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Studies of the radical species in compound ES of cytochrome *c* peroxidase altered by site-directed mutagenesis

(heme enzyme function/electron paramagnetic resonance/affinity chromatography/protein engineering/yeast gene expression)

DAVID B. GOODIN, A. GRANT MAUK, AND MICHAEL SMITH

Department of Biochemistry, 2146 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1W5, Canada

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ABSTRACT **Yeast cytochrome *c* peroxidase reacts with hydrogen peroxide to form an intermediate, compound ES, in which the heme iron atom is converted to a ferryl function (Fe^{4+}=O) and a radical center is generated on a reversibly oxidizable amino acid residue of uncertain identity. As methionine-172 is a possible site of this radical, we have constructed specific variants of cytochrome *c* peroxidase in which methionine-172 is replaced by serine or cysteine. These mutants and the wild-type enzyme have been expressed in *Saccharomyces cerevisiae*, purified, and crystallized. Both mutant enzymes are fully active. A stable, reversible, peroxide-induced intermediate with optical properties characteristic of compound ES is observed for the three forms of the enzyme. The electron paramagnetic resonance spectrum of this intermediate at 93 K for the serine mutant exhibits the narrow free-radical signal and hyperfine structure observed for the wild-type enzyme. However, a broader component of the signal that is observed for the wild-type enzyme at this temperature is absent from the spectrum observed with the serine mutant. These results demonstrate that the narrow component of the free-radical signal observed at 93 K cannot reside at methionine-172. The absence of the broader component of the signal for the serine mutant may reflect the loss of spin density on methionine or, alternatively, could arise from conformationally induced changes in the properties of the radical. The results are consistent with a heterogeneity of radical species in the ES complex.**

Cytochrome *c* peroxidase (CCP; ferrocytochrome *c*: H_2O_2 oxidoreductase, EC 1.11.1.5) is a soluble mitochondrial heme enzyme present in yeast that catalyzes the oxidation of ferrocytochrome *c* by hydrogen peroxide (1). As CCP has been the subject of extensive functional and spectroscopic studies (2) and its three-dimensional structure is now known to high resolution (3), this enzyme has become a model for other related enzymes for which less detailed information is available. Specific molecular mechanisms for the function of CCP have been discussed (4–7) that have relevance to the function and evolution of all heme enzymes. Nevertheless, CCP differs fundamentally from other peroxidases in certain specific and incompletely understood features of its mechanism. In the resting state, peroxidases contain a ferric protoporphyrin IX ($Fe^{3+}P$) prosthetic group (2). These enzymes react rapidly with hydrogen peroxide to form an intermediate that is oxidized by two equivalents above the resting state (1). For horseradish peroxidase and other plant peroxidases, this intermediate (compound I) consists of a ferryl center (Fe^{4+}=O) closely coupled with a porphyrin radical cation (P^+) (8, 9). In contrast, reaction of CCP with hydrogen peroxide produces an oxidized intermediate, compound ES, that is analogous to compound I except that the porphyrin radical cation is absent. Instead, an electron paramagnetic resonance (EPR) signal is observed at $g = 2$ that is only weakly magnetically coupled to the (Fe^{4+}=O) iron center and this signal has generally been attributed to a free radical residing on a reversibly oxidized protein residue (10–12). As compound ES is believed to be an intermediate in the catalytic oxidation of ferrocytochrome *c* by CCP, detailed understanding of this form of the enzyme is of considerable importance.

Two sites are often discussed concerning the identity of the radical species of compound ES. An aromatic residue was first proposed (10) based on difference UV absorption changes (13) and the slow autodestruction of tryptophan and tyrosine residues in compound ES (14). When the three-dimensional structure of CCP became available, Trp-51 was found to be 3.3 Å above and parallel to the distal heme face (3). This positioning is optimal for direct π interaction with the heme. A Trp-51 radical is consistent with evidence indicating the existence of ^{14}N superhyperfine coupling to the radical observed by nuclear modulation of the electron spin echo (15). In addition, Trp-51 is replaced by phenylalanine, a less easily oxidized residue, in all other peroxidases and catalase (2). However, Hoffman and coworkers (12, 16) have performed an extensive study of the EPR and electron nuclear double resonance (ENDOR) properties of the radical species at liquid helium temperature and have concluded that the data are completely inconsistent with the existence of either a neutral or cationic tryptophan radical. Instead, they proposed that a nucleophilically stabilized sulfur radical was consistent with the intrinsic anisotropy of the EPR signal at 4 K. The most likely candidate for such a center is Met-172, which lies 3.7 Å below the proximal heme face (3). Importantly, this residue is substituted by serine at the analogous position of other peroxidases (2) so that, as in the case for Trp-51, the putative oxidizable site is not present in the peroxidases that exhibit the porphyrin-based radical.

The nuclear gene for yeast CCP has recently been cloned and its sequence established (17, 18), so it is now possible to begin studies of its function by using forms of the enzyme altered by site-directed mutagenesis (19). In this paper, we describe the expression of the *CCP* gene contained on a plasmid in *Saccharomyces cerevisiae*. We have constructed mutant forms of the gene altered at the codon for Met-172 and have expressed the mutant genes in yeast. The resulting proteins have been purified and crystallized, and their catalytic and spectroscopic properties have been examined to assess the proposed role of Met-172 in the formation of compound ES. Met-172 was replaced by serine because this substitution may represent the minimum structural alteration necessary to convert the unique properties of CCP ES into

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Abbreviations: bp, base pair(s); CCP, cytochrome *c* peroxidase; *CCP*, gene for cytochrome *c* peroxidase; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; $Fe^{3+}P$, ferric protoporphyrin IX; Fe^{4+}=O, ferryl iron center; kb, kilobase(s); P^+, porphyrin radical cation; pfu, plaque-forming unit.

those of horseradish peroxidase compound I. For example, if Met-172 were reversibly oxidized as a component of the ES intermediate, its replacement by serine could force the enzyme to localize the oxidizing equivalent on another species such as a porphyrin cation radical. Cysteine also was placed at position 172 because this potentially oxidizable residue may confer novel but unknown changes upon the properties or stability of the ES complex.

MATERIALS AND METHODS

Strains and Plasmids. Strains of *Escherichia coli* JM101 (20) and RR1 (21) were used for propagation of plasmids based on pEMBL (22) and YEp13 (23), respectively. *S. cerevisiae* strains W303-1A (α, *ade2*-1, *can1*-100, *trp1*-1, *his3*-11, *his3*-15, *leu2*-3, *leu2*-112, *ura3*-1, GAC$^+$[ψ^o]) and W303-1A A2H were provided by J. Kaput. Strain W303-1A A2H has been constructed from W303-1A by interruption of the genomic *CCP* gene with *HIS3*, resulting in a His$^+$ and presumably CCP$^-$ phenotype (J. Kaput, personal communication).

Mutant Construction and Screening in pEMBL Vectors. A portion of the *CCP* coding sequence contained on the 1.7-kilobase (kb) *Hin*dIII fragment of the yeast shuttle vector YEp13CCP (17) was subcloned into pEMBL8(+) to create the plasmid pEMBL8CCP1. The single-stranded form of this plasmid used for mutagenesis and sequencing was prepared by f1 (IR1) virus superinfection of transformed JM101 as described by Dente *et al.* (22). Synthetic oligodeoxyribonucleotides were made either manually or on an Applied Biosystems 380A DNA synthesizer by phosphite triester methods (24). Two-primer mutagenesis was performed by substituting single-stranded pEMBL8CCP1 DNA for the M13 template in the method of Zoller and Smith (25). This employed mismatched primer extension from the mutagenic oligomers 5′ TGCTCTTNNNGGGGCTC 3′, where NNN was AGC and TGC for the Ser-172 and Cys-172 constructions, respectively. A 17-base-pair (bp) sequencing primer, 5′ ATACTACCCCTGACAAC 3′, hybridizing approximately 100 bp from the 5′ end of the mutagenic oligonucleotide, was used as the second primer. DNA isolated from colonies that screened positive for the mutation was used in a second round of transformation and screening to assure a homogeneous plasmid population. Mutations were verified by sequencing the single-stranded pEMBL8CCP1 DNA with dideoxy chain-terminator methods (27).

Yeast Methods. Yeast expression vectors containing the Met-172, Ser-172, and Cys-172 codons were reconstructed from YEp13CCP by replacement of the 1.7-kb *Hin*dIII fragment. After ligation and transformation, recombinants were screened for the correct insert orientation by *Eco*RI/*Bam*HI restriction mapping. The resulting vectors, YDG13C-CPM172, YDG13CCPS172, and YDG13CCPC172 contain the intact *CCP* gene, its promoter, terminator, and 68-amino acid *N*-terminal signal sequence.

Transformation of yeast strain W303-1A A2H was performed by using the LiCl procedure of Ito *et al.* (28), and transformed colonies were selected for leucine prototrophy. To verify the mutant constructions in yeast, we recovered the expression vectors from transformed yeast cultures and amplified them in *E. coli* RR1. The sequence of the alkali-denatured double-stranded DNA (29) was determined for 800 bp surrounding the mutation site.

Whole-cell lysates were prepared for peroxidase activity measurements by growing 5 ml of yeast cultures to saturation in the appropriate selective medium at 30°C. The cells were washed with 50 mM sodium acetate (pH 5.0) and suspended in 0.5 ml of the same buffer containing 1.25 mM sodium metabisulfite and 1.25 mM EDTA. Cells were broken by mixing with a Vortex for 60 sec at 0°C in the presence of 0.5-mm glass beads. After centrifugation (2 min at 16,000 × *g*), 1–5 μl of the supernatant was used for peroxidase activity measurements.

Large-scale yeast cultures were grown either in sterile 40-liter carboys or in the 100-liter fermentation facility of the University of Victoria. Growth was at 30°C in media containing per liter 10 g of Difco yeast extract, 20 g of Bacto-peptone, 20 g of glucose, 60 mg of adenine, 12.5 mg of tetracycline, and 20 mg of streptomycin (adjusted to pH 6.0 with HCl). A large innoculating culture (20% of the final culture volume) was grown in medium lacking leucine and histidine. The final cultures were aerated at a rate of 0.5 liter·min^{-1} per liter of culture, and growth was continued to saturation (>4 × 10^8 cells per ml).

Enzyme Purification. CCP was purified as described by Nelson *et al.* (30) except that DEAE-Sepharose CL-6B was used instead of DEAE cellulose, and lysis was carried out in 50 mM sodium acetate, pH 5.0/1.25 mM sodium metabisulfite/1.25 mM EDTA. Additionally, cytochrome *c* affinity chromatography (31) was necessary either before or after the final gel filtration step to complete the purification of mutant enzymes. Peroxidase samples were prepared for affinity chromatography by equilibration in 10 mM sodium acetate buffer (pH 6.0). Approximately 1–10 mg of enzyme was loaded onto the column (1 × 5 cm) and washed with the same buffer. CCP was eluted with 500 mM sodium acetate buffer (pH 6.0). Samples of the mutant enzymes having an A_{408}/A_{280} ratio of only 0.6 (constant after repeated gel filtration and DEAE-Sepharose chromatography) were easily purified to homogeneity in one step by passage through the affinity matrix. Crystallization of samples having a ratio of $A_{408}/A_{280} > 1.0$ was accomplished by slowly reducing the concentration of sodium acetate buffer at pH 6.0 from 50 mM to 10 mM (30) by repeated dilution/concentration at 4°C with an Amicon Centricon-10 ultrafiltration device.

Functional and Spectroscopic Measurements. The rate of oxidation of horse heart ferrocytochrome *c* (Sigma type VI) by hydrogen peroxide in the presence of CCP was measured as described by Kang and Erman (32) except that reactions were carried out in argon-saturated 20 mM Tris phosphate (pH 6.0) at 25°C. Optical spectra were recorded at 15°C on a Cary 219 spectrophotometer in 100 mM sodium phosphate (pH 6.0). EPR spectra at X-band were recorded in 3-mm i.d. quartz tubes on a Varian E109 spectrometer with a TE102 microwave cavity and a variable temperature controller. Magnetic fields were measured with a Varian E-500 NMR gaussmeter.

RESULTS

Construction, Expression, and Purification of Mutant Enzymes in Yeast. The DNA sequences of pEMBL8CCP1 containing the wild-type and mutant genes in the region of codon 172 are shown in Fig. 1. Alteration of the Met-172 codon to that of serine and cysteine was performed in the pEMBL-based vector with minimal modification of established methods (25). The observed mutation efficiencies of 2–20% were similar to those obtained with M13 vectors (25). However, the use of *dut*$^-$ *ung*$^-$ *E. coli* (33) to prepare single-stranded pEMBL template containing uracil has recently produced mutant yields of 60% (unpublished data).

The strain of yeast used for CCP expression, W303-1A A2H, contained an integrated interruption of the *CCP* genomic sequence (J. Kaput, personal communication). This interruption was verified by Southern blot analysis of yeast W303-1A A2H genomic DNA digested with *Pst* I, which showed only one band hybridizing to the nick-translated 1.7-kb *CCP Hin*dIII fragment. As expected, this band had a lower mobility than that observed for the gene without the *CCP* interruption. No CCP activity was observed in the whole-cell lysates of this strain. The sensitivity of the CCP

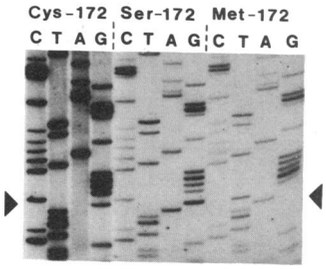

FIG. 1. Sequence analysis of the wild-type and mutant *CCP* genes. Single-stranded pEMBLCCP1 DNA was prepared by f1 (IR1) virus superinfection (22) of transformed *E. coli* JM101 after two consecutive rounds of hybridization screening for the mutation. A 17-bp primer hybridizing 100 bp from the mutagenic site was used for dideoxy chain terminator sequencing (27). The sequences of the wild-type and mutant genes, TGCTCTTATGGGGGCTC for Met-172, TGCTCTTAGCGGGGCTC for Ser-172, and TGCTCTTTGCGGGGCTC for Cys-172, are shown in the region of codon 172 (arrowheads).

assay gives an upper limit of <2% of the activity observed for the wild-type strain W303-1A. However, no difference in the growth rate or aeration sensitivity was observed between strain W303-1A A2H and the progenitor containing the intact *CCP* gene. The absence of an observable phenotype in the host strain indicates that CCP does not serve a vital role under normal growth conditions. This supports the proposed function of the enzyme in a protective role against oxidative damage by endogenous hydrogen peroxide (1).

The expression of *CCP* under control of its own promoter was accomplished by transformation of this host yeast strain with the yeast shuttle vector containing the *CCP* gene. Whole-cell lysates of yeast W303-1A A2H transformed with YDG13CCPM172, YDG13CCPS172, and YDG13CCPC172 containing the *CCP* gene with Met-172, Ser-172, and Cys-172 gave activities of 87%, 32%, and 41%, respectively, compared with that of strain W303-1A containing the intact CCP gene. Thus, for the wild-type gene containing the Met-172 codon, this expression system gives an intracellular level of mature peroxidase that is comparable to that of the wild-type yeast strain. This indicates that transport of the precursor protein to the mitochondria, signal peptide processing, and heme incorporation are properly performed. The reduced levels observed for the mutant genes are not due to lower intrinsic enzyme activity (see below) but to reduced levels of mature peroxidase in the cell.

The wild-type and mutant enzymes were purified to homogeneity from 40- to 100-liter cultures of yeast W303-1A A2H transformed with the appropriate expression vector. Approximately 80 μg, 23 μg, and 8 μg of purified enzyme per liter of yeast culture were obtained for the Met-172-, Ser-172-, and Cys-172-containing enzymes, respectively. Both of the purified mutant enzymes readily yielded needle-shaped crystals with a visual morphology identical to that of the wild-type enzyme. Redissolved crystals of the mutant enzymes had an A_{408}/A_{280} ratio between 1.2 and 1.35, and $NaDodSO_4$/polyacrylamide gel electrophoresis of the mutant enzymes gave a single band with a (relative) mobility identical to that of wild-type CCP. Analysis of cyanogen bromide digests by electrophoresis (15% acrylamide, 8 M urea) gave a band with decreased mobility for the Ser-172 and Cys-172 mutants relative to that observed for the wild-type protein (data not shown). This change was consistent with the loss of the methionine cleavage site between two predicted fragments of $\approx$7.2 kDa and $\approx$2.0 kDa and indicated that the predominant species contained the mutation. Although it is not possible to exclude homologous recombination with the nuclear copy of the gene to generate a low level of the wild-type protein, such events are probably rare, as the crossover must occur in a small 200-bp region between the CCP mutation and the site of interruption by *HIS3*.

Functional and Spectroscopic Properties. The results of steady-state kinetic measurements on wild-type and mutant peroxidase samples are presented in Table 1. CCP exhibits complex non-Michaelis–Menton kinetics as a function of substrate concentration (32), precluding the determination and comparison of K_m and V_{max} values. However, both of the *CCP* mutants catalyze the oxidation of cytochrome *c* with turnover numbers similar to wild-type peroxidase over a 10-fold range of cytochrome *c* concentration. Indeed, the average (relative) rates for both mutant enzymes taken over all substrate concentrations (bottom of Table 1) are well within 1 SD of that observed for the methionine-containing enzyme.

The optical spectra of wild-type and mutant peroxidases in the native state at 15°C and pH 6.0 are presented in Fig. 2 *Upper*, spectra A, B, and C. All of the spectra exhibit similar relative intensities and peak positions with the major differences observed in the 620- to 645-nm region. These differences represent two known interconvertible forms of CCP that have been described (34). In fact, each of the three samples of Fig. 2 could be reversibly prepared completely in the "620-nm" conformation by equilibration in 100 mM sodium acetate (pH 5).

The optical spectra of the hydrogen peroxide induced compound ES intermediates are presented in Fig. 2 *Lower*, spectra D, E, and F. The ES complexes of all three samples exhibit features at 528 nm and 558 nm that are characteristic of the (Fe^{4+}=O) ferryl center as observed for wild-type CCP and in horseradish peroxidase compound II (8, 35). Although the relative intensities of these two transitions differ slightly for the Ser-172-containing enzyme, no evidence is observed for the greatly broadened features that are characteristic of the (Fe^{4+}=O/P^+) ferryl iron porphyrin π-cation radical as observed for horseradish peroxidase compound I (8).

EPR Spectra. EPR spectra at 93 K were recorded for the native and compound ES states of CCP containing methionine or serine at position 172. These spectra are compared with the native and compound I state of horseradish peroxidase in Fig. 3. Most dramatically, the sharp $g = 2$ free-radical signal that is unique to the ES complex of CCP is clearly observed for both the Met-172- and Ser-172-containing enzymes. In addition, the signal for the Ser-172 mutant is distinctly sharper and appears to lack completely the broad sloping wings that are observed in the signal of wild-type CCP at this temperature (16, 36). These signals are examined in more detail at lower field modulation in Fig. 4. In these spectra, the hyperfine structure observed for the narrow component of the signal (36) is essentially identical for both samples.

Table 1. Kinetics of cytochrome *c* oxidation by wild-type and mutant CCP

Cyt *c*, μM	v_o/e, sec^{-1}		
	Met-172	Ser-172	Cys-172
4.8	204.3 (100%)	180.5 (88%)	216.5 (106%)
8.5	208.6 (100%)	206.2 (99%)	239.3 (115%)
17.0	226.0 (100%)	216.6 (96%)	279.2 (124%)
39.6	260.8 (100%)	226.9 (87%)	236.5 (91%)
56.6	277.3 (100%)	268.1 (97%)	239.6 (86%)
Average	100%	94 ± 5%	104 ± 16%

The initial velocities of the catalyzed oxidation of ferrocytochrome *c* expressed as enzyme turnover number normalized by the enzyme concentration (e) were determined as previously described (32). Rates were determined at the specified ferrocytochrome *c* concentrations in the presence of 85 μM hydrogen peroxide/20 mM Tris phosphate (pH 6.0) at 25°C.

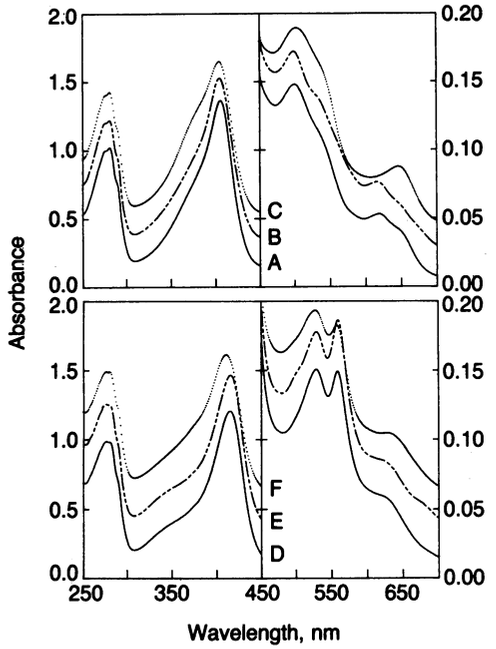

FIG. 2. Optical spectra of wild-type and mutant CCP in the native and compound ES states. Samples were prepared by dissolving crystals of the appropriate enzyme in 100 mM sodium phosphate buffer (pH 6.0) to a concentration of ≈10 μM. The samples were filtered (0.45 μm), and spectra were collected at 15°C. Compound ES was generated by addition of hydrogen peroxide to a final concentration of 50 μM immediately before the spectra were recorded. (*Upper*) Spectra for native enzyme containing Met-172 (spectrum A), Cys-172 (spectrum B), and Ser-172 (spectrum C). (*Lower*) Compound ES spectra for enzymes containing Met-172 (spectrum D), Cys-172 (spectrum E), and Ser-172 (spectrum F). Spectra B, C, E, and F are offset for clarity.

DISCUSSION

Met-172 of cytochrome *c* peroxidase was replaced with serine and cysteine in experiments designed to delineate the role of this residue in the compound ES intermediate. Few effects on the function and properties of the resultant enzymes were observed. Both of the mutant peroxidases are fully active. Optical spectra show that no major differences in the heme electronic structure are observed for either mutant. The altered enzymes exhibit stable hydrogen peroxide-induced intermediates similar to that of wild-type CCP including features characteristic of the ferryl iron porphyrin (Fe^{4+}=O). We conclude that Met-172 is not critical for the functioning of CCP at normal rates. Thus, either Met-172 does not play a role in the enzyme intermediates, or if it does, efficient alternative mechanistic pathways are available in the absence of this residue.

The narrow isotropic $g = 2$ EPR free-radical signal observed at 93 K is present in the ES complex of the enzyme containing either methionine or serine in position 172. The similarity of the hyperfine structure of the two signals indicates that the same radical species is observed in each case. Although the central narrow component of the signal is unaltered, the broad wings observed for wild-type CCP are completely missing in the Ser-172-containing enzyme. This finding is considered evidence that two radical species are present. Other recent reports also have indicated that the radical species of compound ES may be complex, existing at more than one site or in more than one form. Studies of intraprotein electron redistribution have suggested two forms or conformational states of the radical center that differ in their relative energetics (5, 6). Other evidence has come directly from the EPR properties of the radical. Hoffman *et al.* (16) have observed changes in the signal with temperature,

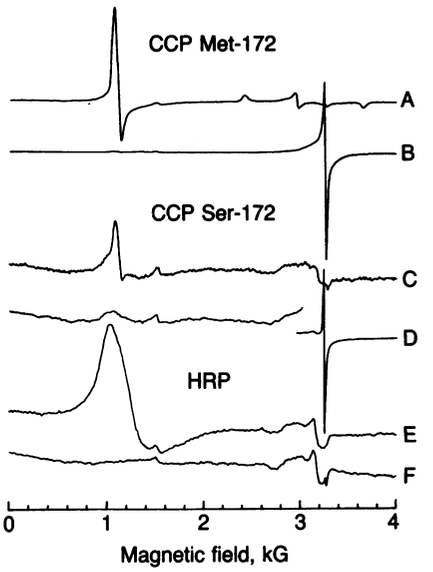

FIG. 3. EPR spectra recorded at 93 K for the native and compound ES forms of CCP containing Met-172 and Ser-172. Samples were dissolved in 70 mM sodium phosphate buffer, pH 6.0/30% glycerol. Compound ES was generated by adding hydrogen peroxide to a final concentration of 1 mM immediately before freezing to 77 K. Experimental conditions were 50 mW microwave power at 9.18 GHz, 10-G field modulation at 100 KHz. Samples: A, native state of Met-172 CCP at 1 mM and 1.25×10^3 recorder gain; B, Met-172 CCP compound ES, 4.2×10^2 gain; C, native state of Ser-172 CCP at 0.1 mM and 1.25×10^4 gain; D, Ser-172 CCP compound ES at 1.25×10^4 gain (left-hand spectrum) and at 3.2×10^3 gain (right-hand spectrum); E, native state of horseradish peroxidase (HRP) at 0.6 mM and 1.25×10^4 gain; F, HRP compound I at 1.25×10^4 gain.

in which the $g_{\|} = 2.05$ component broadens and becomes difficult to observe at 77 K. This was attributed to a change in the $g_{\|}$ distribution of the radical with temperature. However, Hori and Yonetani (36) have recently argued that the EPR spectrum of compound ES was derived from more than one magnetically distinct species. They suggested that the broad anisotropic EPR signal observed at 4 K is overshadowed or replaced by a distinct isotropic signal containing hyperfine structure as the temperature is raised to 77 K. At this temperature, the anisotropic signal is greatly broadened and only observable as sloping wings superimposed upon the narrow radical signal. The two signal components have

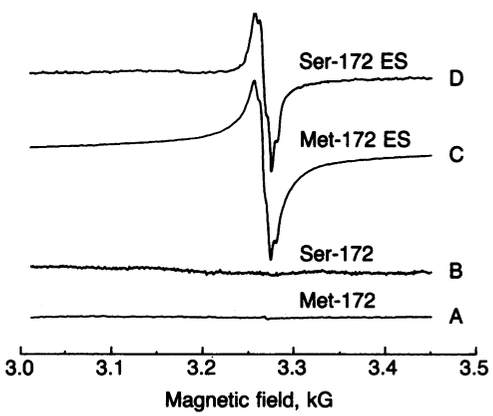

FIG. 4. EPR spectra at low-field modulation for wild-type and Ser-172 CCP. Conditions are as described in Fig. 3 except that a 2-G field modulation was used. Samples: A, 1 mM Met-172 CCP in the native state at 2×10^3 recorder gain; B, 0.1 mM Ser-172 CCP native state at 1.25×10^4 gain; C, Met-172 CCP compound ES at 1.6×10^3 gain; D, Ser-172 CCP compound ES at 1.25×10^4 gain.

different microwave saturation behavior that is consistent with an efficient spin-relaxation mechanism for the broad signal. It is noted, however, that the relative contribution of these two components to the total spin concentration has never been determined, and it is possible that the narrow isotropic signal represents only a small fraction of the total unpaired spin density in the ES complex.

The identity of the radical species has been the subject of intense discussion for a number of years. Considerable evidence has been obtained that supports a residue other than tryptophan as the major radical site of the ES complex (12, 16). At 4 K, the observed EPR signal had a large intrinsic anisotropy with $g_{\parallel} = 2.05$ and $g_{\perp} = 2.01$, which is inconsistent with expected π-radical g-shifts of no larger than 0.01 from the free electron value of 2.0023. In addition, ENDOR transitions of approximately five β-like protons coupled to the radical species were observed, but no evidence for ^{14}N hyperfine resonances was obtained, indicating that no spin density resides on nitrogen, as expected for a radical of the tryptophan nitrogen heterocycle. These properties were considered more consistent with a sulfur-based radical.

Optical studies of compound ES also have been reported that failed to support the involvement of a tryptophan radical. Optical difference techniques have afforded an absorption spectrum of the free radical site that exhibits a weak absorption band at 570 nm (5). This band occurs at a wavelength that is inconsistent with an observed neutral tryptophan radical (420–490 nm) or a cationic tryptophan radical (600 nm) (37). Finally, a recent study of compound ES by difference magnetic circular dichroism produced no clear evidence for changes in the tryptophan centers of the protein (26).

Based on the results of this work, we propose that compound ES contains two radical species such that the unpaired electron is delocalized over more than one center or is heterogeneous with respect to which center is oxidized. In this view, the narrow radical signal containing hyperfine structure observed at 93 K cannot reside at Met-172 because the signal is observed in the Ser-172 mutant. As noted by Hori and Yonetani (36), this component of the signal may thus represent an oxidized tryptophan or tyrosine residue. However, its identity, the relative contribution that it makes to the oxidizing equivalents of the ES complex, and the role that it plays in the function of CCP will most likely await the characterization of enzyme variants at Trp-51. In the wings of the isotropic signal is a second radical species that exhibits a broad EPR signal at 93 K. The properties of this signal, which is easily observed at 4 K, are inconsistent with a tryptophan radical. This component is absent in the spectrum of CCP-containing Ser-172 and, thus, may represent unpaired spin density on Met-172. Alternatively, it is possible that Met-172 is not involved directly in the radical center, but that its replacement in some way indirectly affects the properties of the broad signal such that it is no longer observed at 93 K. This could be due to a change in spin-relaxation rate, in the $g_{\parallel}$ distribution, or in the stability of the radical brought about by a conformational perturbation caused by the amino acid substitution. Replacement of the buried methionine with the smaller serine residue may result, for example, in the incorporation of a water molecule to fill the unoccupied space. Although this may change the hydrogen bonding network and the dielectric constant in this region, it is not possible to predict the resulting effects on the EPR signal properties without more knowledge about the location of the radical. These possibilities should be easily testable by EPR experiments at liquid helium temperature on samples of the enzyme altered by site-directed mutagenesis at both Met-172 and/or Trp-51.

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