THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 262, No. 16, Issue of June 5, pp. 7719-7724, 1987
Printed in U.S.A.

The Peroxide Complex of Yeast Cytochrome c Peroxidase Contains Two Distinct Radical Species, Neither of Which Resides at Methionine 172 or Tryptophan 51*

(Received for publication, December 29, 1986)

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The nature of the free radical species observed in the peroxide complex of yeast cytochrome c peroxidase is described for protein variants containing amino acid substitutions at Met-172 and Trp-51. As was the case with Met-172 mutations (Goodin, D. B., Mauk, A. G., and Smith, M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 1295–1299), Trp-51 can be substituted to give active enzyme. Phe-51-containing enzyme has a higher turnover rate than the original enzyme and exhibits an altered pH dependence. The properties of the isotropic and axial components (Hoffman, B. M., Roberts, J. E., Kang, C. H., and Margoliash, E. (1981) *J. Biol. Chem.* 256, 6556–6564; Hori, H., and Yonetani, T. (1985) *J. Biol. Chem.* 260, 349–355) of the EPR signal of the wild-type enzyme-peroxide complex, studied as a function of H_2O_2 stoichiometry, support proposals (Goodin *et al.* (1985) and Hori and Yonetani (1985), see above) that two distinct radical species are formed, and spin quantification shows that the isotropic radical is always formed in substoichiometric amounts. The peroxide complexes for proteins containing amino acid substitutions at either Met-172 or Trp-51 exhibit somewhat larger than normal levels of the isotropic radical signal. In addition, these mutants are unlike wild-type enzyme in that the axial EPR signal associated with the peroxide complex is seen only at 10 K and not at 90 K. Thus, neither amino acid can be considered to be the molecular species responsible for either radical signal, but both mutations appear to affect the physical properties of the axial signal representing the major radical species.

Among the many metalloenzymes that are known, yeast cytochrome c peroxidase (ferrocytochrome-c:hydrogen-peroxide oxidoreductase, EC 1.11.1.5) is unusual in that it and an unrelated ribonucleotide reductase (4) are the only two enzymes that are presently believed to involve a protein-centered free radical species as a part of their reaction mechanism. The heme iron center of the resting ferric enzyme reacts rapidly with hydroperoxides to form a relatively stable complex (ES)[1] that has been oxidized by two charge equivalents (5). One of these equivalents is accounted for in the ferryl (Fe^{4+}=O) center of the oxidized heme. The oxidized ES complex returns to its resting state by oxidation of two molecules, one after the other, of its reducing substrate, ferrocytochrome c. Whereas the oxidized complex for many other peroxidases, notably horseradish peroxidase compound I, retains the second oxidizing equivalent on the porphyrin macrocycle as a π cation radical (6), the ES complex of cytochrome c peroxidase contains a unique free radical EPR signal that has long been ascribed to a reversibly oxidizable amino acid residue (7–9). This free radical is magnetically isolated from the heme iron (9), and its properties are thus inconsistent with a porphyrin-based radical (2). Two potentially oxidizable residues, Trp-51 and Met-172, have been identified based on their close positioning relative to the heme center (10, 11) and the physical properties of the radicals they might produce (2, 7, 9, 12–14).

The EPR properties of the ES radical have been well studied (2, 3, 7–9, 14). At temperatures below 25 K, the observed radical appears as an axial signal with $g_\perp = 2.006$ and $g_\parallel = 2.034$ (2, 3) which is difficult to saturate, indicating an efficient spin relaxation mechanism. It has been argued that the properties of this signal are inconsistent with a radical of an aromatic residue but are possibly attributable to the intrinsic anisotropy of a nucleophilically stabilized sulfur radical (2, 9). At temperatures above 25 K, this axial signal is greatly broadened, presumably by spin lattice relaxation, so that only broad wings around $g = 2$ are observed (3). Superimposed on this broad resonance is an isotropic signal at $g = 2.004$ containing hyperfine structure that is easily saturated and is orientation-independent. These properties led Hori and Yonetani (3) to propose that ES contains more than one distinct radical species.

In a previous study, we have replaced Met-172 of cytochrome c peroxidase by site-directed mutagenesis to examine its role in the ES complex (1). It was found that Met-172 could be replaced to give active enzyme. EPR spectra at 90 K of the ES complex of cytochrome c peroxidase containing Ser-172 showed the presence of the isotropic signal, but lacked the broad wings. These results were consistent with a heterogeneity of radical species, but it was not possible to determine definitively if the axial signal was abolished by replacement of Met-172 or otherwise altered in its properties so that it was no longer observed at 90 K. In this study, we present additional evidence for two distinct radical species in ES, assess the relative contribution made by the isotropic signal, and

* This work was supported by operating grants from the Medical Research Council of Canada (to M. S. and A. G. M.) and the National Cancer Institute of Canada (to M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ES, cytochrome c peroxidase-peroxide complex; WT CCP, cytochrome c peroxidase containing no alterations from the sequence of the cloned gene; W, watt.

further investigate the EPR properties at 10 K for cytochrome c peroxidase containing mutations at Met-172 or Trp-51.

MATERIALS AND METHODS

Construction and Expression of Mutant Cytochrome c Peroxidase—The site-directed mutagenesis methods of Zoller and Smith (15) were used as previously described (1) for the construction of cytochrome c peroxidase mutants in the vector pEMBLCCP1 with the following exceptions. The mutation efficiency was increased significantly by adapting methods of Kunkel (16) for incorporation of uracil into the single-stranded template DNA. Briefly, 1 ml of an overnight culture (grown ampicillin) of the dut⁻ ung⁻ *Escherichia coli* RZ1032 (17) freshly transformed with pEMBLCCP1 was diluted into 20 ml of the growth medium and incubated for 1 h at 37 °C. No additions of uridine to the growth medium were made. The culture was superinfected at a multiplicity of infection of 40 with f_1 (IR1) bacteriophage followed by incubation at 37 °C for 4–6 h before isolation of single-stranded DNA as earlier described (18). Template DNA containing uracil was checked for a high differential survivability in ung⁻ and ung⁺ *E. coli* strains as described by Kunkel (16), and efficiencies were typically 10^4-fold higher in RZ1032 than in JM101 (19). In the mutagenesis reactions, ratios of the mutagenic oligonucleotide to primer were reduced from 20:1 as described by Zoller and Smith (15) to 2:1 or 1:1, and the second primer was omitted. Mutagenesis efficiencies of 60–80% were consistently obtained, and this enabled the construction of families of mutations at a given amino acid by using mixed oligonucleotides. Mutagenic oligonucleotides, 25 bases in length, were synthesized on an Applied Biosystems 380A DNA synthesizer. Four primers were used for the construction of Trp-51 mutants which contained the following base mixtures for the Trp-51 codon at the center of the primer: C/T, C/T, T; A/G, A/G, A/T; G/A, C/T, G; and C/T, G/A, C/G. Following mutagenesis (15) and transformation of JM101 with as much as 0.25 pmol of DNA from the mutagenesis reaction, plasmid DNAs from ampicillin-resistant colonies were sequenced (20) to screen for desired amino acid replacements.

Protein Expression and Purification—Using methods described earlier (1), mutants of the *CCP* gene (the cytochrome c peroxidase gene) contained on the 1.7-kilobase *Hin*dIII fragment of pEMBLCCP1 were subcloned into the yeast vector YEp13CCP which was used to transform yeast strains DG5-4 (*a, leu2, his3, CCP::HIS3*) or DG5-10 (*a, leu2, his3, ura3, CCP::HIS3, PEP4::URA3*). These yeast strains were constructed by crossing ZA515 (*α, bar1, leu2, PEP4::URA3*) with a strain W303-1AX (*a, ura3, his3, CCP::HIS3*) which was in turn derived from W303-1A A2H used previously (1). The *pep4* phenotype was verified using the method of Jones (21). Mutant *CCP* genes were recovered from transformed yeast cultures and sequenced to verify the mutations. Yeast whole cell lysates were prepared for activity analysis and Western blot as earlier described (1).

Purification of cytochrome c peroxidase from 40-liter cultures of transformed yeast was performed by published methods (1, 22) with the following exceptions. Some mutants of cytochrome c peroxidase appear to be unstable during cell autolysis in ethyl acetate. For this reason, yeast cells were broken by mechanical fracture. Approximately, 1 kg of air-dried yeast was suspended in enough buffer A (consisting of 50 mM sodium acetate (pH 5.0), 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride) to make the suspension pourable. The mixture was frozen by pouring into liquid nitrogen and fractured by grinding three times for 1 min in a stainless steel blender. The freezing/grinding step was repeated once, and 3 liters of buffer A were added. After thawing and centrifugation at 10,000 × *g* for 20 min, the clear supernatant was loaded onto a 5 × 15-cm DEAE-Sepharose CL-6B column equilibrated in 50 mM sodium acetate (pH 5.0). From this point, the protein purification followed the method previously published (1, 22).

Functional and Spectroscopic Measurements—Steady-state kinetics of the cytochrome c peroxidase-catalyzed oxidation of horse heart cytochrome c by H_2O_2 were performed and analyzed as described by Kang and Erman (23) and were carried out at 25 °C in 20 mM Tris phosphate buffer, 113 μM H_2O_2, >95% reduced cytochrome c at 20 μM, and approximately 300 pM cytochrome c peroxidase.

EPR spectra at 90 K were collected at 9.18 GHz using 100 kHz-field modulation in 3-mm inner diameter quartz tubes on a Varian E109 spectrometer using a TE102 microwave cavity. EPR spectra at 10 K were collected on a similar spectrometer at 9.12 GHz equipped with an Air Products Helitran cryostat located in the Chemical Biodynamics Division of the Lawrence Berkeley Laboratory. Spin quantification was accomplished by numerical double integration of digitized spectra using a $K_2NO(SO_3)_2$ solution standardized optically (24).

RESULTS

Isotropic and Axial Signals Represent Distinct Species—EPR spectra collected at 90 K are presented in Fig. 1 showing the effects of varying amounts of H_2O_2 on the signals of cytochrome c peroxidase. As expected upon addition of an equimolar quantity of H_2O_2, the ferric enzyme was fully oxidized as observed by the complete disappearance of the axial ferric iron EPR signal at $g = 6.0$. The appearance of the axial signal of the ES complex was observed as an increase in EPR signal intensity in the "wings" of the $g = 2$ region under conditions of high microwave power. Addition of a 10-fold excess of H_2O_2 failed to cause an increase in the amplitude of this axial signal over the level observed with equimolar addition. However, the magnitude of the isotropic signal at $g = 2$ observed at low power did not saturate at equimolar quantities of H_2O_2 but continued to increase significantly as the H_2O_2 to cytochrome c peroxidase ratio was raised from 1 to 10.

This observed superposition of two signal species was also reflected in their power saturation dependence. It is known that the axial and isotropic signals have different microwave power saturation behavior in which the isotropic component begins to saturate at levels of approximately 1 mW, whereas signal intensity in the wings fails to saturate at levels up to 100 mW (3). Although Hoffman *et al.* (2) have suggested that this behavior may arise from different components of a single radical with different spin relaxation rates and slow cross-relaxation, Hori and Yonetani (3) have proposed that the two signals are attributable to distinct magnetic species. Shown in Fig. 2 is the microwave power dependence of the 90 K EPR signal intensity observed at the magnetic field position of the maximum in the isotropic signal at $g = 2.004$. A different dependence was observed when the H_2O_2 to cytochrome c peroxidase ratio was raised from 0.25 to 2.5. This observation strongly supports the notion that a mixture of radical species exists in which a greater contribution of the more easily

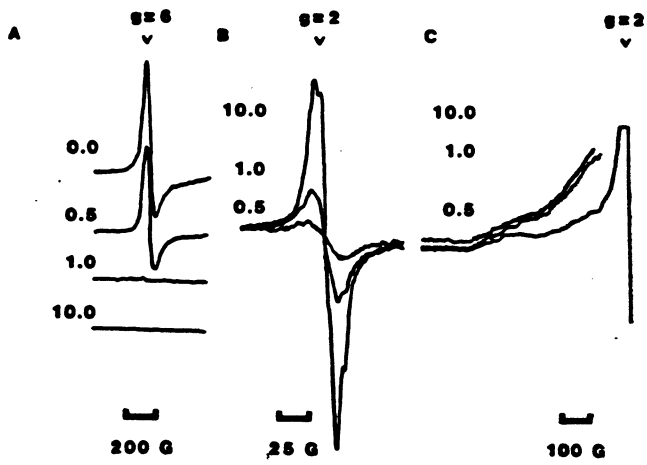


FIG. 1. Effect of H_2O_2 stoichiometry on EPR signals of ES. EPR spectra were collected at 90 K for 200 μM cytochrome c peroxidase solutions in 100 mM sodium phosphate (pH 6.0), 30% glycerol, containing various amounts of added H_2O_2. Spectra in *A* were collected using 1-mW microwave power, 20-G field modulation, and a receiver gain of 1.25×10^4; spectra in *B*, 1-mW microwave power, 2-G field modulation, and a receiver gain of 2.5×10^4; spectra in *C*, 200-mW microwave power, 20-G field modulation, and a receiver gain of 3.2×10^3. The *numbers* to the *left* of each scan represent the ratio of H_2O_2 to final concentration cytochrome c peroxidase. The *upper scan* in *C* was offset slightly to distinguish it from the scan below it.

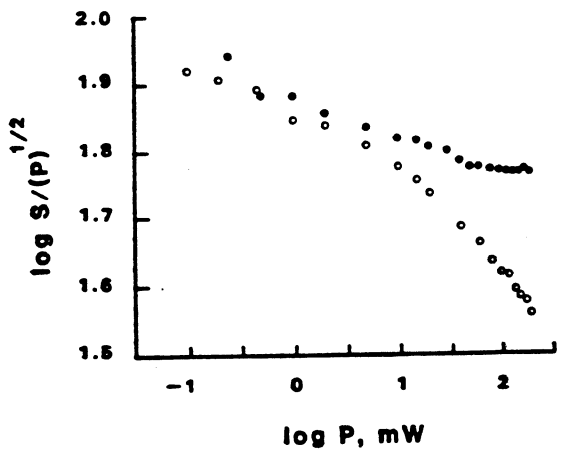


FIG. 2. **Microwave power dependence of EPR signal intensity at two ratios of [H_2O_2] to [cytochrome c peroxidase].** EPR signal intensity (S) was measured at 90 K as the peak-to-peak amplitude of the isotropic signal at 3240 G as a function of microwave power (P). Both samples contained cytochrome c peroxidase at a concentration of 100 μM in 100 mM sodium phosphate (pH 6.0) and 30% glycerol. Two curves are shown: for [H_2O_2]/[cytochrome c peroxidase] = 0.25 (●) and for [H_2O_2]/[cytochrome c peroxidase] = 2.5 (○).

TABLE I

EPR spin quantification of the isotropic component of the ES EPR signal at 90 K

Spectra were collected at 6-μW microwave power and 10-G field modulation. The spin concentration ([S]) was determined by double integration using 152 μM $K_2NO(SO_3)_2$ as a spin standard.

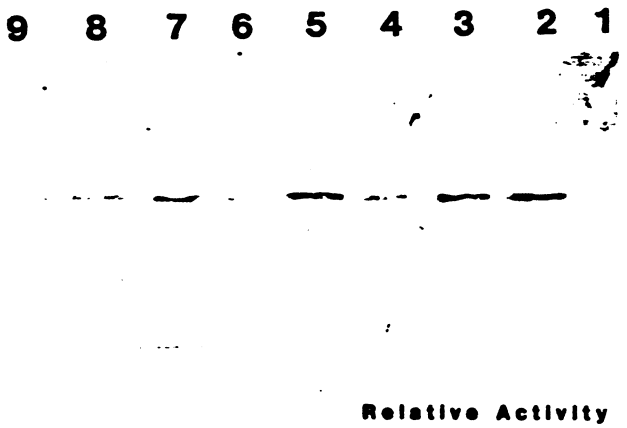
	[CCP]*	[H_2O_2]	[H_2O_2]/[CCP]	[S]	[S]/[CCP]
	μM	μM		μM	
WT CCP	200	2000	10.0	16.3	0.082
WT CCP	200	200	1.0	9.6	0.048
CCP(F51)	121	188	1.6	12.0	0.099

* CCP, cytochrome c peroxidase.

saturable isotropic signal is generated at high H_2O_2 to cytochrome c peroxidase ratios.

A measurement was made of the contribution that the isotropic signal made to the total spin composition of the ES complex. Measurements on the signal observed at 1.5 K (8) have previously led to the conclusion that the ES complex contains one EPR detectable spin per molecule. This represents the total number of spins present and would include the unknown contribution of the isotropic radical species. Presented in Table I are the results of spin quantifications obtained by double integration of only the narrow isotropic component of the ES signal at 90 K under conditions of low power. It is clear that the isotropic signal represented less than 10% of a spin per mol of ES even when a 10-fold excess of H_2O_2 was used. Thus, the axial EPR signal is clearly the major spin species of the ES complex.

Activity and Properties of Cytochrome c Peroxidase Mutants at Trp-51—A number of amino acid substitutions at Trp-51 of cytochrome c peroxidase were generated and examined for expression in transformed yeast (1). As shown in Fig. 3, Western blot analysis of transformed yeast whole cell lysates indicated a wide variation in the level of cytochrome c peroxidase. The total enzyme activity measured in whole cell lysates (Fig. 3) showed qualitative agreement with the variation in the protein level observed by Western blot. This variation was reproducible in that the relative levels depended only on the identity of the amino acid replacing Trp-51 and not upon growth conditions or yeast strain. Similar variations were observed in the yeast strains DG5-4 and DG5-10. These



	Relative Activity
1. CCP⁻	0%
2. WT-CCP	100%
3. Ser-172	78%
4. Cys-172	26%
5. Phe-51	174%
6. Met-51	5%
7. Thr-51	90%
8. Cys-51	9%
9. Lys-51	5%

FIG. 3. **Relative cytochrome c peroxidase activities and Western blot of transformed yeast whole cell lysates.** Yeast whole cell lysates were assayed for cytochrome c peroxidase activities as earlier described (1). For Western blotting, 5 μl of each cell lysate, corresponding to 50 μl of cell culture, was run on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After transfer to nitrocellulose and blocking (37), the filter was probed using rabbit anti-cytochrome c peroxidase serum and detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (38). *Sample 1* was from yeast strain DG5-4 transformed with YEp13 containing no cytochrome c peroxidase sequence (39), *samples 2–9* were from yeast strain DG5-4 transformed with YEp13CCP containing either wild-type cytochrome c peroxidase gene sequence or the specified mutation.

strains were derived from the earlier described host W303-1A A2H, and DG5-10 contained a *URA3* interruption of *PEP4*, resulting in vacuolar protease deficiency (25). These variations in protein level and total activity may thus represent differences in the intrinsic enzyme stability, in the mitochondrial membrane transport, or in heme binding properties of the precursor apoprotein. Nevertheless, these observations demonstrate that a number of different amino acids can replace Trp-51 to give cytochrome c peroxidase with catalytic activity.

Samples of cytochrome c peroxidase containing Phe-51 (CCP(F51)) and Trp-51 (WT CCP) were purified as earlier described (1). The optical spectrum of CCP(F51) gave an A_{408}/A_{280} ratio of 1.38. This was slightly higher than 1.32 observed for WT CCP but was otherwise similar to that of WT CCP. Addition of H_2O_2 to CCP(F51) produced an absorbance spectrum that was characteristic of the ES complex (26) (data not shown) but with somewhat broadened transitions at 519 and 548 nm relative to those of WT ES. These properties are in agreement with those observed by Fishel *et al.* (27) for cytochrome c peroxidase containing Phe-51 produced in *E. coli*.

The purified protein containing Phe-51 exhibited an initial turnover rate at pH 5.6 with horse heart cytochrome c that was approximately 5 times higher than the Trp-51-containing

enzyme, as also observed by Fishel *et al.* (27). Shown in Fig. 4 is the pH dependence of the initial turnover rate in 100 mM phosphate buffer. In addition to the overall increased turnover rate, CCP(F51) exhibited a markedly different pH dependence in which the maximal rate was observed at pH 6.5 rather than 5.0 as observed for WT CCP and CCP(S172).

EPR spectra of the native ferric forms of WT CCP, CCP(C172), and CCP(F51) at 10 K are shown for the $g = 6$ region in Fig. 5. These spectra are roughly similar in the position of their turning points. EPR spectra (not shown) for CCP(S172) are essentially identical to that shown for CCP(C172). The apparent broadening of the component of the signal at $g = 6.65$ (980 G) in the spectrum of WT CCP of Fig. 5 was not always observed and is somewhat variable from preparation to preparation. However, for CCP(F51), an additional set of turning points is apparent as two shoulders on either side of the $g = 6$ transition.

EPR Properties of Mutant Cytochrome c Peroxidase-Peroxide Complexes—EPR spectra at 90 K for WT CCP, CCP(F51), and their ES complexes are shown in Fig. 6. The ES complexes were generated by the addition of a slight excess

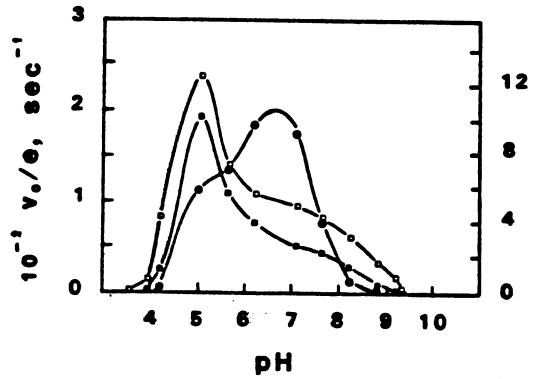


FIG. 4. **Initial enzyme turnover rate as function of pH for WT CCP, CCP(S172), and CCP(F51).** Enzyme kinetics were performed as described by Kang and Erman (23) using conditions described under "Material and Methods." Samples were: WT CCP (□), CCP(S172) (■), and CCP(F51) (●) (*right scale*).

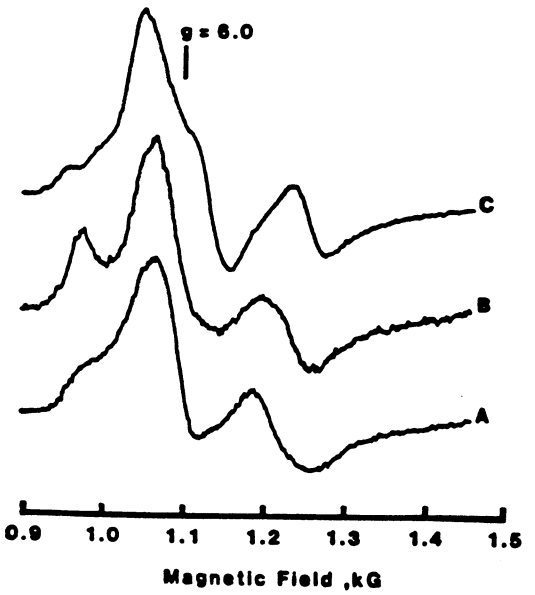


FIG. 5. **EPR spectra at 10 K for ferric cytochrome c peroxidase samples.** Spectra were collected for 100 μM cytochrome c peroxidase in 100 mM sodium phosphate (pH 6.0) using 1-mW microwave power and 2-G field modulation. *Sample A*, WT CCP; *sample B*, CCP(C172); *sample C*, CCP(F51).

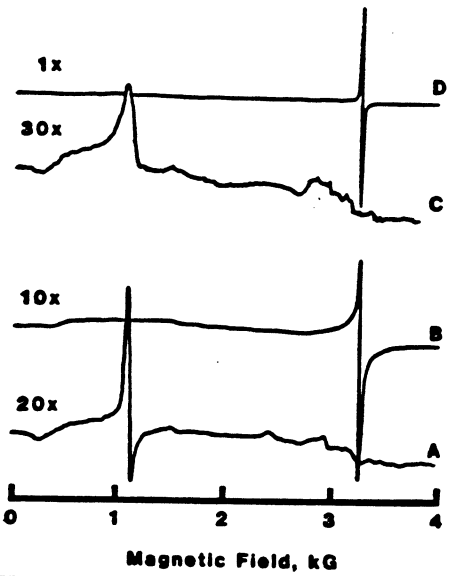


FIG. 6. **EPR spectra at 90 K of WT CCP and CCP(F51) and their peroxide complexes.** Spectra were collected using 50-mW microwave power and 10-G field modulation for 121 μM cytochrome c peroxidase in 100 mM sodium phosphate (pH 6.0) and 30% glycerol and a small excess of H_2O_2. *Sample A*, WT CCP; *sample B*, WT ES complex; *sample C*, CCP(F51); *sample D*, CCP(F51) ES complex. The relative receiver gains are displayed at the *left* of each scan.

of H_2O_2. As clearly seen in the $g = 2$ region, the ES complex of CCP(F51) contained the narrow isotropic component of the ES radical signal. The hyperfine structure observed for this signal under low field modulation conditions (3) was also present. Potentially more significant are the differences observed in the wings of the free radical EPR signals of Fig. 6. The axial EPR signal associated with the ES complex, observed as broad wings at 90 K, is clearly observed in the $g = 2$ region of WT ES. However, this is not the case for CCP(F51) ES, where no broad wings are evident under these conditions. This result is remarkably similar to the effect observed previously (1) when Met-172 was replaced by serine. In addition, this effect was observed in the 89 K EPR data of Fishel *et al.* (27) for the *E. coli*-derived cytochrome c peroxidase containing Phe-51, although some indication of an axial signal remained.

From the relative gain settings of Fig. 6, it is apparent that the amplitude of the narrow component of the signal is greater for CCP(F51) than observed in the sample of WT CCP. Spin quantification of this isotropic signal for CCP(F51) ES (Table I) showed that it was nevertheless present at less than 10% of the total cytochrome c peroxidase concentration. This conclusion was verified by double integration of the two spectra of Fig. 6. The spin concentration of the isotropic signal observed for CCP(F51) ES at 90 K represented only 17% of that obtained by integrating the spectrum of WT ES over 1500 G to include as much contribution from the wings as possible. Fishel *et al.* (27) have observed that the total integrated signal intensity at 89 K was similar for the ES complex of both Trp-51- and Phe-51-containing enzymes. These authors suggested that the narrow signal for the Phe-51 mutant had increased by an amount that compensated for that lost from the axial signal. However, our data indicate that the isotropic signal of CCP(F51) ES does not increase sufficiently to account for the "missing" intensity in the wings of the axial signal. Thus, it is not possible to establish clearly from the EPR data at 90 K if the axial signal has been eliminated or otherwise altered by these mutations. It must be pointed out, however, that little quantitative significance should be attached to the integration of the broad signals

observed at 90 K. Significant errors may be introduced in such calculations (28) arising from small base-line changes or from a significant contribution from the axial wings if they extend well beyond 1500 G from the signal center.

EPR spectra at 10 K of the ES complexes generated from samples of WT CCP, CCP(S172), CCP(C172), and CCP(F51) are shown in Fig. 7. Each of these spectra shows the presence of varying amounts of the axial EPR signal of the ES radical. For some samples, addition of exact stoichiometric amounts of H_2O_2 appeared to result in the incomplete formation of the ES complex, as judged by residual levels of the $g = 6$ ferric EPR signal; and these samples had smaller than normal amplitudes of the axial ES EPR signal. However, by addition of a slight excess of H_2O_2 (1.5-fold) followed by rapid (<15 s) freezing, samples of CCP(F51) ES were prepared that had essentially the same signal intensity as observed for equimolar WT ES. It has been noted that the ES complex for cytochrome *c* peroxidase containing Phe-51 is less stable than the wild-type complex (27), and this may explain the observed variability in these samples. Nevertheless, the EPR results of Fig. 7 clearly show that the axial signal species can be observed at 10 K for the ES complex containing amino acid replacements for either Met-172 or Trp-51.

DISCUSSION

The data presented above, aided by the study of forms of the enzyme altered by site-directed mutagenesis, allow a number of conclusions to be drawn concerning cytochrome *c* peroxidase and its peroxide complex. Neither Met-172 or Trp-51 is necessary for cytochrome *c* peroxidase to function as a peroxidase. However, substitution of Trp-51 produces observable effects on the initial rate (Fig. 4 and Ref. 27) and pH dependence of its kinetics as well as a small structural perturbation in the symmetry of the ferric heme EPR signal (Fig. 5). The axial and isotropic EPR signals of the ES complex are observed to be formed independently, and the axial signal represents the major species. Neither of these signals can be attributed to either Met-172 or Trp-51, although replacement of either residue appears to alter the properties of the axial signal so that it is difficult to observe at 90 K.

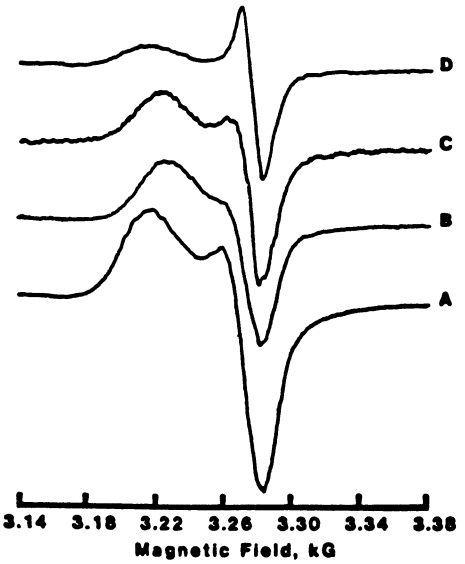


FIG. 7. **EPR spectra at 10 K for peroxide complex of mutant cytochrome *c* peroxidase.** Spectra were collected for 100 μM cytochrome *c* peroxidase in 100 mM sodium phosphate (pH 6.0) and 100 μM H_2O_2 using 10-μW microwave power and 2-G field modulation. *Sample A*, WT ES complex, 1×10^4 receiver gain; *sample B*, CCP(F51) ES complex, 1×10^4 receiver gain; *sample C*, CCP(S172) ES complex, 2×10^4 receiver gain; *sample D*, CCP(C172) ES complex, 1×10^4 receiver gain. The appearance of a narrow signal component near the free electron *g* value superimposed on the axial signal of CCP(C172) ES is probably due to the presence of a small amount of a similar signal observed in this sample prior to oxidation with H_2O_2.

The alteration in the magnitude of the steady-state rate and its pH dependence observed upon substitution of Trp-51 are not fully understood. Although it has been shown that a group with a pK = 5.5, possibly His-52, must be unprotonated for the efficient reaction of native cytochrome *c* peroxidase with H_2O_2 (29), the changes observed in these overall steady-state rates may include other factors such as binding and release of cytochrome *c* and intermolecular as well as intramolecular electron transfer steps. Thus, these reaction components must be studied individually to assess which are responsible for the changes observed upon substitution of Trp-51. These studies are in progress. The observed kinetics, however, indicate that the rate-limiting step in the mechanism has been altered by the substitution. If the kinetic model that has been proposed (23) is correct, the rate-limiting step involves electron transfer within the cytochrome *c* peroxidase-cytochrome *c* complex rather than cytochrome *c* binding or reaction of cytochrome *c* peroxidase with H_2O_2.

The EPR properties of the native enzyme (Fig. 5) indicate that CCP(F51) contains an additional conformational substate that can be distinguished by a slightly different asymmetry of the heme center. The apparent anisotropy of this signal in cytochrome *c* peroxidase has been earlier described (8) as arising from the contribution at pH 6 of two different forms of the iron center having slightly different symmetries. One component is represented by the central line at $g = 6.0$ arising from high spin ferric iron in a purely axial environment. This is superimposed on a smaller signal containing two turning points flanking the $g = 6$ signal at $g = 5.18$ and 6.65 (980 and 1260 G) and represents a distinct form of axial iron with a small rhombic distortion. Presumably, cytochrome *c* peroxidase exists in solution as a mixture of two conformations which result in small symmetry differences in the heme environment. The present data indicate that CCP(F51) exists as a mixture not only of the axial and rhombic forms present for WT CCP, CCP(S172), and CCP(C172), but contains an additional minority conformation with an intermediate degree of rhombic distortion. Trp-51 is hydrogen-bonded to a water molecule that resides 2.4 Å above the distal heme iron (10). Although this water molecule has been described as ligating to the iron atom with a slightly longer than normal distance (10), recent reports have shown that resonance Raman spectra of ferric cytochrome *c* peroxidase at pH 6 are more characteristic of a five-coordinate than a six-coordinate heme protein (30, 31). It is clear that substitution of Trp-51 by Phe-51 will cause a change in the hydrogen bonding of this water, and its effects may be subtly reflected in the anisotropy of the ferric EPR signals.

From the EPR data on the ES complex, we conclude that the narrow isotropic and broad axial EPR signals observed for the ES complex represent distinct species as earlier proposed (1, 3), that the isotropic signal species is formed independently of the axial species, and that it usually represents only a small fraction of the total number of spins associated with the ES complex. A similar signal has been observed in substoichiometric levels in samples of myoglobin oxidized with H_2O_2 (32). It is therefore likely that under physiological conditions, the isotropic species does not figure prominently in the composition or function of the ES complex. It may represent a residue oxidized by H_2O_2 in a side reaction or result from the spontaneous decomposition of ES. The latter interpretation is not likely, however, as we have observed that

in samples of WT ES stored at room temperature for approximately 15–20 min, there is no increase in the ratio of the isotropic to axial signal components. It is also possible that the same molecular species gives rise to both signal components. This could occur if two conformations of the species existed that would result in the radical having different properties. Indeed, the existence of two conformational forms of the radical has been proposed to explain properties of the equilibrium intramolecular distribution of oxidizing equivalents in ES (33, 34). However, although it would be easy to imagine two radical conformations having different spin relaxation properties, it would be more difficult to explain the greatly different anisotropies unless these properties were determined by spin-spin dipolar interaction with the ferryl heme center. Arguments have been previously presented (2) that such an interaction would not be large enough to produce the observed anisotropy.

The presence of the EPR signals of the ES complex in samples of cytochrome *c* peroxidase altered by site-directed mutagenesis demonstrates that neither Met-172 or Trp-51 contains the molecular species of either of the observed radicals. This conclusion is consistent with a magnetic circular dichroism study in which no evidence for alteration of tryptophan residues was observed in the ES complex (35). The increased levels of the minority isotropic spin species observed for CCP(F51) ES and CCP(S172) ES (1) may result from a more rapid decay of an ES complex that is less stable than that of WT CCP (27), in the increased accessibility of a residue oxidized by H_2O_2 during a side reaction, or in the presence in the mutant proteins of a different contribution from distinct conformations of the radical site.

The absence of detectable axial EPR signals at 90 K for the ES complexes of mutants at Met-172 or Trp-51 which are nevertheless observed at 10 K must result from an alteration in the properties of the axial signal such that it is not easily observed at 90 K. For example, the mutations may cause an increase in the spin lattice relaxation rate of the axial spin species to the point that the signal is broadened beyond detection at 90 K. This could be the result of a conformational alteration of the radical site; however, the similar effects of two different mutations, near to but on opposite sides of the heme, suggest that the properties of the radical may be influenced by the heme itself. Mössbauer studies (36) have shown that the ferryl centers of heme proteins usually exist as an $S = 1$ state with a large zero-field splitting of approximately 28 K so that the magnetic states may not be significantly populated at low temperature. A weak interaction between the ES radical species and the thermally excited states of this center may provide the efficient and highly temperature-dependent spin relaxation characteristic of the axial radical. The two mutations near the heme may have simply altered the magnitude of the zero-field splitting of the ferryl center and, as a result, the temperature dependence of the observed EPR properties. The temperature dependence of the magnetic properties of these mutant enzymes needs to be studied to evaluate these effects.

Acknowledgments—We thank Prof. Wm. Powrie, Dr. M. P. Klein, and Prof. K. Sauer for making EPR facilities available, T. Atkinson for oligonucleotide synthesis, and Dr. G. Ammerer for his gift of yeast strain ZA515. We are grateful to Dr. L. Fishel and Prof. J. Kraut for providing a copy of their manuscript in advance and to Prof. T. Yonetani for useful discussions.

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