taking into account the dead volume of the mixer (20 μL) and Teflon tubing (120 μL) as well as the average velocity of the drive syringes (2–8 mL/s), we estimate a freezing time ranging from 150 to 450 ms for the small samples.

For the larger ENDOR sample tubes, Teflon tubing was used to direct the flow of mixed components into ENDOR tubes that had been precooled in the isopentane bath and then were poised above the isopentane bath as the CcP mixture was shot into them. These ENDOR tubes were immersed in the freezing mixture immediately following loading with about 1 mL of the protein–H_2O_2 mixture; the entire operation from mixing until immersion took about 1 s.

EPR–ENDOR Methods. ENDOR measurements were conducted on a commercial X-band Bruker ER-420 EPR spectrometer with a home-built ENDOR cavity and a double Dewar system used for helium-temperature EPR–ENDOR (Scholes, 1979). The EPR and ENDOR spectra shown in this paper were obtained by Y.L., X.T., and C.P.S. in the laboratory of C.P.S. Some preliminary EPR measurements at 4.2 and 77 K were done at UCLA by L.A.F. as described in Fishel et al. (1987). Magnetic field-modulated ENDOR study of CcP compound I was done at pumped helium temperature (2.1 K) under conditions of adiabatic rapid passage with 10–100 μW microwave power, 100-kHz field modulation of approximately 4 G ptp for strongly coupled protons and 1.0 G ptp for weakly coupled protons, and an ENDOR RF amplitude of about 0.5 G ptp. The ENDOR RF source was a Hewlett-Packard 8601A generator–sweeper whose frequency output was repetitively swept by a voltage ramp provided by a Tracor 570 signal averager. EPR and ENDOR traces were collected in the memory of the signal averager and then stored on disk by a Z-80-based microcomputer. Pulsed EPR for measuring saturation recovery T_1's was done with a modification to the Bruker ER 420 spectrometer which is described by Scholes et al. (1984, 1989). For EPR on smaller, quick-frozen samples, we used a more standard EPR system, IBM ER-200D, having a 4102-ST X-band EPR cavity and an APD Cryogenics LTR-3-110 Helitran System to control the temperature in the 4.6–50 K range. Data were stored and manipulated for this latter work by an AT&T PC 6300 personal computer used in combination with the EW EPR Software routine and IBM DAC board (Morse, 1987). g values of the small samples were measured by reference to a DPPH sample with known g = 2.0036 value (Poole, 1983), and appropriate minor corrections were made between sample and DPPH for differences in field (measured to within 0.1 G) and EPR frequency (by an XL Microwave 8–12-GHz frequency counter). From our ability to determine the position of the absorption derivative zero crossing, we estimate that we can measure the g values of narrow radical signals with this latter apparatus to within ±0.0003. Our computer systems had double integration routines available, and 1 mM cupric perchlorate samples were used as integration standards. However, our experiments and apparatus were primarily designed to measure spectroscopic parameters such as ENDOR frequencies, g values, and hyperfine couplings. They were not designed for precise spin quantitation because the larger ENDOR samples differed somewhat from each other in sample volume and the smaller quick-frozen samples contained Teflon tubing necessary for quick sample loading. We estimate a factor of 2 error in spin quantitation.

Theoretical and Structural Background for ENDOR

The first-order expression for proton ENDOR frequencies is

$$\nu_{ENDOR} = \nu_{NMR} \pm A/2 \quad (1)$$

where ν_{ENDOR} is the ENDOR frequency, ν_{NMR} is the NMR frequency of free protons (about 14 MHz at the magnetic fields used here), and A is the first-order hyperfine coupling. Proton ENDOR features usually occur in pairs centered about the free proton NMR frequency so that the splitting between a pair is A. The hyperfine coupling constant, A, reflects a direct contact interaction with unpaired electron spin density that arrives at the proton nucleus through covalency or conceivably a through-space dipolar interaction with more distant sources of electron spin (Scholes, 1979). Assuming that the compound I radical is on tryptophan, larger, predominantly covalent couplings would be from protons either on the indole ring or on the α- or β-carbons. "Strongly coupled" protons have large splittings (taken as greater than 10 MHz here), which reflect a close proximity of the proton to sources of spin density. "Weakly coupled" protons (couplings less than 4 MHz) would be farther removed from the source(s) of electron spin density; their hyperfine interaction may involve a larger contribution from magnetic dipolar interactions[3] and a weaker covalent contribution.

Trp-191 resides on the proximal side of the heme in a position where it lies parallel to the proximal His-175 (at about 4-Å distance), makes van der Waals contact with the heme

Table II: Interatomic Distances (Å) from Atoms on Trp-191 to Selected Atoms on His-175, Heme, Met-230, Met-231, and Asp-235

His-175	Trp-191		heme	Trp-191	
NE	CH2	4.0	CHA	CH2	3.5
CE	CZ2	3.8	C1A	CH2	3.8
ND	CE2	3.9	C4D	CH2	3.9
CD	CZ3	4.2	Fe	CH2	5.1
CG	CE3	4.6	Fe	CZ2	5.2
Met-230	Trp-191		Met-230	Trp-191	
S	NE	4.2	S	CD1	3.9
S	CD1	4.5	S	NE	4.6
S	CE2	4.4	S	C6	5.0
Asp-235	Trp-191		Asp-235	His-175	
$O^{\delta 2}$	NE	2.8	O	ND	2.9

edge and with methionine sulfur on Met-230, and hydrogen bonds to the carboxyl group of Asp-235 (Figure 1). The latter carboxyl group also hydrogen bonds to ND of proximal His-175. Relevant labeling of Trp-191 atoms, heme, and His-175 atoms is given in Figure 1. Table II lists distances from atoms on Trp-191 to several porphyrin carbons, the heme iron, atoms of the proximal His-175, sulfurs of Met-230 and -231, and $O^{\delta 2}$ of Asp-235 (Wang et al., 1990). As shown in Figure 1 and in Table II, the porphyrin carbons at about 3.5 Å from Trp-191 and His-175 carbons at about 4.0 Å are the closest neighbors having protons that could interact with a radical on this tryptophan but which are not covalently linked to it. For a proton 3.5 Å distant from an unpaired electron, we would expect a maximum dipolar interaction of 3.6 MHz by the dipolar formula of footnote 3 (with $\theta = 0$). However, because of the possible delocalization of the electron spin on the indole ring, the dipolar couplings to such a noncovalently linked proton would probably be less than 3.6 MHz. If the radical were preferentially localized on the more distant six-membered portion of the indole ring, it is conceivable that protons at the α or β Trp-191 carbons connecting the indole ring to the peptide backbone might also be weakly (rather than strongly) coupled.

Results

EPR Results: Compound I Radical Signal. When examined by EPR, the reaction product of the CcP enzyme with H_2O_2 exhibited a primary or majority compound I radical in almost all of the mutants shown in Table I. The exceptions were Trp-191 → Phe (Mauro et al., 1988; Scholes et al., 1989) and Asp-235 → Asn. Figure 2, recorded with large samples prepared for ENDOR study, provides a comparison of such EPR spectra at gains chosen to give approximately the same ptp derivative height. On comparison with that of CcP(MI), the compound I EPR signal from the Met-230 and Met-231 mutants was perturbed *but not eliminated*; notably, the feature at $g = 2.038$ became progressively less sharp and tended to a lower g value in the order CcP(MI) > Met-231 → Leu > Met-230 → Ile (or Met-230 → Tyr) > Met-230,231 → Leu,Leu. Smaller, more rapidly frozen EPR samples were made from the Met-230 → Ile, Met-231 → Leu, Met-230,231 → Leu,Leu and Trp-191 → Phe mutants, and these showed no difference in line shapes or relative intensities from those

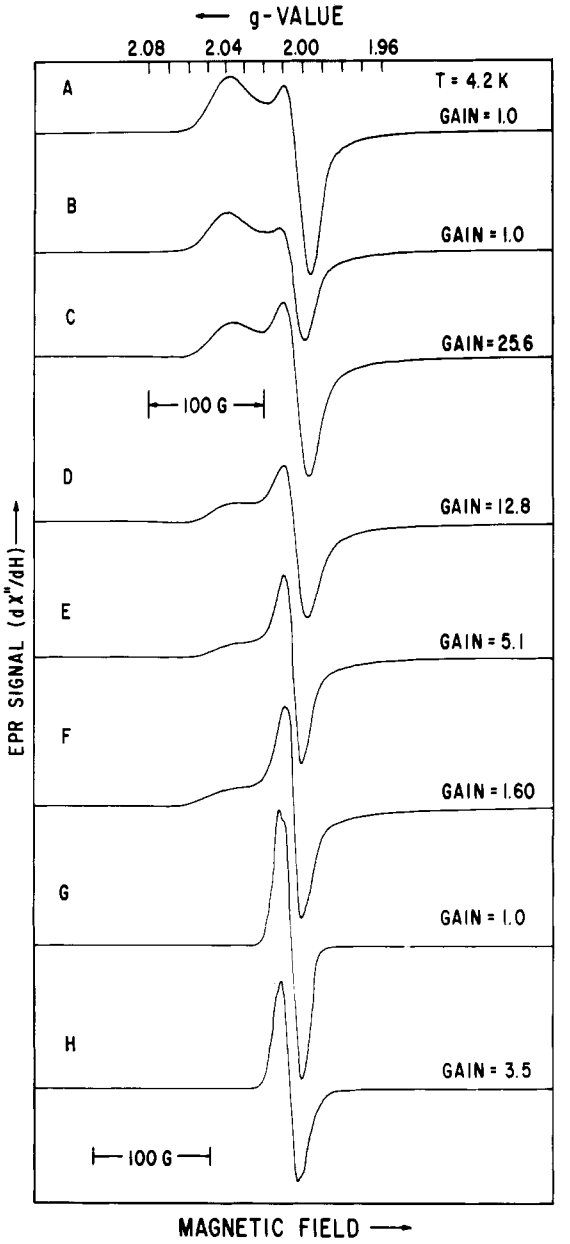

FIGURE 2: EPR spectra ($d\chi''/dH$) of compound I signals from CcP(MI) and mutant forms whose identities are (A) CcP(MI) (750 μM), (B) Trp-223 → Phe (535 μM), (C) Met-231 → Leu (225 μM), (D) Met-230 → Ile (225 μM), (E) Met-230 → Tyr (225 μM), (F) Met-230,231 → Leu,Leu (750 μM), (G) Trp-191 → Phe (750 μM),and (H) Asp-235 → Asn (500 μM) in deuterated solvent. These spectra were taken at 4.2 K over a range of 500 G. Microwave power was approximately 100 μW; 100-kHz field modulation was 4 G ptp. Each spectrum was from eight accumulations of 50-s duration each. Gains were chosen to provide approximately the same overall ptp height; the relative gains are indicated with each spectrum.

of the respective more slowly frozen larger counterparts. At liquid helium temperatures the integrated spin intensity of the doubly mutated Met-230,231 → Leu,Leu was about 50% that of CcP(MI) while the single-methionine mutants, taking into consideration the starting protein concentration, consistently had an intensity that was 10–20% that of the CcP(MI). These methionine mutants may have had a larger contribution from the narrow radical centered at $g = 2.00$, but they still retained the broad primary compound I radical signal.

Of the other mutants surveyed in Table I (not at Met-230/231, Asp-235, or Trp-191) but at other methionines, cysteines, tyrosines, and tryptophans not touching Trp-191,

[3] A first-order point–dipole approximation for the hyperfine coupling of an electron to a proton is $hA_{dipole} = g_{eff}\beta_e g_n \beta_n (3\cos^2\theta - 1)/r^3$, where g_{eff} is the electronic g value, β_e is the electronic Bohr magneton, β_n is the nuclear Bohr magneton, g_n is the nuclear g value = 5.58 for protons, θ is the angle between the applied magnetic field and the vector between the electron spin and the proton, r is the unpaired electron to proton distance, and h is Planck's constant.

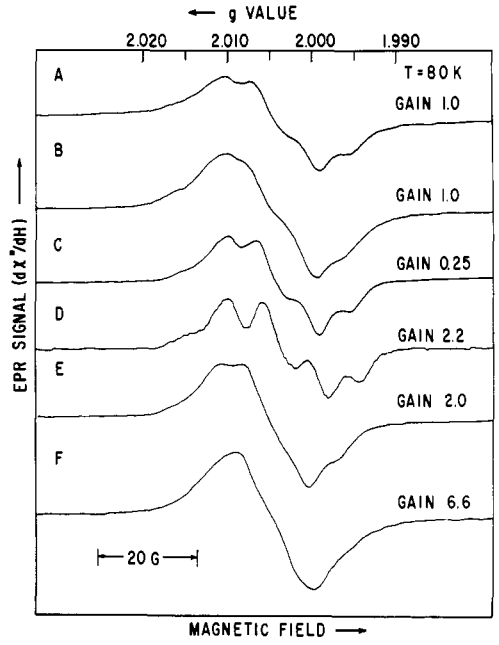

FIGURE 3: EPR spectra ($d\chi''/dH$) taken at 80 K over 100-G range near $g = 2.00$ to show the narrow free radical signal from the following forms: (A) CcP(MI) (750 μM); (B) Met-230,231 → Leu,Leu (750 μM); (C) Trp-191 → Phe (750 μM); (D) dark-adapted photosystem II at a concentration of 5 mg in 1 mL; (E) Asp-235 → Asn (500 μM, deuterated solvent); (F) Asp-235 → Asn (500 μM, protonated solvent). Spectra were taken with 100-μW power, field modulation of 0.75 G ptp, and 16 accumulations of 10-s duration.

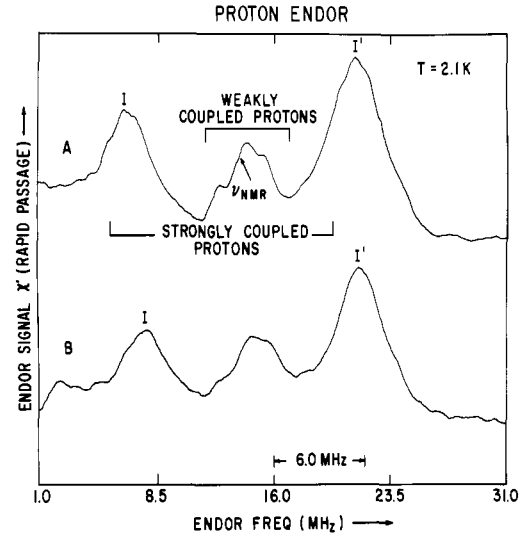

FIGURE 4: Proton ENDOR signals from (A) CcP(MI) (750 μM) and (B) the Met-230,231 → Leu,Leu (750 μM) mutant, as taken over a 1–31-MHz frequency range. Spectra were obtained with ENDOR RF of about 0.5 G ptp, 100-kHz field modulation = 4 G ptp, 2-s sweeps over the above frequency range, and microwave power = 100 μW. Each spectrum represents 200 accumulations. These spectra were obtained at $g = 2.046$.

the line shapes were typical of the CcP compound I majority radical signal with no outstanding contribution from a narrow signal. The mutants Met-119 → Ile, Tyr-39 → Phe, and Tyr-36,42 → Phe,Phe gave low intensities, approximately 10–20% of the integrated radical intensity of CcP(MI). In these samples which showed a weak compound I signal there was no concomitant evidence for remaining ferric heme. (A ferric heme signal could occur either because the heme did not readily react with H_2O_2 or because the heme quickly reverted to ferric form following decay of the compound I radical.)

EPR Results: *Narrow Signal.* A narrow radical signal near $g = 2.00$ with some resolved hyperfine structure is frequently found in association with the compound I EPR signal. We have typically found in CcP(MI) a 20% contribution from the narrow signal, most easily measured at 80 K where, unlike the compound I signal, it remains unbroadened. Even at liquid helium temperatures the Trp-191 → Phe and the Asp-235 → Asn mutants yielded only narrow radical signals (Figure 2G,*H*), which in a first-derivative presentation appear quite large. However, the integrated intensity of the former was about 20% of the intensity of compound I signal and of the latter about 10%. These mutants had no broad, rapid passage signal at 2.1 K, such as has been found in horseradish peroxidase, due to a porphyrin radical tightly coupled to ferryl heme (Schulz et al., 1984). The T_1 at both 2.1 and 4.2 K, as measured by saturation recovery, stayed constant at about 10 ms for the Trp-191 → Phe mutant (Scholes et al., 1989). The T_1, measured in this work for the Asp-235 → Asn mutant, significantly increased from about 1 ms at 4.2 K to 70 ms at 2.1 K.

Unlike the highly temperature-dependent line shape of the compound I radical signal (Scholes et al., 1989), there was little line-shape change[4] in either of these narrow radical signals between 4.2 and 80 K. At 80 K the radical signal (Figure 3C) from Trp-191 → Phe had a *g* value at its zero crossing of 2.0046 ± 0.0003 [not the 2.001 previously reported by Scholes et al. (1989)]. The radical signal in photosystem II is given for comparison in Figure 3D. The radical of Asp-235 → Asn had a *g* value of 2.0050 ± 0.0003 at 80 K. From the Trp-191 → Phe mutant [shown in Scholes et al. (1989)] there was resolved hyperfine structure with splittings of about 7 G, while from the Asp-235 → Asn mutant hyperfine structure with approximately the same splittings was resolved but only in deuterated solvent. Because their compound I signal which accounts for the majority of spins had substantially broadened at 80 K (Scholes et al., 1989), similar narrow minority radical signals were observable[5] from all of the other samples.

In Figure 3 we compare the narrow radical signals from CcP(MI), Met-230,231 → Leu,Leu photosystem II, Trp-191 → Phe, and Asp-235 → Asn. At 80 K no difference in EPR line shape was found between the EPR signals from small, quickly frozen samples or from the more slowly frozen ENDOR samples. For samples of the Asp-235 → Asn mutant which were allowed to stand at ice temperature for up to 1 h after being mixed with H_2O_2 there was a diminishment of radical EPR signal and an increase in ferric heme signal. On comparison to CcP(MI) or to any other mutant, the Trp-223 → Phe mutant had a smaller ratio of its narrow radical signal to its majority compound I signal by at least a factor of 2.

ENDOR Spectra. The proton ENDOR spectrum of CCP(MI) has strongly coupled features, labeled I,I′ and split well away from ν_{NMR}, which have hyperfine couplings on the order of 14 MHz (Scholes et al., 1989). These protons were initially thought to be on β-carbons adjacent to methionine

[4] The zero-crossing *g* value of the Asp-235 → Asn radical was observed to increase from 2.001 ± 0.0005 at 5 K to 2.002 ± 0.0005 at 20 K to 2.004 ± 0.0005 at 50 K while there was no such *g* value change from the Trp-191 → Phe radical.

[5] The EPR signals from two separate 100 μM samples of CcP(MI) were compared, the first made with a 1:1 molar ratio of H_2O_2 to protein and the second with a 10:1 molar ratio of H_2O_2 to protein. At 4.2 K the compound I EPR signals from these two samples and their integrated intensities were identical. At 80 K we observed the narrow radical signal from both of these samples and found the signal heights identical but obtained slightly better resolution of hyperfine detail from the sample made with the 1:1 ratio of H_2O_2 to protein.

Table III: Compound I Proton Hyperfine Couplings Determined by ENDOR

	strong couplings		weak couplings	
sample	features	couplings (MHz)	features	couplings (MHz)
CcP(MI)	I,I′	14.2 ± 0.5[a] (separation of I,I′ in Figure 4A at g = 2.046)	x,x′	0.27 ± 0.05 (possibly exchangeable)[d]
			y,y′	0.99 ± 0.10 (possibly exchangeable)[d]
			z,z′	1.80 ± 0.10[d]
			††,†††′	3.00 ± 0.10[d]
			†,†′	3.72 ± 0.15[d]
Met-230,231 → Leu,Leu	I,I′	13.5 ± 0.5[b] (separation of I,I′ in Figure 4B at g = 2.046)	x,x′	0.27 ± 0.05[e]
			y,y′	1.03 ± 0.10[e]
			z,z′	1.88 ± 0.10[e]
			,′	2.70 ± 0.10[e]
			,′	4.12 ± 0.15[e]
Asp-235 → Asp	α,α′	13.3 ± 0.5[c] (separation of α,α′ in Figure 7A,B at g = 1.990)	1,1′	0.33 ± 0.05[f]
			2,2′	0.93 ± 0.10[f]
			3,3′	2.08 ± 0.10 (exchangeable)[f]
			4,4′	3.03 ± 0.15 (exchangeable)[f]
	β,β′	7.4 ± 0.5 (at g = 1.990)		

[a] For CcP(MI) there are at least four sets of features within I,I′ having couplings of 12.1 ± 0.5, 14.1 ± 0.15, 16.0 ± 0.5, and 17.6 ± 1.0 MHz. Features a, b, and c in Figure 5A are those with couplings of 12.1, 14.1, and 16.0 MHz, respectively. The 12.1-MHz features exchanges with D_2O. [b] From the Met-230,231 → Leu,Leu mutant there was only this one broad nonexchangeble feature within I,I′ with coupling of 13.5 MHz. [c] There are at least three sets of features within α,α′ having couplings of 12.8 ± 0.5, 14.8 ± 0.5, and 18.3 ± 1.0 MHz. None is exchangeable with D_2O. [d] From Figure 6A at g = 2.032. [e] From Figure 6D at g = 2.032. [f] From Figure 7C,D at g = 1.990.

sulfur (Hoffman et al., 1979, 1981). (Our Met-230,231 → Leu,Leu mutant was initially prepared to test this.) Sivaraja et al. (1989) have since assigned these protons to tryptophan by using ENDOR combined with perdeuterated tryptophan. Figure 4, taken at g = 2.046, where there was no likelihood of overlap with any narrow minority signal at g = 2.00, compares the overall ENDOR spectra of CcP(MI) with that of the double mutant Met-230,231 → Leu,Leu. These two spectra appeared grossly similar when observed over the wide 1–31-MHz frequency sweep; however, closer observation of the strongly coupled proton features centered at 7 or at 21 MHz revealed differing features. Figure 5 shows such features centered at 7 MHz (where $\nu_{ENDOR} = \nu_{NMR} - A/2$). The features a, b, and c in Figure 5 have previously been reported for CcP(MI) and a Trp-51 → Phe mutant (Scholes et al., 1989), and their couplings are listed in the footnote to Table III. Corresponding details have also been noted (not shown) from the Zeeman partners of the 7-MHz features which occur near 21 MHz (where $\nu_{ENDOR} = \nu_{NMR} + A/2$). At least one of the features of CcP(MI) (labeled a in Figure 5A) was found to be exchangeable on deuteration with D_2O solvent (Figure 5B), as previously noted by Sivaraja et al. (1989). As an example of a perturbation to an aromatic residue near but not touching Trp-191, the ENDOR features of the Trp-223 → Phe mutant (Figure 5C) were unchanged from those of CcP(MI). In contrast, the ENDOR signals (Figures 5D–F) near 7 MHz from the Met-230 and/or -231 mutants lacked the detail and possibly some of the features of those of CcP(MI). Especially for the double Met-230,231 → Leu,Leu mutant (which had a large underlying EPR signal), this difference was definitely not due to any simple lack of ENDOR sensitivity.

Weakly coupled proton ENDOR spectra were also observed (Figure 6 and Table III), extending a few megahertz away from the free proton NMR frequency. Some of the features close to the free proton frequency were perturbed by deuteration (Figure 6B), indicating that some of the weakly coupled features near the free proton frequency are deuterium exchangeable. Details of certain weakly coupled proton ENDOR features were changed in the Met-230 and/or -231 mutants. Features indicated with asterisks (*, **) from CcP(MI) appeared to change to features denoted with daggers (†, ††) from the Met-230,231 → Leu,Leu mutant. These features with asterisks and daggers were not exchangeable with deuterium.

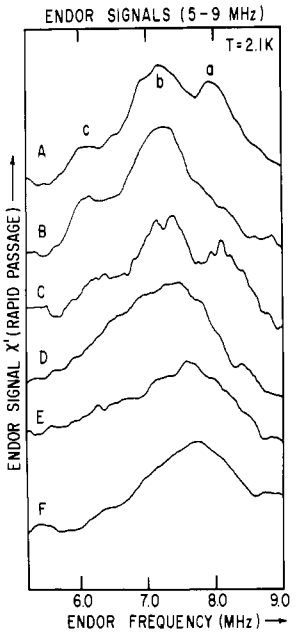

FIGURE 5: This figure compares ENDOR features from the signal near 7 MHz (see previous Figure 4). Samples were as follows: (A) CcP(MI), protonated solvent (750 μM); (B) CcP(MI), deuterated solvent (300 μM); (C) Trp-223 → Phe (535 μM) (structure on peaks a and b from this spectrum is noise); (D) Met-230,Met-231 → Leu,Leu (750 μM); (E) Met-231 → Leu (225 μM); (F) Met-230 → Tyr (225 μM). All spectra were taken at g = 2.045 except the double mutation Met-230,Met-231 → Leu,Leu (D), for which the ENDOR spectrum was taken at g = 2.055 because ENDOR features can emerge with better resolution when ENDOR is performed well out on a g value extremum. The spectra of CcP(MI) (A, B), Trp-223 → Phe (C), and Met-230,Met-231 → Leu,Leu (D) were taken with 200 2-s data accumulations. Because their underlying EPR signals were weaker, the spectra (E and F) from the single-Met mutants were taken with approximately 1000 2-s accumulations. All spectra except B were taken with an EPR cavity whose EPR frequency was approximately 9.40 GHz where the value of ν_{NMR} was about 14.0 MHz; spectrum B was taken with an EPR cavity whose resonance frequency was 9.13 GHz, where the value of ν_{NMR} was about 13.6 MHz. To compensate for this difference in ν_{NMR}, spectrum B was shifted by 0.4 MHz to higher frequency. Otherwise, the conditions are as in Figure 4.

We have not found an ENDOR signal from the Trp-191 → Phe mutant because of the cross relaxation within its EPR line (Scholes et al., 1989). However, the narrow radical EPR

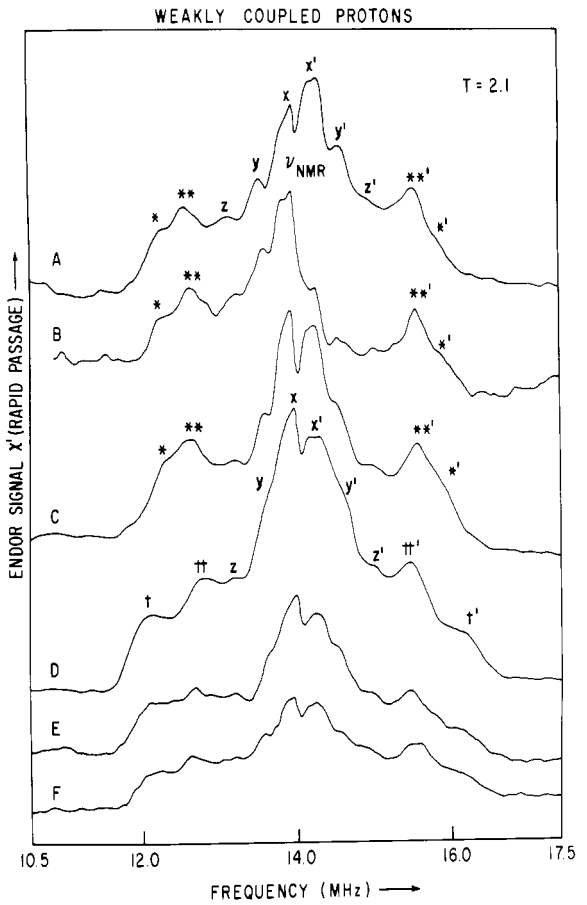

FIGURE 6: This figure is a comparison of weakly coupled proton ENDOR features near the free proton ENDOR frequency as taken at $g = 2.032$. Samples were as follows: (A) CcP(MI), protonated solvent (750 μM); (B) CcP(MI), deuterated solvent (300 μM); (C) Trp-223 → Phe (535 μM); (D) Met-230,231 → Leu,Leu (750 μM); (E) Met-231 → Leu (225 μM); (F) Met-230 → Tyr (225 μM). These spectra were taken with RF of about 0.5 G ptp, 100-kHz field modulation of about 0.75 G ptp, 5-s sweeps over a range of 10.5–17.5 MHz, and about 200 data accumulations. Notice the contrast of the outlying asterisked (*, **) features of CcP(MI) with their counterparts (†, ††) from the Met-230,231 → Leu,Leu double mutant (D). The single-Met mutants (E, F) had weaker underlying EPR signals; hence, their ENDOR signal to noise is poorer.

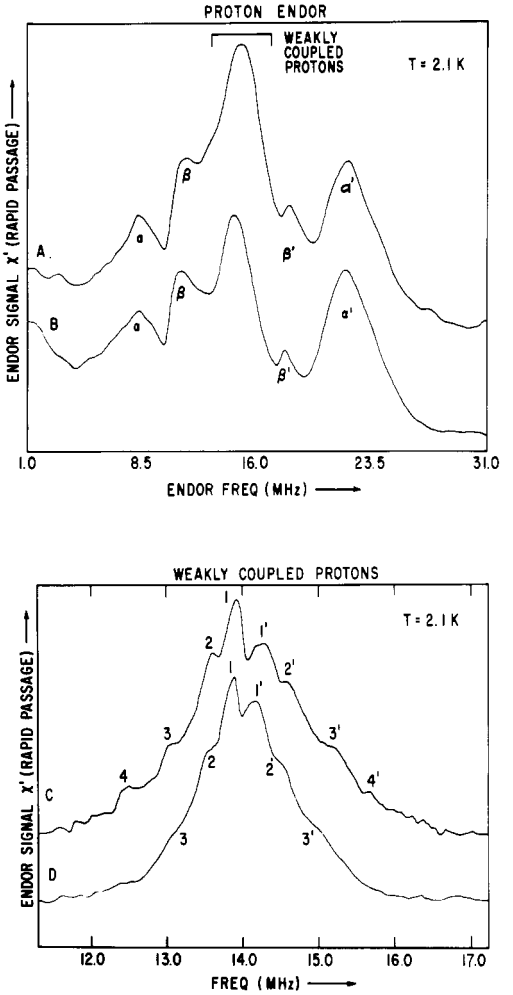

FIGURE 7: This figure shows the ENDOR spectra of the Asp-235 • Asn mutant and compares spectra from the radical as prepared in protonated vs deuterated buffer. Concentration was 500 μM. The ENDOR spectra from the Asp-235 → Asn mutants were taken at approximately 20 G above the EPR first-derivative zero crossing ($g = 1.990$), to attain optimal resolution. ENDOR spectra taken over a 1–31-MHz range are shown in (A) from the Asp-235 → Asn mutant in protonated buffer and in (B) from the Asp-235 → Asn mutant in deuterated buffer. These spectra were taken with RF of about 0.5 G ptp, 100-kHz field modulation of about 3.2 G ptp, 1-s sweeps over the 1–31-MHz range, a microwave power of about 30 μW, and about 3000 data accumulations. ENDOR spectra taken over a 11–17-MHz range are shown in (C) from the Asp-235 → Asn mutant in protonated buffer and in (D) from the Asp-235 → Asn mutant in deuterated buffer. These latter spectra were taken with RF of about 0.5 G ptp, 100-kHz field modulation of about 0.4 G ptp, 2-s sweeps over the 1–31-MHz range, a microwave power of about 30 μW, and about 1000 data accumulations.

signal from the Asp-235 → Asn mutant gave strongly and weakly coupled proton ENDOR signals (Figure 7 and Table III). The strongly coupled protons with average coupling of 13.3 MHz (α,α') occurred approximately in the range where couplings were observed for compound I, but slower sweeps over the α and α' features revealed several proton hyperfine couplings whose details were not the same as those seen from compound I (Figure 5A). There were additional features (β,β') with a coupling of 7.4 MHz definitely not exhibited by compound I. The relatively small hyperfine couplings (all less than 10 G, or 28 MHz) observed from EPR or ENDOR are consistent with an aromatic radical (Gordy, 1980, pp 516–529). In contrast to an alkyl radical with spin density localized on one or a few carbons and with several proton hyperfine couplings larger than 10 G (Gordy, 1980, p 497), such an aromatic radical would have spin density delocalized over many atoms. The markedly different intensities of the central features of the Asp-235 → Asn mutant in protonated and deuterated solvent showed that the central feature had a large contribution from weakly coupled, exchangeable protons. The narrow radical seen in spectra of this mutant is thus more accessible to exchangeable solvent protons than the compound I radical of the parent enzyme. Detailed ENDOR spectra (Figure 7C,D) taken over a narrow frequency range near ν_{NMR} have revealed the explicit features 1,1′, 2,2′, 3,3′, and 4,4′ with couplings given in Table III; features 3,3′ and 4,4′ were exchangeable. These weakly coupled features from the Asp-235 → Asn mutant are not the same features observed for the majority compound I signal.

Discussion

Influence of Trp-191 on CcP Structure and Function. Figure 1 graphically shows that the Trp-191, porphyrin, and proximal histidine are close enough for magnetic interaction and possibly even for direct, intraprotein electron transfer. Recent structural evidence supports the possibility that an electronic interaction can occur between heme iron and Trp-191; binding of the electron-donating NO ligand to ferric heme

has resulted in considerable electron difference density around the Trp-191 position (Edwards et al., 1988; Edwards & Poulos, 1990). Although previous work has shown that the mutation of Trp-191 to Phe caused the loss of the compound I radical signal (Scholes et al., 1989), the 1:1 stoichiometry for the reaction of the ferric enzyme with peroxide is preserved in the Trp-191 → Phe mutant (Mauro et al., 1988; Erman et al., 1989). The substitution of Phe for Trp-191 is accommodated by adjustments of the protein backbone, but without major alteration in the protein conformation or change in the iron coordination sphere (Wang et al., 1990; Smulevich et al., 1988). The implication of these observations is that the substitution of Phe for Trp-191 results, following binding of peroxide, in the oxidation of one or more alternative sites on the enzyme. Transient optical spectra indicate that the initial reaction of the Trp-191 → Phe mutant with peroxide oxidizes the porphyrin, creating a transient prophyrin cation radical (Erman et al., 1989). However, at 20 °C the porphyrin radical decays within 50 ms, which indicates possible subsequent oxidation by the porphyrin radical of a nearby aromatic side chain, such as tyrosine in the mutant protein. The midpoint potential for porphyrin oxidation from Fe(IV) to porphyrin radical runs between +1.3 to +1.5 V in tetraphenylporphyrins bearing various electron-donating and -withdrawing substituents (Kadish et al., 1976). A comparison of spectra C and D of Figure 3 shows that the similarity in EPR spectra between the tyrosine radical of photosystem II and the radical observed in CcP(MI) when Trp-191 is mutated to Phe is striking.

Influence of Met-230 and -231 on Compound I Radical. Because of their proximity to Trp-191 (Figure 1B), the polarizable sulfur atoms of Met-230 and -231 are in a position to influence a Trp-191 radical. The sulfur of Met-230 in particular (Figure 1B) may play a part in positioning the indole ring of Trp-191. The EPR and ENDOR features of single- and double-methionine mutants showed that changes at Met-230 and/or Met-231 also resulted in a spectroscopic perturbation. It is clear that these mutations both can cause perturbations that are physically adjacent to Trp-191 and that the mutants exhibit similar effects on the EPR and ENDOR spectra. In the ENDOR spectra of these methionine mutants (Figure 5) there is loss in detail from those strongly coupled protons most likely residing on the indole ring or on the α- or β-carbons connecting it to the protein backbone. The perturbation to the weakly coupled protons, particularly the appearance of new features, noted in Figure 6 by † and ††, in place of those with * and ** in the parent compound I spectrum, implies changes in hyperfine interactions with protons more distant from the radical center. The weakly coupled protons are the ones whose dipolar couplings are most sensitive (through the formula of footnote 3) to change in orientation.

It has been reported that no spin density resides on methionines (Sivaraja et al., 1989). As exemplified by characteristic g values and especially by hyperfine couplings, our EPR and ENDOR results establish that the Trp-191 conformation needed to establish its electronic structure is affected by its Met-230 and Met-231 neighbors. Hyperfine changes, resulting from mutations at Met-230 and/or Met-231, indicate that spin density and local structure is altered. Hyperfine change could, in fact, be for any of the following reasons: (1) spin densities at individual tryptophan carbons are perturbed; (2) the relative orientation of hyperfine and g tensors is perturbed; (3) the relative orientation of the indole π cloud and the CH bonds of α- and β-protons on Trp-191 is altered (Gordy, 1980, pp 216–217); (4) distances and angles that specify orientation with respect to more distant dipolar-coupled protons not on Trp-191 are changed. The first three reasons would be relevant to strongly coupled protons, while the last three would be relevant to weakly coupled protons. In overview, the altered EPR and ENDOR signals of compound I from the mutants at methionines 230 and 231 may indicate loss of sulfurs that normally serve specifically to stabilize compound I charge density localized predominantly on Trp-191, or they may simply indicate that these two methionines stabilize the packed interior protein structure so as to stabilize the position of Trp-191.

Influence of Asp-235 on Compound I Radical. The Asp-235 → Asn mutation perturbs both the orientation of Trp-191 and the reactivity of the heme iron (Wang et al., 1990; Smulevich et al., 1988). Asp-235 constitutes the central element in a proximal hydrogen-bonding network that links the carboxylate side chain with His-175 and Trp-191 (Figure 1A). Work of Wang et al. (1990) has shown that substitution of Asn for Asp-235 disrupts this hydrogen-bonding network, with at least two consequences: First, the loss of the hydrogen-bonding interaction between Asp-235 and the indole N of Trp-191 results in the approximately 180° rotation of the indole side chain, so that it forms a hydrogen bond with the backbone carbonyl of Leu-177 (Wang et al., 1990). Despite the change in the orientation of the indole ring, the distances between the Trp-191 residue, the porphyrin, and His-175 are largely conserved. Second, the hydrogen-bonding interaction between His-175 and the carbonyl oxygen of Asn-235 is considerably weaker than the interaction between the carboxylate of Asp-235, thus decreasing the electron density on Fe (Smulevich et al., 1988).

In the present work we have shown that the majority compound I radical signal is also absent when Asp-235 is replaced by Asn. In view of the clear experimental evidence for a different site for the narrow minority radical in the Trp-191 → Phe mutation, we propose that the narrow radical signal observed in Asp-235 → Asn also is no longer located at Trp-191. The general similarity in the EPR line shape of the narrow radical signal in the Asp-235 → Asn and the Trp-191 → Phe mutants might indicate a non-Trp-191 location for the narrow radical. The accessibility of the narrow radical in the Asn-235 mutant to exchangeable protons implies that the radical resides near the exterior of the protein. As observed for the Asn-235 narrow radical, the 12–18-MHz coupling of the features labeled α,α' is comparable to the couplings for the para (3,5) protons of most tyrosine radicals, and the 7.4-MHz coupling of the β,β' features is comparable to the coupling observed for the meta (2,6) protons of the tyrosine radical (Bender et al., 1989; Barry et al., 1990). Generally, the β-methylene carbons of the tyrosine radical in photosystem II or ribonucleotide reductase have been found with couplings > 20 MHz, and we do not see couplings > 20 MHz from the narrow radical of the Asn-235 mutant. However, it is possible that the conformation of a tyrosine radical about its β-methylene carbon can be disordered, hence blurring the features of the β-methylene protons.

On the other hand, the possibility cannot be complete ruled out that the narrow radical in the Asn-235 mutant is still near the Fe(IV) center, perhaps even at Trp-191, and exhibits a more typically aromatic radical signal simply because of the new orientation of the indole ring. The major difference in hyperfine-coupled protons between the compound I radical and the narrow radical of the Asp-235 → Asn mutant is the presence of the β,β' proton couplings exhibited by the latter. The short T_1 and its temperature dependence are evidence in favor of a location indicating proximity of the radical to the Fe(IV). The differently oriented radical still at Trp-191 would have to be more accessible to solvent than the compound I

radical, but it can be shown that the general area of the Trp-191 indole in the Asp-235 → Asn mutant is still well sequestered from solvent (Wang et al., 1990).

The lack of the broad compound I radical signal in the Asp-235 → Asn mutant may result from any or all of the following: (1) Loss of the hydrogen-bonding interaction between the carboxylate of Asp-235 and the indole N of Trp-191. This interaction in the unmutated CcP(MI) probably serves to stabilize the formal positive charge created by oxidation of the indole ring and to promote oxidation of Trp-191 by the porphyrin radical; loss of this interaction would prevent oxidation of Trp-191 by the porphyrin radical in the Asn-235 mutant. (2) Loss of the hydrogen-bonding interaction between the Asp-235 carboxylate and His-175. The decreased electron density on the iron in the Asn-235 mutant would make oxidation of the porphyrin by peroxide less favorable energetically (Kadish et al., 1976) and might even prevent the formation of the porphyrin radical observed in the Trp-191 → Phe mutant. (3) The change in the orientation of Trp-191, which would lead to loss of an electron-transfer pathway.

Control of Radical Site by Protein Environment. The well-documented existence of a minority narrow radical in compound I of CcP and CcP(MI) in addition to the characteristic broad compound I radical provides evidence for a tendency toward the oxidation of a second moiety other than Trp-191, even in the unperturbed enzyme (Hori & Yonetani, 1985; Scholes et al., 1989). In this regard, recent pulse radiolysis studies have shown that in solution tyrosine is rapidly oxidized by a tryptophan radical, due to a favorable 400-mV difference in redox potential (Shen et al., 1987; Merenyi et al., 1988). Apparently, however, the local environment in CcP favors the initial oxidation of Trp-191 over tyrosine residues. One possible explanation is that the interaction between the carboxylate of Asp-235 and the indole NH of Trp-191 stabilizes the indole radical sufficiently to prevent subsequent oxidation of tyrosine. The present results with the Asp-235 → Asn mutant, as well as those reported previously for the Trp-191 → Phe mutant (Scholes et al., 1989), suggest that perturbation of the indole ring of Trp-191, or its replacement with a phenyl ring, favors the oxidation of a site or sites other than Trp-191. The similarity between the EPR line shapes of the radical in these two mutations and that observed for the tyrosine radical in photosystem II suggests that the radical location is shifted to a tyrosine exclusively when Trp-191 or its interaction with Asp-235 is modified. The slightly different appearance of several of the radicals in Figure 3, as well as the distinctly different temperature-dependent characteristics of the radicals in Asn-235 and Phe-191 mutants, may imply that the narrow radical can form on one of several tyrosines, depending on the particular mutation. We have not had notable success yet in eliminating the narrow radical by site-directed mutagenesis of a particular residue, and this could imply that the narrow radical could reside on one of several tyrosines.

Additional Mutants. Possible radical sites listed in Table I and not at Trp-191, Met-230/231, or Asp-235 were also mutated. These were found to have little influence on the EPR line shape, although the integrated intensity of the mutants Tyr-39 → Phe, Tyr-36,42 → Phe,Phe, and Met-119 → Ile was smaller than that of CcP(MI). No crystal structures have been determined for any of these mutants. The effects these mutations may have on the optical (UV/vis spectroscopic), three-dimensional, and kinetic properties, including intermolecular electron transfer of the mutant CcP proteins, are topics for future investigation.

In Summary. Our most general finding is that only mutations that alter Trp-191 directly, or the Asp-235, Met-230, or Met-231 of its immediate environment, produce significant alterations to the compound I radical EPR line shape and ENDOR signals. This finding provides evidence that it is Trp-191 and no other tryptophan which is the site of the compound I radical. Furthermore, the detailed EPR or ENDOR changes which occur because of these mutations have given insight, when combined with three-dimensional or kinetic information, into the electronic structure of the radical and the perturbing influences on the radical.

ACKNOWLEDGMENTS

Mr. Harold Taylor performed valuable technical repairs and modifications to the ENDOR system. We thank Prof. Helmut Beinert for helpful discussions about rapid freezing of proteins in liquid nitrogen/isopentane. Dr. Jimin Wang kindly provided X-ray coordinates prior to their publication. L.A.F. thanks the following at UCLA for kindly permitting him to use an EPR spectrometer and for providing advice and assistance at various stages of this research: Jane and Charles Strouse, Leslie Momoda, and Garrard Aka. We thank Huguette Pelletier (protein preparation), Louise Schmidt (oligonucleotides), and David Lewin for excellent technical assistance. Mr. Lloyd D. Tisdale (UCSD) built the mixer apparatus for the stopped-flow system. We are grateful for the kind gift of dark-adapted spinach photosystem II from Prof. G. Brudvig and Dr. C. Buser, Department of Chemistry, Yale University. We are grateful to Ms. Jessica W. Wolpaw for a critical reading of the manuscript and to Ms. Janet F. Bank for sample handling.

Registry No. CcP, 9029-53-2; Trp, 73-22-3.

REFERENCES

Anderson, D. H. (1986) Doctoral Dissertation, Department of Chemistry, University of California, San Diego.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Barry, B. A., & Babcock, G. T. (1988) *Chem. Scr. 28A*, 117–122.

Barry, B., El-Deeb, M. K., Sandusky, P. O., & Babcock, G. T. (1990) *J. Biol. Chem.* (in press).

Beck, W. F., & Brudvig, G. W. (1987) *Biochemistry 26*, 8285–8295.

Bender, C. J., Sahlin, M., Babcock, G. T., Barry, B. A., Chandrashekar, T. K., Salowe, S. P., Stubbe, J., Lindström, B., Petersson, L., Ehrenberg, A., & Sjöberg, B.-M. (1989) *J. Am. Chem. Soc. 111*, 8076–8083.

Bray, R. C. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Eisenhardt, R., Gibson, Q. H., & Lonberg-Holm, K. K., Eds.) pp 195–203, Academic Press, New York.

Butler, W. F., Calvo, R., Fredkin, D. R., Isaacson, R. A., Okamura, M. Y., & Feher, G. (1984) *Biophys. J. 45*, 947–973.

Coulson, A. F. W., & Yonetani, T. (1972) *Biochem. Biophys. Res. Commun. 49*, 391–398.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Edwards, S. L., & Poulos, T. L. (1990) *J. Biol. Chem. 265*, 2588–2595.

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

Edwards, S. L., Kraut, J., & Poulos, T. L. (1988) *Biochemistry 27*, 8074–8081.

Erman, J. E., Vitello, L. B., Mauro, J. M., & Kraut, J. (1989) *Biochemistry 28*, 7992–7995.
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.
Goodin, D. B., Mauk, A. G., & Smith, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1295–1299.
Gordy, W. (1980) in *Theory and Applications of Electron Spin Resonance*, pp 216–217, 497, 516–529, John Wiley & Sons, New York.
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.
Kadish, K. M., Morrison, M. M., Constant, L. A., Dickens, L., & Davis, D. G. (1976) *J. Am. Chem. Soc. 98*, 8387–8390.
Kim, K., & Erman, J. E. (1988) *Biochim. Biophys. Acta 954*, 95–107.
Kulmacz, R. J., Ren, Y., Tsai, A.-L., & Palmer, G. (1990) *Biochemistry 29*, 8760–8771.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
Lang, G., Spartalian, K., & Yonetani, T. (1976) *Biochim. Biophys. Acta 451*, 250–258.
Maniatas, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mauro, J. M., Fishel, L. A., Hazzard, J. T., Meyer, T. E., Tollin, G., Cusanovich, M. A., & Kraut, J. (1988) *Biochemistry 27*, 6243–6256.
Merenyi, G., Lind, J., & Shen, X. (1988) *J. Phys. Chem. 92*, 134–137.
Morse, P. D. (1987) *Biophys. J. 51*, 440a.
Oosterhuis, W. T., & Lang, G. (1973) *J. Chem. Phys. 58*, 4757–4765.
Poole, C. P., Jr. (1983) in *Electron Spin Resonance*, 2nd ed., p 442, J. Wiley & Sons, New York.
Prince, R. C. (1988) *Trends Biochem. Sci. 13*, 286–288.
Scholes, C. P. (1979) in *Multiple Electron Resonance Spectroscopy* (Dorio, M. M., & Freed, J. H., Eds.) pp 297–328, Plenum Press, New York.
Scholes, C. P., Janakiraman, R., Taylor, H., & King, T. E. (1984) *Biophys. J. 45*, 1027–1030.
Scholes, C. P., Liu, Y., Fishel, L. A., Farnum, M. F., Mauro, J. M., & Kraut, J. (1989) *Isr. J. Chem. 29*, 85–92.
Schulz, C. E., Rutter, R., Sage, J. T., Debrunner, P. G., & Hager, L. P. (1984) *Biochemistry 23*, 4743–4754.
Shen, X., Lind, J., & Merenyi, G. (1987) *J. Phys. Chem. 91*, 4403–4406.
Sivaraja, M., Goodin, D. B., Smith, M., & Hoffman, B. M. (1989) *Science 245*, 738–740.
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.
Tartof, K. D., & Hobbs, C. A. (1987) *Focus (Bethesda Res. Lab.) 9* (2), 12.
Wang, J., Mauro, J. M., Edwards, S. L., Oatley, S. J., Fishel, L. A., Ashford, V. A., Xuong, N. H., & Kraut, J. (1990) *Biochemistry 29*, 7160–7173.
Whittaker, M. M., & Whittaker, J. W. (1988) *J. Biol. Chem. 263*, 6074–6080.
Whittaker, M. M., & Whittaker, J. W. (1990) *J. Biol. Chem. 265*, 9610–9613.
Yonetani, T., Schleyer, H., & Ehrenberg, A. (1966) *J. Biol. Chem. 244*, 3240–3243.