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$_2$O$_2$-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$^{191}$ $\rightarrow$ Phe. The results definitively 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$_2$O$_2$-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 $\pi$-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 $\pi$-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$^{191}$ lies about 5.6 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$^{191}$ (14). The single-crystal EPR study of Hori and Yonetani (10) further implicated Trp and implied that the residue must be either Trp$^{191}$ 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 thiourea radical cation (R$_2$SSR$_2$)$^{+}$ 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$^{191}$ (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$^{191}$ 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 pLACCp 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$^{191}$ $\rightarrow$ Phe mutant. The protein samples used in this analysis were:

1) ES(H$_2$H, H$_2$O), perprotonated (natural isotopic abundance) enzyme, CcP(MKT), in H$_2$O buffer;
2) ES(d$_4$-Met,H$_2$O), prepared in H$_2$O buffer from the methionine auxotroph grown on l-methionine-d$_4$ (CD$_3$-methionine);
3) ES(Ser)$^{172}$, prepared in H$_2$O buffer from Ser residue 230.

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3) ES(d₄-Trp,H), from the tryptophan auxotroph grown on dl-tryptophan-d₄ in H₂O buffer;
4) ES(d₅-Trp,D), as with ES(d₅-Trp,H), but exchanged into D₂O buffer; and
5) ES(F51), natural isotopic abundance enzyme, CpC(MKT) containing the mutation Trp⁵₁ → Phe, in H₂O 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 (gₓ = 2.04) whose ENDOR study we neglected at ∼9 GHz (X-band) (8, 10, 16). A 'H ENDOR pattern obtained from the signal of the wild-type sample ES(H,H) (Fig. 1A) is divided into two distinct types of protons centered at the proton Larmor frequency, vₓ = vᵧ ± A'/2, where vᵧ ∼ 53 MHz for a microwave frequency of ∼35 GHz. The doublet splittings represent the proton hyperfine interactions; for ES they range from A'H ∼ 20 MHz to A'H ∼ 3 MHz. In addition, there is a “distant-ENDOR” peak at vᵧ. One feature often observed at Q-band, a difference in intensity of the νₓ and νᵧ 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 'H ENDOR spectrum matches the X-band spectrum in (16).

The 'H ENDOR spectrum of the ES(d₅-Met,H) sample is identical to that of ES(H,H), immediately confirming the conclusion that the proton hyperfine couplings of the radical are not associated with any of the six Met residues of the protein.

The '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 'H ENDOR spectrum of ES(d₄-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 vᵧ and the intense proton doublet with A'H ∼ 15 MHz. This latter doublet is eliminated when enzyme sample ES(d₅-Trp,H) is subjected to H-D exchange to produce ES(d₅-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 exchangeable protons is not an artifact of sample preparation, instrument variability, or both; the loss of the 'H ENDOR spectrum in the sample ES(d₅-Trp,D) is accompanied by the appearance of a strong 'H ENDOR signal (Fig. 1E). The deuteration pattern is much less resolved than the proton signals because of quadrupolar interactions of the deuterons 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 νᵧ = 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 (gₓ = 2.04) EPR signal of CcCP 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-
able N-H proton of the indole ring. However, the LEFE measurements (17) and our own preliminary \(^{15}N\) Q-band ENDOR data indicate that the only nitrogen associated with R\(_t\) has a low spin density. The complete elimination of nonexchangeable \(^{1}H\) ENDOR signals by deuteronating 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\(^{91}\). Of the seven Trp residues in CcP, Trp\(^{51}\) and Trp\(^{91}\) are 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\(^{91}\) 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\(^{91}\), not Trp\(^{51}\).

The present study has unambiguously identified the primary site of the ES radical with Trp, most likely Trp\(^{91}\) 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.

REFERENCES AND NOTES

10. H. Hori and T. Yonetani also report an isotropic signal with \(g = 2.004\) that appears as a minor component (~10%) of the total EPR intensity [J. Biol. Chem. 260, 349 (1985)]. This signal is not observed in the 2 K EPR spectra of samples used in the present study. Because of this, all ENDOR signals described here [and previously (8, 16)] are associated with the majority (\(g = 2.04\)) signal. Indeed, at the \(g\)-values used for the ENDOR measurement (\(g > 2.01\)), the isotropic signal could not be monitored, and thus the present results do not address the identity of this minority signal.

25. The ENDOR spectra presented in Fig. 1 are representative of dozens taken for each sample under varying conditions and at fields throughout the EPR absorption envelope as part of a project to analyze the hyperfine interaction tensors, much as was done in (24).
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A SPECIFIC METHOD FOR ENTRY INTO MEIOTIC PHASE 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-rat 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 postmeiotic 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 NUMEROUS OF PROTO-ONCOGENE PRODUCTS FALL INTO FIVE FUNCTIONAL CATEGORIES THAT APPARE 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 spermatoozoa (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-