$(2^1A_g) \leftarrow S_0$ transition in DPB gains its one-photon oscillator strength through vibronic coupling with S_2, involving a b_u in-plane bending mode (*1*). Our observation that the molecule is essentially planar in both states, and that band 1 is parallel polarized, confirms this interpretation, at least for the vibronic levels accessed in the present experiment (*9*).

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Identification by ENDOR of Trp^{191} as the Free-Radical Site in Cytochrome c Peroxidase Compound ES

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The chemical identity of the amino acid free-radical site that represents one of the two oxidizing equivalents stored in the H_2O_2-oxidized intermediate (compound ES) of the mitochondrial heme enzyme, cytochrome c peroxidase (CcP) has been sought for almost a quarter of a century. Site-directed mutagenesis alone cannot yield this answer. Low-temperature 35-gigahertz (Q-band) electron nuclear double resonance (ENDOR) spectroscopy was used to examine compound ES prepared from proteins containing specifically deuterated methionine or tryptophan, as well as the amino acid replacement $Trp^{51} \rightarrow$ Phe. The results definitely identify the site of the radical in compound ES as tryptophan, most likely Trp^{191}.

THE FULLY OXIDIZED STATE OF CcP, called compound ES, contains an unusual free-radical species whose identity has long been sought (*1*). The H_2O_2-dependent oxidation of ferrocytochrome c is catalyzed by CcP (*2, 3*) and, like horseradish peroxidase (HRP) and catalase (*4*), CcP can store two oxidizing equivalents after treatment with peroxide. In this fully oxidized state, called compound I in the case of HRP and catalase, one of the oxidizing equivalents is stored as the oxy-ferryl [Fe(IV) = O] state of the heme iron with spin $S = 1$ (*4, 5*). In compound I the second equivalent is stored as the π-cation radical of the porphyrin macrocycle (*4, 6*), but in ES it exists as a reversibly oxidized amino acid residue that gives an electron paramagnetic resonance (EPR) signal whose axial g-tensor [$g_{\parallel} = 2.04$ and $g_{\perp} = 2.01$ (*7–10*)] is unlike organic radicals composed of first-row elements. The ES state of CcP is one of only four known instances in which an enzyme makes use of a stable amino acid radical. The others are ribonucleotide reductase (*11*), prostaglandin H synthase (*12*), and signal II of photosystem II (*13*), each of which contains a Tyr π-radical.

Several species have been proposed as the free-radical site (R·) of ES. In the high-resolution crystal structures of CcP (*14*) and ES (*15*), the indole ring of Trp^{51} lies about 3.6 Å above and parallel to the heme pyrrole ring II. On this basis, it was suggested that R· is the Trp radical formed by hydrogen: atom abstraction from the nitrogen of Trp^{51} (*14*). The single-crystal EPR study of Hori and Yonetani (*10*) further implicated Trp and implied that the residue must be either Trp^{51} or Trp^{191}; the latter was first noted as a possible site in (*3*). However, paramagnetic resonance data [g-values observed in EPR (*6*); hyperfine splittings obtained from ENDOR spectra (*7, 16*); and the linear electric field (LEFE) response of the signal (*17*)] were interpreted to be inconsistent with R· being an isolated aromatic free radical, and magnetic circular dichroism (CD) measurements detected no effects attributable to radical formation at Trp (*18*). As an alternative, we proposed that the EPR signal is sulfur-based and is associated with a nucleophilically stabilized Met radical. Met^{172}, which is located two residues away from the proximal iron ligand, His^{175}, and is in contact with the proximal heme face, seemed an ideal candidate to carry at least some of the spin density associated with the ES EPR signal. A pair of Met residues, 230 and 231, also were considered as an alternative radical site (*15*), in consonance with our initial proposal that R· is a dimeric thioether radical cation $(R_2SSR_2)^+$ in which two proximate Met sulfurs share the charge created by the loss of one electron (*8*).

Recent site-directed mutagenesis studies in which Met^{172} (*19*) was replaced by Ser and Trp^{51} (*20, 21*) or Trp^{191} (*22*) were replaced by Phe have been used to probe the identity of the radical. Unfortunately, the mutagenesis approach has given definitive answers to the wrong question. A site-specific modification of CcP that leaves the radical site intact may be taken to prove that the site in question is not associated with the radical, which indeed is the case for Trp^{51} and Met^{172}. However, if modification of a site changes or abolishes the radical EPR signal, this in no way constitutes a proof that the radical resides at the site in question—it merely shows that the enzyme in the ES state has been perturbed. An example of this type is the modification of Trp^{191}, which lies perpendicular to the heme and in contact with the His ligand to iron. Replacement of Trp^{191} by Phe decreased, but did not eliminate, enzymatic activity and eliminated the radical signal (*22*). It appears unlikely that continued mutagenesis studies could identify the location of the radical with logical certainty.

To unambiguously determine the identity of the radical site, we used a structurally nonperturbative technique, isotopic labeling plus ENDOR spectroscopy, to probe the radical site. The enzyme containing Met-Lys-Thr on the amino terminus, CcP-(MKT), was produced in *Escherichia coli* by the plasmid pLACCCP under control of the *lac* promoter (*23*). Purified protein made by this method exhibited normal functional properties, and gave optical and EPR spectra in the native and compound ES states that were unaltered from those of the protein isolated from yeast. Auxotrophic strains of *E. coli* deficient in Trp or Met biosynthesis were used to produce enzyme selectively deuterated at Trp or Met; these samples also were subjected to H-D solvent exchange. This approach was augmented by examination of the $Trp^{51} \rightarrow$ Phe mutant. The protein samples used in this analysis were:

1) ES(H,H), perprotonated (natural isotopic abundance) enzyme, CcP(MKT), in H_2O buffer;

2) ES(d_3-Met,H), prepared in H_2O buffer from the methionine auxotroph grown on L-methionine-d_3 (CD_3-methionine);

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3) ES(d_8-Trp,H), from the tryptophan auxotroph grown on DL-tryptophan-d_8, in H_2O buffer;

4) ES(d_8-Trp,D), as with ES(d_8-Trp,H), but exchanged into D_2O buffer; and

5) ES(F51), natural isotopic abundance enzyme, CcP(MKT) containing the mutation $Trp^{51} \rightarrow$ Phe, in H_2O buffer.

We recorded EPR and ENDOR spectra using a 35-GHz (Q-band) EPR spectrometer (*24, 25*). We note that preparing the necessary amounts of enzyme would have been much more difficult, and in some cases impossible, were it not for the small sample volumes (about 10 to 20 μl) required for a Q-band spectrum.

The 2 K EPR spectrum of ES at Q-band frequency shows only the axial R· signal ($g_{||}$ = 2.04) whose ENDOR we studied at ~9 GHz (X-band) (*8, 10, 16*). A ^{1}H ENDOR pattern obtained from the signal of the wild-type sample ES(H,H) (Fig. 1A) exhibits numerous doublets from distinct types of protons centered at the proton Larmor frequency, $\nu_\pm = \nu_H \pm A^H/2$, where ν_H ~53 MHz for a microwave frequency of ~35 GHz. The doublet splittings represent the proton hyperfine interactions; for ES they range from $A^H \sim 20$ MHz to $A^H \sim 3$ MHz. In addition, there is a "distant-ENDOR" peak at ν_H. One feature often observed at Q-band, a difference in intensity of the ν_+ and ν_- halves of the spectrum, reflects details of the spin relaxation that are not important here. Other than this asymmetry, and a slightly better overall resolution, the Q-band ^{1}H ENDOR spectrum matches the X-band spectrum in (*16*).

The ^{1}H ENDOR spectrum of the ES(d_3-Met,H) sample is identical to that of ES(H,H), immediately forcing the conclusion that the proton hyperfine couplings of the radical are not associated with any of the six Met residues of the protein.

The ^{1}H ENDOR spectrum of ES(F51) (Fig. 1B) is similar to that for ES(H,H) (Fig. 1A). However, the peaks are better resolved in Fig. 1B, and it appears that at least one proton doublet in Fig. 1A, with A^H ~10 MHz, either is eliminated in Fig. 1B or has a sharply changed A^H and falls within the grouping with couplings of 12 to 20 MHz. This observation suggests that the radical site is largely unchanged by this mutation, but clearly demonstrates that it is perturbed as previously observed (*20*).

The spectra of Fig. 1, C and D, definitively identify the radical site a Trp residue. In the ^{1}H ENDOR spectrum of ES(d_8-Trp,H) (Fig. 1C), all proton signals have been suppressed by deuteration of the Trp constitutive proton sites, with the exception of the distant-ENDOR signal at ν_H and the intense proton doublet with A^H ~15 MHz. This latter doublet is eliminated when enzyme sample ES(d_8-Trp,H) is subjected to H-D exchange to produce ES(d_8-Trp,D) (Fig. 1D). Considering that deuteration of the constitutive protons is not complete, the spectrum in Fig. 1D is only consistent with the loss of intensity from all resolved proton doublets of Fig. 1A.

The loss of proton resonances upon deuteration of constitutive and exhangeable protons is *not* an artifact of sample preparation, instrument variability, or both; the loss of the ^{1}H ENDOR spectrum in the sample ES(d_8-Trp,D) is accompanied by the appearance of a strong ^{2}H ENDOR signal (Fig. 1E). The deuteron pattern is much less resolved than the proton signals because of quadrupolar interactions of the deuteron nuclei [I(D) = 1; I(H) = 1/2], but the overall shapes and breadths of the patterns in Fig. 1 are in agreement when properly scaled for the difference in nuclear g-factors. Moreover, selective labeling at Trp allows a 1:1 correspondence between individual H and D signals (*26*). Note that the D signals would have been centered at $\nu_D \lesssim 2$ MHz in an X-band spectrometer and likely would have been too weak to characterize.

The results in Fig. 1, A through D, demonstrate that the paramagnetic species associated with the major ($g_{||}$ = 2.04) EPR signal of CcP compound ES is a Trp radical. The likely assignment is as a π-cation radical, but the CD measurements (*18*) and characteristics of the proton hyperfine tensors (*16*) are not easily accommodated by this interpretation. Thus we do not totally dismiss the possibility of an aliphatic radical produced by hydrogen-atom abstraction from the β-carbon of Trp, analogous to an early (incorrect) proposal for the radical of ribonucleotide reductase. The large hyperfine coupling to the exchangeable proton indicates that it is covalently bonded to an atom having a large spin density. The obvious assignment would be as the exchange-

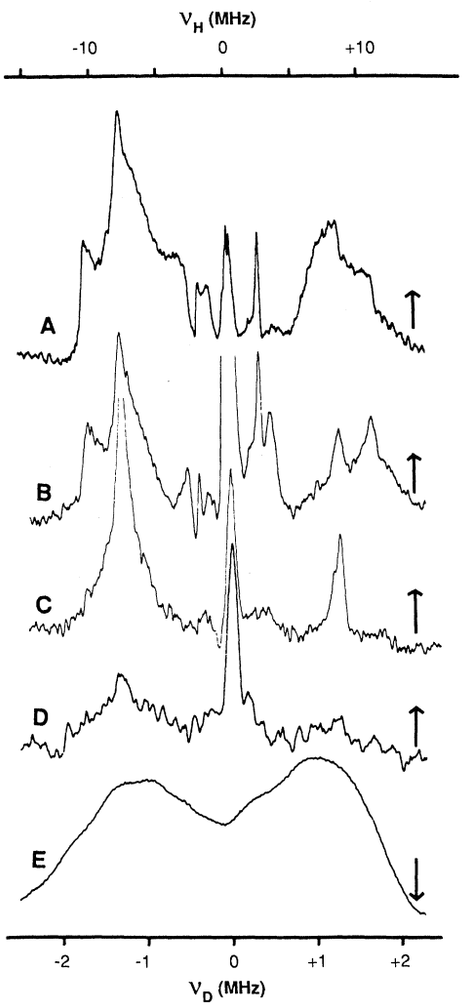

Fig. 1. Q-band ^{1}H and ^{2}H ENDOR spectra at $g_\perp$ = 2.01 of the ES compound of CcP. Proton spectra: (**A**) ES(H,H); (**B**) ES (F51); (**C**) ES(d_8-Trp,H); (**D**) ES(d_8-Trp,D). Deuteron spectrum: (**E**) ES(d_8-Trp,D). The proton spectra are presented as $\delta\nu_H = \nu - \nu_H$ (top scale); the proton Larmor frequency is ν_H = 53.2 to 53.4 MHz, depending on the spectrum. The deuteron ENDOR is presented as $\delta\nu_D = \nu - \nu_D$ (bottom scale); the deuteron Larmor frequency is ν_D = 7.7 MHz. Because nuclear hyperfine coupling constants for H and D are related by $A^H/A^D = g^H/g^D$ = 6.514, the scales have been adjusted by this factor. Thus a given coupling would cause the same splitting on the H and D spectra, and features in the H and D spectra can be compared directly. Conditions: T = 2 K; microwave frequency = 35.3 GHz; microwave power = 50 μW; 100-kHz field modulation, 3.2 G; radio frequency (rf) power = 20 W; rf scan rate 2 MHz/s [except (E), 3 MHz/s]. Number of scans: (A, B) 100, (C) 450, (D) 200, and (E) 250. Samples of ES for study were ~1.5 mM, in 100 mM potassium phosphate buffer (pH 6.0) made up to 30% glycerol (v/v). Ferric CcP was oxidized to ES by addition of standardized H_2O_2 in slight stoichiometric excess; the oxidized protein was rapidly frozen and stored at 77 K. Deuteron exchange was carried out by diluting concentrated protein into D_2O buffer (at least 100-fold dilution) incubating overnight, and then concentrating the sample to the original concentration using a Micro-ProDicon concentator (Bio-Molecular Dynamics). CcP containing labeled Met was isolated from cultures of *Escherichia coli* JF1754 (hsdR, lac, gal, metB, leuB, and HisB) containing the plasmid pLACCCP and grown for 36 hours at 37°C in defined media M9 (*24*) containing (per liter) 1 ml of glycerol, 0.6 g each of amino acid except 1.0 g each of His, Leu, and Trp and 0.5 g of L-Met-d_3 (CD_3-methionine), 99.1% by atom D (MSD Isotopes), and 100 μg of ampicillin. Enzyme labeled at Trp was prepared from cultures of JA300 (F^-, thr, leuB6, B1, trpC1117, rpsL, hsdR, and hsdM, λ^-) containing the same plasmid and grown in media containing (per liter) 5 g of yeast extract, 5 g of NaCl, 1 ml of glycerol, 1 g each of amino acid except 0.46 g of L-Trp-2′, 4′, 5′, 6′, 7′-d_5 or 1.0 g of DL-Trp-d_8, 97.6% by atom D (MSD Isotopes), and 100 μg of ampicillin. The modified media used for this strain was necessary for significant and reliable expression of the enzyme.

able N-H proton of the indole ring. However, the LEFE measurements (*17*) and our own preliminary ^{14}N Q-band ENDOR data indicate that the only nitrogen associated with R· has a low spin density. The complete elimination of nonexchangeable ^{1}H ENDOR signals by deuterating Trp indicates that there is no appreciable spin density on any other moiety bearing a constitutive proton. This result permits interaction of the radical spin with the $Fe^{IV} = O$ moiety of the heme or with a neighboring carboxyl group (Asp^{235}), but apparently precludes substantial delocalization onto other moieties, such as the protoporphyrin ring, the axial His, or the pair of Met residues (230 and 231) that surround Trp^{191}. Of the seven Trp residues in CcP, Trp^{51} and Trp^{191} are by far nearest to the heme active site at 4.1 and 5.1 Å, respectively (*14*). The others lie between 15 and 28 Å from the heme and are poor candidates for the radical center because of *g*-values (*7–10*) and the LEFE results (*17*) strongly suggest that the radical must be near the heme. Moreover, according to the single-crystal EPR study (*10*), Trp^{51} and Trp^{191} are the only Trp residues properly oriented with respect to the heme. Because the $Trp^{51} \rightarrow$ Phe mutation causes only minor perturbations in the ^{1}H ENDOR pattern, the radical site can only be Trp^{191}, not Trp^{51}.

The present study has unambiguously identified the primary site of the ES radical with Trp, most likely Trp^{191}, and thus answers one long-standing question. It does not explain the apparently contradictory results obtained in paramagnetic resonance and CD studies. The search for these explanations represents the next challenge provided by compound ES of CcP.

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26. M. Sivaraja *et al.*, unpublished results.

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Specific Expression of Nuclear Proto-Oncogenes Before Entry into Meiotic Prophase of Spermatogenesis

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The expression of proto-oncogenes representative of several functional categories has been investigated during development of mouse male germ cells. The c-*raf* proto-oncogene and three members of the c-*ras* gene family were expressed in mitotically active stem cells, throughout the prophase of meiosis and to varying extents in post-meiotic cell types. In contrast, the nuclear proto-oncogenes c-*fos*, c-*jun*, and c-*myc* were specifically expressed at high levels in type B spermatogonia. High levels of c-*myc* and c-*jun* RNAs were also detected in spermatocytes early in the prophase of meiosis. The type B spermatogonia represent the last mitotic cell division before entry into meiotic prophase; therefore, these nuclear proto-oncogenes may be involved in altering programs of gene expression at this developmental transition.

A NUMBER OF PROTO-ONCOGENE products fall into five functional categories that appear to constitute elements in signal transduction pathways: growth factors, tyrosine protein kinases, guanosine triphosphate (GTP)–binding proteins, serine and threonine protein kinases, and nuclear proteins. The ability of activated oncogenes to induce abnormal cell growth suggests that their normal cell progenitors, the proto-oncogenes, may function to regulate the growth and differentiation of normal cells. The identification of some proto-oncogenes as known growth factors (*1*), growth factor and hormone receptors (*2*), and transcription factors (*3*) supports the function of these genes as cellular regulatory elements, but the normal physiologic functions of most proto-oncogenes remain unknown. Analysis of the expression and function of these genes within defined normal cell populations provides an approach to identifying genes that may be involved in regulation of cell differentiation, as well as in control of cell growth.

The development of male germ cells in the testis provides a system that is particularly well suited for analysis of gene expression within a cell lineage because cell types can be isolated that represent a spectrum of developmental stages ranging from mitotically proliferating stem cells to mature spermatozoa (*4*). At birth the male germ cells are represented by the primitive type A spermatogonia that differentiate into the renewing stem cell population of type A spermatogonia. In the mouse, type A spermatogonia undergo four mitotic divisions and differen-

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