Electron Paramagnetic and Electron Nuclear Double Resonance of the Hydrogen Peroxide Compound of Cytochrome c Peroxidase*

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We have collected electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectra from the hydrogen peroxide compound of yeast cytochrome c peroxidase, termed ES, employing EPR microwave frequencies of 9.6 and 11.6 GHz. We have measured and analyzed the temperature dependence of the spin-lattice relaxation rate (1/T₁) of the paramagnetic center of ES over the temperature range 1.9 to 4 K. In addition, an upper bound to exchange coupling between the ferryl heme and EPR-visible centers of ES has been calculated and expressions for the dipolar interaction between a ferryl heme and a free radical have been derived. These results all confirm that the EPR signal of ES is not associated with an aromatic amino acid radical, and in particular not with a tryptophanyl radical. This conclusion has led us to consider an explanation of the EPR signal in terms of a nucleophilically stabilized methionyl radical.

Peroxidases catalyze substrate oxidations by hydrogen peroxide through the action of an enzymic intermediate which is oxidized by two equivalents above the ferric heme resting state (1, 2). The intermediate in horseradish peroxidase and catalase, called Compound I, is widely believed to contain a heme prosthetic group in which the iron has been oxidized to an oxo-ferryl (Fe(IV)) state with S = 1 and the porphyrin macrocycle has been oxidized to the cation free radical (1-4). It may be schematically represented as:

\[ (-Fe^{IV})^+ \]

Yeast cytochrome c peroxidase forms an intermediate, termed ES, which is isoelectronic to Compound I, but of quite different characteristics (2, 4-7). The optical (5), EPR (8-10), Mössbauer (11, 12), and ENDOR spectra (10) and the static magnetic susceptibility (13) of ES all indicate that the heme iron is in the oxo-ferryl (Fe(IV)) state with S = 1, and that the second oxidizing equivalent resides in a spatially removed paramagnetic species with S = \( \frac{1}{2} \):

\[ (-Fe^{IV})^+ + (\bar{R}) \]

The paramagnetic center, \( \bar{R} \), was originally suggested to be the stable free radical formed from an aromatic amino acid side chain (8), and somewhat later a heme-bound peroxy radical (14). Recent x-ray diffraction (15) and amino acid sequence (16) studies of cytochrome c peroxidase have found the indole ring of tryptophan-51 lying over pyrrole ring II, about 3.6 Å above and nearly parallel to the heme plane, with the indole nitrogen atom NE1 translated parallel to the heme plane by a distance of about 2 Å. On this basis, it was suggested that \( \bar{R} \) is the tryptophanyl radical formed by H-atom abstraction from NE1. This suggestion has been supported by an examination of the amino acid sequence homologies between yeast cytochrome c and horseradish peroxidases (16, 17). It was concluded that tryptophanyl residue 51 of cytochrome c peroxidase is represented by a less reactive phenylalanine in horseradish peroxidase (16), a substitution which may account for the differences between the enzymic intermediates formed by the two proteins.

However, the \( g \)-values observed in EPR (9) and the hyperfine splittings obtained from ENDOR spectra (10) were interpreted to exclude any type of aromatic free radical as the source of the ES EPR signal. Instead, we proposed that the EPR signal is sulfur-based, quite possibly in the form of a dimeric thioether radical cation (H₂SS₄)⁺ in which two proximate methionyl sulfurs share the charge created by the loss of one electron (10, 18). This suggestion also is supported by reference to the crystal structure (15) and the amino acid sequence (16). Methionine residue 171 is located two residues away from the proximal histidine (residue 174) and its sulfur atom is 5.6 Å from the heme plane in the resting state ferric protein (15), well within a distance where direct interaction with the prosthetic group is possible. Furthermore, this methionine residue is represented by a less reactive serine in horseradish peroxidase (16, 17).

To resolve the discrepancy, we have measured the EPR spectrum of ES at 9.6 and 11.6 GHz, have recollected ENDOR spectra at the ordinary x-band frequency of 9.6 GHz and have obtained spectra at 11.6 GHz as well, and have measured the spin-lattice relaxation rate of \( \bar{R} \). We have also calculated upper bounds to the exchange couplings between ferryl heme and radical and have derived expressions for the dipolar interaction between a ferryl heme and a radical. These results all confirm that the EPR-visible paramagnetic center of ES cannot be a tryptophan and also indicate that this center is not a peroxy radical. Thus, they again lead us to examine the possibility that the radical in ES is sulfur-based.

**Experimental Procedures**

Bakers' yeast cytochrome c peroxidase was prepared as described elsewhere (19). Samples of ES were prepared from the enzyme in its ferric state (~1.5 mM) in 10 mM sodium acetate buffer containing 50% glycerol (v/v) (pH 6.0) by the addition of \( \text{H}_2\text{O}_2 \) in slight stoichiometric excess. They were then rapidly frozen and stored at 77 K.

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1 The abbreviations used are: ES, hydrogen peroxide compound of cytochrome c peroxidase; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; HFS, hyperfine splitting; \( \bar{R} \), EPR observable paramagnetic center of ES.
ENDOR experiments were performed by immersing the microwave cavity in a bath of superfluid helium (T < 2.1 K). The ENDOR spectrometer operating at ~9.6 GHz with 100 kHz detection is described elsewhere (20). Its performance was upgraded by the addition of a Narda GaAs FET microwave amplifier model 60201. Magnetic field modulation at frequencies other than 100 kHz was carried out with a function generator, Macintosh Corp. 65-Watt audio power amplifier, and PAR model 128A phase-sensitive detector. Operation at 11.6 GHz required replacement of the cavity, the appropriate waveguide elements in the bridge, and the installation of a Varian N0, VA-287M Klystron.

Typical conditions for ENDOR were ~2 µW microwave power, field-modulation amplitude of 3 to 4 G, and radiofrequency field of 11 G in the rotating frame. The ENDOR of ES was observed as a decrease in the dispersion mode EPR signal. The dispersion mode was employed because the signals were more intense than in absorption and the uninformative ‘matrix’ ENDOR peak center at $\nu_H$ is suppressed. Simulated powder EPR spectra were calculated by Program SIM 14 (Quantum Chemistry Program Exchange, No. 265); powder ENDOR simulations were carried out analogously and will be described elsewhere. Molecular orbital calculations utilized a program implementing the Hückel-McLachlan technique (21).

Saturation studies were performed with magnetic field modulation of 1 kHz and minimum amplitude, thus ensuring slow passage condition (22). This is particularly critical because of the long relaxation times (vide infra). Microwave power was varied with a Hewlett-Packard No. X382A precision attenuator. As the microwave power, $P$, is varied, the slow passage EPR signal strength, $S$, of a protein sample typically follows the relationship (23)

$$S = \frac{P^{1/2}}{1 + P}$$

with $\delta$ approaching the value of unity expected for inhomogeneous broadening. The value of $P^{1/2}$ for a given temperature and magnetic field can be obtained either from the half-power point of a plot of $SP^{1/2}$ versus log $P$ or, more conveniently, by identifying $P^{1/2}$ with the $P^2$ (uncorrected) obtained by the method of Castner (24). For any given temperature, the two procedures do not give the same value of $P^{1/2}$. However, within the experimental error of ±1% in $P^{1/2}$, the temperature dependencies that we obtain by the two methods are identical. Thus, consistent use of either procedure is satisfactory for this purpose. We also directly measured the spin-lattice relaxation time, $T_1$, by the procedure of Mailer and Taylor (25) under conditions of rapid adiabatic passage. The dispersion EPR signal for these conditions lags behind the field modulation of frequency $\omega_0$ by an angle $\phi$ given by

$$\tan(\phi - 90^\circ) = \omega_0 T_1$$

Phase angles were measured from the phase-sensitive detector. The use of this technique probably gives spin lattice relaxation rates which are accurate to within a factor of two.

RESULTS

EPR

Fig. 1c presents the derivative of the absorption-mode EPR signal of ES at 77 K, in the acetate buffer/glycerol solution. The signal is stoichiometric with the heme (8, 9), and as originally noted by Yonetani et al. (8), is radical-like rather than displaying the characteristics of a heme-resonance. However, careful examination shows that the signal is not isotropic, as originally reported, but rather that the resonance envelope is characteristic of an $S = \frac{1}{2}$ species with axial g-tensor: $g_|| = 2.09; g_\perp = 2.01$. The $g_\perp$ region of Fig. 1a is broad and poorly defined, although noticeably more distinct than in spectra from samples without glycerol, and the shape is suggestive of a distribution of $g_\perp$ values. The 77 K spectrum does not saturate at available microwave powers (~200 mW). Upon cooling an ES sample to helium temperatures, $g_\perp$ decreases remarkably (Fig. 1b). In addition, the spectrum is considerably sharper than that at 77 K, suggesting that the $g_\perp$ distribution has been frozen out. In the absence of glycerol, the resolution is not as good as that of Fig. 1b, but the $g$-values are the same (10); presumably, intermolecular dipolar interactions introduced by protein aggregation upon freezing these samples decreases the resolution.

Because the compound ES contains both an $S = 1$ heme and a radical center, it is important to establish whether the separation between $g_\parallel$ and $g_\perp$ features is field-dependent. This would not be the case if the spectrum envelope arises from a field-independent interaction between the two sites (26). Fig. 1c presents an 11.6 GHz spectrum of ES at 4.2 K. The increased splitting between $g_\parallel$ and $g_\perp$ is appropriate for a field-dependent interaction. The 4.2 K spectra at both frequencies are best computer-simulated with the same $g$-values: $g_\parallel = 2.05, g_\perp = 2.01$.

Attempts to computer simulate Fig. 1b show that a component Gaussian line and a linewidth tensor of $W_1 = 25$ G and $W_2 = 20$ G give a good fit to the parallel region of a 9.6 GHz spectrum, but the signal in the perpendicular region has a broader high field edge than predicted, and a satisfactory overall fit could not be obtained. Although these observations might suggest that the resonance may be homogeneously broadened by spin-relaxation, for example by exchange interactions with the heme, both relaxation and ENDOR measurements presented below indicate slow relaxation and inhomogeneous broadening (22, 27). Thus, the shape of the spectrum more likely results from a distribution in HFS or apparent g-values, as is also suggested by the temperature dependence of the EPR signal. The $g$-values could vary with temperature and solvent if they are sensitive to a conformational equilibrium and/or distribution which is temperature-dependent and also influenced by the environment.

The possibility that $R$ is the neutral tryptophan radical may be tested by comparing the observed ES EPR spectrum with theoretical spectra predicted for this species, since spectra for analogous compounds manifest the existence of large $^{14}$N HFS. We have performed molecular orbital calculations on indole radicals using the convenient and highly reliable Hückel-McLachlan technique (21, 28). The resulting $\pi$-electron spin densities and corresponding (29, 30) nuclear HFS tensors are listed in Table I, both for the cation radical obtained by simple electron loss and for the neutral radical obtained by H-atom abstraction. The $^{14}$N HFS for the cation radical are small and not expected to be resolved in the EPR spectrum. However, the neutral radical has a large tensor component associated with the normal to the radical plane. Despite the fact that no totally successful simulation of the

![Figure 1](https://example.com/figure1.png)
Theoretical hyperfine couplings for tryptophanyl radicals

Calculations were performed by the Huckel-McLachlan technique (21) using the appropriate coulomb and bond integrals as defined and tabulated by Streitweiser (28). The cation radical is formed from tryptophan by simple electron removal and the neutral radical by H-atom extraction: cation radical, \( a(N1) = 1.5 \); neutral radical, \( a(N1) = 0.5 \). All carbon \( a \) values = 0.0 except for \( a(C3) = -0.5 \) (inductive effect of alkyl side-chain); all bond integrals = 1.0. No significant effects were observed upon variation of the C-N bond integrals.

<table>
<thead>
<tr>
<th>Position</th>
<th>Cation radical</th>
<th>Neutral radical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coupling type</td>
<td>( \sigma )-Spin density</td>
</tr>
<tr>
<td>N(1)</td>
<td>( ^{14}N ) 0.04</td>
<td>(1.1, 1.1, 6.7)</td>
</tr>
<tr>
<td>C(2)</td>
<td>( \alpha ) 0.42</td>
<td>(-13, -37, -25)</td>
</tr>
<tr>
<td>C(3)</td>
<td>( \beta ) 0.39</td>
<td>(14 ( \leq A \leq 55 ))</td>
</tr>
<tr>
<td>C(5)</td>
<td>( \alpha ) 0.10</td>
<td>(-3, -9, -6)</td>
</tr>
<tr>
<td>C(6)</td>
<td>( \alpha ) 0.04</td>
<td>(1, 3, 2)</td>
</tr>
<tr>
<td>C(7)</td>
<td>( \alpha ) 0.09</td>
<td>(-3, -8, -5)</td>
</tr>
<tr>
<td>(8)</td>
<td>( \alpha ) 0.01</td>
<td>(0, 1, 1)</td>
</tr>
</tbody>
</table>

* Proton HFS were calculated from the predicted spin density on the nearest aromatic carbon as follows (29). For \( \alpha \)-protons, \( A_{\alpha}(MHz) = -30, -90, -60 \)\( \gamma \). For \( \beta \)-protons, \( A_{\beta}(MHz) = 140 \cos^2 \beta \), where \( \beta \) is the dihedral angle between the C-H bond and the \( p_i \) orbital on the aromatic carbon. Nitrogen HFS were obtained from the nitrogen spin density using \( A_{N}(MHz) = 29, 28, 168 \)\( \gamma \). The \( \Delta \)-direction corresponds to the normal to the plane of the indole ring.

**Spin-lattice Relaxation**

The temperature dependence of the spin-lattice relaxation rate \( (1/T_1) \) for the ES radical center was measured by the method of progressive saturation. The half-saturation power, \( P_{1/2} \) (see under "Experimental Procedures") obeys the proportionality (23, 24)

\[
\frac{1}{T_1} \propto P_{1/2}
\]

and temperature variations of \( 1/T_1 \) can be studied through variations in \( P_{1/2} \). Note that we find samples frozen in the absence of glycerol to be harder to saturate, because of the effects of intermolecular dipolar interactions, and thus relaxation measurements were not performed on such samples. Saturation curves for ES were obtained over the range of 1.9 to 4 K and for two different positions on the EPR spectrum envelope. In one case, we measured the signal height at the magnetic field corresponding to the \( g_i \), maximum in the unsaturated derivative spectrum, in the second, the peak to peak amplitude of the derivative feature at \( g_i \). The results are presented in Fig. 2. At the lowest temperatures, \( P_{1/2} \) is the same for the two fields, but differences occur as the temperature is raised. Thus, cross-relaxation is slow and the spin-lattice rate varies across the spectrum, with the \( g_i \) region less readily saturated (shorter \( T_1 \)) than \( g_j \).

Saturation techniques are convenient for measuring the temperature variation of \( 1/T_1 \) through Equation 3, but not for obtaining the actual relaxation rates. We thus measured \( 1/T_1 \) for the perpendicular field position by the rapid adiabatic passage technique of Maier and Taylor (25). The results are plotted in Fig. 2. We note, in particular, that the temperature dependence of \( (1/T_1)_L \) obtained independently by the two procedures agrees remarkably well. Vertically aligning the \( P_{1/2} \) and \( 1/T_1 \) curves in Fig. 2, one obtains the proportionality constant in Equation 2, \( 1/T_1 = (1.6 \times 10^8 s^{-1} W^{-1}) \times P_{1/2} \) (W) under the experimental conditions employed.

The spin-lattice relaxation rate of an isolated S = ½ paramagnetic center at low temperatures \((T < 4.2 K)\) is expected to have the form (27)

\[
\frac{1}{T_1} = aT + bT^n
\]

with the first term arising from the direct process involving a single lattice vibration, and the second arising from a Raman process involving two vibrations. The dashed line in Fig. 2 is obtained from this equation employing \( n = 9 \), the normally expected value (27), through use of the coefficients given in Table II. The correspondence to the relaxation data from the \( g_i \) region is moderately satisfactory, but coefficients are orders of magnitude larger than expected for the relaxation mechanisms involved. Attempts to describe the \( g_i \) data through use of Equation 4 with \( n = 9 \) indicates that the difference in relaxation behavior between \( g_i \) and \( g_j \) (anisotropy in \( 1/T_1 \)) would be associated with the Raman term. However, the \( g_i \) curve is not shown because it is somewhat less satisfactory than for \( g_j \); for \( T > 2.5 K \), \( P_{1/2} \) appears to increase slightly faster than the limiting slope permitted by the \( T^n \) term. It has recently been found that relaxation rates for some ferromagnets obeys Equation 4 with \( n = 6.33 \), but not \( n = 9 \) (31).

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**Figure 2.** Saturation behavior of ES as a function of temperature: open symbols correspond to \( g_i \), and solid symbols correspond to \( g_j \). Values of \( P_{1/2} \) obtained by continuous wave saturation (○, □, ■, ◄, left ordinate, with circles and squares belonging to two independent experiments. Values of \( 1/T_1 \), measured by the Maier-Taylor technique (25) (▲), right ordinate. Curves are calculated from Equation 4 (—) for \( g_i \), and from Equation 5 (—) for \( g_j \) and \( g_i \) using the parameters listed in Table II. See text and Table I for explanation.

**Table II**

Spin-lattice relaxation rate parameters for compound ES

These parameters generate the theoretical curves of Fig. 2.

<table>
<thead>
<tr>
<th>Relaxation process</th>
<th>Orientation</th>
<th>( a )</th>
<th>( b^n )</th>
<th>( \Delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raman (Equation 4)</td>
<td>( g_i )</td>
<td>140</td>
<td>0.25</td>
<td>K</td>
</tr>
<tr>
<td>Orbach (Equation 5)</td>
<td>( g_i )</td>
<td>150</td>
<td>2.6 ( \times 10^7 )</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>( g_i )</td>
<td>125</td>
<td>2.0 ( \times 10^6 )</td>
<td>28</td>
</tr>
</tbody>
</table>

* Units of \( b \): Equation 4, \( s^{-1} K^2 \); Equation 5, \( s^{-1} \).
The steeply rising relaxation curves of Fig. 2 cannot be described in this manner. Interaction between radical and ferryl heme would add to Equation 4 a term exponential in inverse temperature, exp(−Δ/T). This term represents relaxation through phonon-induced transitions to the excited zero-field levels of the Fe(IV), and thus Δ is expected to correspond to the zero-field splitting, Δ = 30 K (12). When such Orbach processes are present, they usually dominate the relaxation rate at all but the lowest temperatures, and so we have also analyzed the relaxation data with the equation (27)

$$\frac{1}{T_1} = aT + b e^{-Δ/T}$$  \hspace{1cm} (5)

The solid lines in Fig. 2 are obtained from this equation, using the parameters listed in Table II. These parameters were not obtained from a least squares fitting procedure, but rather by adjusting them for a satisfactory correspondence between experiment and theory, subject to the constraint that the activation temperature, Δ, be equal, within experimental error, for the two data sets. The two experimental curves are well reproduced with Δ = 27 ± 2 K, in good agreement with expectations arising from Mossbauer spectroscopic measurements (11, 12). This analysis leads to the conclusion that the contribution to 1/T1 from the direct process is effectively independent of orientation, and that the Orbach process is 7 to 8 times more effective when H0 lies along the unique axis of the g-tensor. On balance, the analysis of the relaxation data in terms of the Orbach mechanism (Equation 5) seems somewhat more satisfactory than the alternative (Equation 4).

**ENDOR**

ENDOR is performed by inducing nuclear transitions with a radio frequency field while keeping the external static magnetic field, H0, fixed. When cross-relaxation is slow, as in ES, the ENDOR signal arises from that subset of molecules with orientations such that EPR occurs in the vicinity of H0 (32, 33). Field positions in the parallel region will give single crystal-like spectra which arise from free radical centers with H0 along g0. In contrast, positions in the perpendicular region of the EPR spectrum give rise to an ENDOR pattern which is essentially a superposition from molecules with H0 lying in the plane normal to g0.

ENDOR signals from ES might in principle be associated with 1H or 15N. The normal ENDOR pattern for a set of magnetically equivalent protons is a pair of lines separated in frequency by the hyperfine coupling constant, A', and mirrored about the free proton Larmor frequency, νH = gμBH0/h (13.62 MHz at 3200 G) (27, 32, 33):

$$νH = νp \pm νH$$  \hspace{1cm} (6)

A feature of the EPR spectrum characterized by a particular g-value appears at a field which is proportional to the spectrometer microwave frequency, ν(M): H0 = ν(M)/gβ. Thus, when H0 corresponds to a given feature of the EPR spectrum, the center frequency of the proton-ENDOR spectrum varies likewise, while the splitting A' is independent of microwave frequency. In contrast, a single set of equivalent 15N nuclei normally exhibits a four-line ENDOR pattern centered at A'√2/2 (27, 32, 33):

$$νN = A'\sqrt{2} \pm P^N \pm νp$$  \hspace{1cm} (7)

The splittings depend on the 15N Larmor frequency, which is small (νp = 0.984 MHz at 3200 G), and also on P^N, the quadrupolar coupling constant, but, unlike the proton ENDOR signals, the center position of the 15N pattern is independent of spectrometer microwave frequency.

To fully characterize the hyperfine couplings in the ES radical, ENDOR spectra were recorded at numerous positions across the EPR spectrum, using spectrometers operating at 8.6, 9.6, and 11.6 GHz. Fig. 3 displays typical 2 K ENDOR patterns; spectra a and b were taken with the standard frequency, 9.6 GHz, and spectra c and d were taken with a higher microwave frequency (11.6 GHz). The spectra presented cover the frequency range up to 34 MHz; no signals other than those shown were observed for any field position, at radio frequencies of up to ~100 MHz.

The signals presented are substantially stronger and better resolved than previously (10). This results largely from more favorable relaxation rates in the glycerol medium. However, in order to achieve the excellent signal/noise ratios in Fig. 3, it was necessary to sweep the ENDOR frequency at a rate of ~2 MHz/s. This slightly shifts the ENDOR pattern in the direction of sweep (~0.3 MHz) and slightly broadens the resonances in that direction. These sweep artifacts reflect relaxation processes in the coupled electron-nuclear spin system which have a rate comparable to the sweep rate, and may be corrected for by averaging the peak positions of spectra swept in opposite directions. In the following discussion, the text invariably refers to corrected values, while the free-proton frequency, νH, indicated in Fig. 3, is the apparent, shifted position.

The ENDOR patterns of Fig. 3, a and b, are roughly symmetric about νH = 13.4 MHz, the free proton frequency expected for ES in a spectrometer with microwave frequency of 9.6 GHz, indicating that the signals are totally proton resonances. The lower signal strengths of the low frequency half of the spectrum can be accounted for by well established mechanisms (32-34). However, the proton intensity in the frequency range ν ≤ 7 MHz overlaps the range of 15N ENDOR frequencies predicted from Equation 7 through use of the Hückel-McLachlan electronic structure calculation for a...
tophanyl cation radical (Table I). We therefore rebuilt our spectrometer to operate at 11.6 GHz to shift the proton resonances to higher frequency and expose this region of the spectrum. Fig. 3, c and d, presents ENDOR spectra of ES taken at 2 K in the 11.6 GHz spectrometer. The patterns are indeed centered symmetrically around the free proton frequency, \( \nu_p = 17.5 \) MHz, expected for this instrument. Moreover, there is no suggestion of \(^{14}\)N resonances in the low frequency edge of the spectrum. Thus, we conclude that the ENDOR signals arise exclusively from protons, with no observable contribution from \(^{14}\)N.

Two distinct groups of proton ENDOR signals may be discerned in Fig. 3. The pair of intense outer features appears to arise from proton sets with similar coupling constants, 10 \( \leq A \leq 22 \) MHz; the lower intensity inner features are associated with other sets having \( A \leq 4 \) MHz. The relative intensity of the resonances with small couplings can be enhanced as shown in Fig. 4. This figure presents an enlargement of the frequency range containing these peaks, with spectrum \( a \) taken at a parallel field position and spectrum \( b \) at a perpendicular field. No fewer than five separate pairs of resonances from proton sets with small coupling are resolved. The intense features associated with the strongly coupled protons are not as well articulated in Fig. 3, but it appears that no fewer than five coupling constants can be discerned.

The degree of anisotropy of the proton hyperfine couplings gives evidence regarding the nature and identity of the radical site. As described above, the anisotropy may be examined by comparing pairs of ENDOR spectra taken at parallel and perpendicular field positions. For the protons with larger couplings, spectra \( a \) and \( b \) and spectra \( c \) and \( d \) in Fig. 3 constitute such pairs; for the protons with smaller couplings, spectra \( a \) and \( b \) in Fig. 4 are best examined. Table III presents all the best-estimate proton coupling constants for the ES free radical, taken both in parallel and perpendicular orientations. The couplings change little with orientation as confirmed by spectra taken at intermediate field positions. The minimal effects of orientation indicate that the strongly coupled protons are \( \beta \)-protons; \( \alpha \)-protons have highly anisotropic couplings (29). Calculations indicate that the smaller couplings, although probably also from \( \beta \)-protons, might arise from dipolar interactions with protons about 5 Å away.

**Heme-radical Spin Coupling and the EPR Spectrum**

**Analysis**

Interpretation of the EPR signal of ES in terms of the properties of an isolated radical is not appropriate if the large value of \( g_R = g_R - g_s = 0.06 \) is not an intrinsic characteristic of the radical, but actually results from dipolar or exchange interactions with the ferryl heme. We now show that neither interaction is responsible for the observed ES \( g \) tensor, for clarity considering the dipolar and the isotropic exchange coupling mechanisms separately.

**Dipolar Coupling** — The \( S = \frac{1}{2} \) free-radical observed in EPR and ENDOR and the paramagnetic \( (S = 1) \) ferryl(Fe(IV)) heme centers will undergo a magnetic dipole-dipole interaction of the form (27, 30)

\[
H_d = -\mu_n (\mathbf{g}_R \cdot \mathbf{g}_e) (\mathbf{g}_R \cdot \mathbf{e}) \left[ \frac{\mu_n^2}{R^3} - 3 (\mathbf{g}_R \cdot \mathbf{e}) (\mathbf{g}_R \cdot \mathbf{e}) \right]
\]

where \( \mu_n \) and \( \mathbf{g}_e \) are the magnetic moments of radical and heme. For simplicity, we consider these moments to be interacting point dipoles separated by a vector \( R = R_{e} \), of magnitude \( R \) and direction given by the unit vector \( \mathbf{e} \). This is suitable for obtaining a semiquantitative estimate of the dipolar interaction.

Mössbauer (11, 12) and magnetic susceptibility (13) studies show that the orbitally nondegenerate \( S = 1 \) ground state of the ferryl-heme also has the spin-degeneracy removed by a large zero-field splitting term, with a zero-field splitting parameter of \( D \approx 20 \) cm\(^{-1} \). At the low temperature of our ENDOR measurements, \( T < 4 \) K, only the ground spin-state level with \( S = 1, m_s = 0 \) is populated significantly (11). As a consequence, in the absence of an applied field the heme appears to be nonmagnetic, and there is no first-order dipolar interaction between the ferryl heme and the free-radical center.

However, as a higher-order effect, the magnetic field applied during an EPR or ENDOR experiment, \( \mathbf{H} = H_0 \mathbf{b} \) of magnitude \( H_0 \) and direction \( \mathbf{b} \) induces a heme magnetic moment of the form (12)

\[
g_{e} = -\left( \mu_\perp \begin{pmatrix} 0 \\ \mu_\perp \end{pmatrix} \right) \mathbf{b} + \mathbf{b} \mathbf{b}
\]

where

\[
\mu_\perp = \frac{\mathbf{g}_e^2 \mathbf{b}^2 H_0}{[\mathbf{b}^2] + (\mathbf{g}_e^2 \mathbf{b} H_0)^2} - 0.5
\]

with the final approximation being appropriate for the ferryl-heme of ES at x band: \( D \approx 20 \) cm\(^{-1} \); \( g_e^2 \approx 2.25; g_e^2 \beta H_0 \approx \frac{1}{2} \)

**TABLE III**

Proton coupling constants for compound ES

| Coupling constants listed as \( A_i \) measured from spectra with \( H_0 \) set to the low-field (\( \parallel \)) edge of the EPR spectrum, those listed as \( A_i \), \( A_i \) set to the high-field edge (See Fig. 1). |
|---|---|
| \( A_i \) | \( A_i \) |
| Strongly coupled protons (Fig. 3) | 21.6 ± 0.5 | 21.4 ± 0.5 |
| | 18.9 | 18.9 |
| | 16.0 | 16.0 |
| | 13.4 | 13.8 |
| | 11.4 | 12.0 |
| Weakly coupled protons (Fig. 4) | 3.54 ± 0.05 | 4.63 ± 0.05 |
| | 2.95 | 3.01 |
| | 1.76 | 1.83 |
| | 0.96 | 0.95 |
| | 0.47 | 0.44 |
Thus, the actual induced heme magnetic moment is lower by a factor of about 30 than the "normal" value, \( g_{\text{en}} \).

The major effect of the dipolar interaction between a free-radical and the induced moment of a ferryl heme is not a symmetric broadening of the free-radical EPR signal. Instead, because \( \mu_r \) is proportional to the strength of the external field \( (\text{Equation 9b}) \), the heme-ferryl radical dipolar interaction introduces an angle-dependent contribution to the \( g \)-value of the radical. This would have the form

\[
g_\text{eff}(\theta, \phi) = g_{\text{en}} g_\text{r} \left[ 1 - \frac{1}{D/2} \right]
\]

where \( g_\text{r} \) is the \( g \)-value of the noninteracting radical, and \( G_\text{r}(\theta, \phi) \) is a function of the polar coordinates \( (\theta, \phi) \) of the external magnetic field; it can have values, \(-2 \leq G_\text{r} \leq 1\), and depends parametrically on \( \eta \), the angle between the \( e_r \) unit vector and the heme normal.

With an expression available for \( \delta g_\text{ex} \), we can examine the possibility that the ES free-radical is associated with tryptophan 51, and that the anomalous result, \( \delta g_{\text{ex}} = 0.05 \), arises from dipolar interactions with the ferryl heme. The x-ray diffraction study of cytochrome c peroxidase described above (15) would place nitrogen NE1 on the indole ring at a distance \( R \approx 4 \) Å from the iron. In order to obtain an upper bound to the possible dipolar contribution to the \( g \)-tensor, we assign unit spin density to nitrogen on NE1, and obtain from Equation 10 that the largest contribution of the dipolar interactions to the free-radical \( g \)-tensor must have a value \( \delta g_{\text{ex}} < 0.005 \). This limit is an order of magnitude smaller than \( \delta g_{\text{ex}} \) for the ES free-radical at liquid helium temperatures.\(^2\) The use of more realistic, delocalized spin density distribution on the radical (see Table 1) and oxy-ferryl iron (35) would leave the limit essentially unchanged. Moreover, this interaction would decrease with increasing temperature, and thus cannot explain the increased value of \( g_\text{r} \) at 77 K (Fig. 1). Thus, the unusual \( g \)-tensor does not originate in dipolar coupling to the heme.

**Exchange Coupling**—The combination of Mössbauer and static magnetic susceptibility measurements shows that an exchange coupling, if present, must be small compared to the ferryl-heme zero-field splitting parameter, \( D \). Moreover, the calculations just presented show that anisotropic exchange arising from the dipolar interaction can be ignored. Thus, the exchange interaction can safely be taken as an isotropic cosine coupling, which we write: \( H_{\text{ex}} = J S_\text{r} S_\text{h} \). Schulz et al. (36) have discussed the eigenstates of a weakly coupled ferryl heme/free-radical system. In an analogous fashion, we will show that the major effect of the exchange coupling is to modify the \( g \)-tensor of the free radical. Taking the isolated radical to have an isotropic \( g \)-value, \( g_\text{r} = 2 \), a first-order calculation shows that an exchange interaction would produce an axial \( g \)-tensor in which the unique axis lies along the heme normal:

\[
\begin{align*}
g_{\text{eff}} &= 2 - 2g_{\text{en}} J/2D \\
g_{\text{eff}} &= 2 - 2g_{\text{en}} J/2D
\end{align*}
\]

The parallel component is unaffected by exchange while the perpendicular components most probably are reduced (anti-ferromagnetic exchange, \( J > 0 \)); an increase of \( g \) (ferromagnetic exchange, \( J < 0 \)) is possible although unlikely. These results may be contrasted with the more familiar situation of exchange coupling between spin systems in which crystal field splittings are large compared to spin-orbit coupling and also exchange (37).

This prediction is in qualitative disagreement with the actual \( g \)-tensor of the ES free radical for which \( g_r \approx 2 \) and the unique tensor component, \( g_\text{eff} \), is larger than 2. Since the disagreement does not depend on assumptions as to the magnitude nor even as to the sign of \( J \), we can thus eliminate exchange couplings between heme and radical as the source of the unusual ES \( g \)-tensor. On this basis, we may further estimate an upper limit to \( J \) by noting that the exchange-induced \( g \)-shift, \( \delta g_{\text{ex}} \), must be less than the observed shift of \( g_\text{r} \), namely, \( \delta g_{\text{ex}} < \delta g_{\text{en}} = 0.01 \). Using the estimates, \( D = 30 \) K and \( g_{\text{en}} = 2.25 \), we obtain \( J \leq 0.15 \) K. This may be contrasted with the case of horseradish peroxidase Compound I, whose highly anisotropic \( g \)-tensor, with principal values of roughly 1.4, 2.3, and 2 has been interpreted in terms of an anisotropic exchange tensor with principal values of \(-2, -2, \) and \(-2 \) K (36).

An independent argument as to the exchange coupling in ES can be obtained by comparing the spin-lattice relaxation measurements of ES and those of Compound I of horseradish peroxidase. The EPR signal of the latter has been reported to relax via an Orbach process (Equation 5) with exponent \( \Delta = 29 \pm 4 \) K (36), effectively the same as that obtained here for ES through a fit to the equation. If ES likewise relaxes by the Orbach mechanism, then to a first approximation the relaxation rates for the two proteins should be related by the square of the respective exchange integrals between radical and ferryl heme (27). From the suggested value of \( 1/T_1 \approx 10^8 \) s\(^{-1} \) for horseradish peroxidase at 3.4 K (36) and 1/T, for ES at the same temperature, taken from Fig. 2, it appears that the relaxation rate for ES is less than that for horseradish peroxidase Compound I by roughly two orders of magnitude for \( g_r \), and slightly less for \( g_\text{eff} \). Using the ratio of relaxation rates and the exchange coupling for horseradish peroxidase, one obtains \( J < 0.2 \) to 0.4 K for ES. Given the approximate nature of the comparison and of the estimated 1/T, for horseradish peroxidase, the level of agreement between this estimate and that obtained above from the ES \( g \)-value is probably better than might be expected. Thus, the relaxation measurements clearly confirm that exchange coupling in ES is at most quite weak. Moreover, were more precise measurements of 1/T, to show that Raman (Equation 4) rather than Orbach (Equation 5) processes are dominant, this would reduce the upper limit to the exchange integral still further.

**DISCUSSION**

The properties of the \( \mathcal{R} \) radical may be summarized for convenience as follows: (i) it is, in effect, magnetically independent of the ferryl heme; (ii) it has a molecular \( g \)-tensor with large intrinsic anisotropy; (iii) the observed hyperfine interactions are describable by the \( \beta \) proton-like coupling constants in Table II; (iv) there is no evidence of spin-density on nitrogen.

Is \( \mathcal{R} \) an Aromatic \( \pi \)-Radical?—The characteristics of the ES EPR signal do not support an identification with the aromatic \( \pi \)-electron free-radical of a tyrosyl, histidyl, or tryptophan residue. First, as originally noted by Wittenberg et al. (9), the \( g \)-shifts are too large for such a radical. Phenoxo radicals normally have \( g \)-shifts much smaller than that of ES, with \( |\delta g_\text{en}| < 0.01 \) for all \( \mathcal{R} \) (38), and the tyrosyl radical, either neutral or ionized, is not reported to have an unusual
anisotropy (39). Nitrogen-heterocycle radicals such as histidyl and tryptophanyl would likewise have the typically small \( g \)-radical \( g \)-shifts, \( |g| < 6.01 \) (40). Indeed we are unaware of any aromatic \( \pi \)-radical composed of the elements C, H, O, and N for which any \( g \)-tensor component is as large as 2.01, much less 2.05. Furthermore, \( g \)-values of \( \pi \)-radicals do not exhibit the marked temperature dependence displayed by ES. Finally, one expects \( |g| > |g| \) for a planar \( \pi \)-radical, contrary to observation for ES (41). The calculations presented above validate these arguments, since they show that the observed \( g \)-tensor and its temperature dependence are an intrinsic characteristic of the radical and not the result of radical-heme couplings.

The proton ENDOR results also indicate that the ES radical site is not a planar aromatic \( \pi \)-radical. The ENDOR spectra show that the ES radical site probably contains no fewer than five \( \beta \)-type protons and give no evidence of \( \alpha \)-type protons, whereas a radical ion formed from an aromatic amino acid should have only two \( \beta \)-protons and several \( \alpha \)-protons. In addition, the values of the proton coupling constants found in ENDOR studies of tryrosyl (39) and histidyl (43) radicals are incompatible with those observed for ES. Estimated proton HFS tensors for both neutral and cation tryptophanyl radicals are listed in Table I. With one exception, neither radical has a ring proton with a HFS tensor element of 10 to 22 MHz, although the ENDOR spectrum of ES shows that there must be a number of such couplings. The exception, H(2), has an intermediate element close to these values (25 MHz), but also another element of \( \sim 35 \) to 40 MHz, larger than any observed. It also is likely that both radical forms would have a \( \beta \)-proton coupling larger than observed here.

These arguments are based on predictions of \( g \)-tensor and proton hyperfine tensor values which have been partly generated from studies with model compounds and partly from theoretical considerations. This approach is nicely supported by recent studies on the enzyme ribonuclease reductase, whose EPR spectrum is very different from that of ES. It has all the features predicted for a tyrosyl radical, including a nearly isotropic \( g \)-tensor and large \( \beta \)-proton HFS, and recent isotope studies have proven this assignment to be correct (44).

The apparent absence of spin density on nitrogen further argues against identifying the ES signal with tryptophanyl radicals. As discussed above, the HFS calculated for the neutral tryptophanyl radical (Table I) should give rise to observable manifestations in the EPR spectrum, contrary to what is observed with ES. In addition, the calculated HFS, along with quadrupole couplings typical of bicoordinate nitrogen (45), lead to prediction from Equation 7 of a \( ^{14} \)N ENDOR pattern centered at about 25.5 MHz and some 4 to 6 MHz wide, thus extending the ENDOR signal to about 28 MHz. However, careful examination of the high frequency edges of ENDOR spectra taken at 8.66, 9.60, and 11.66 GHz shows no \( ^{14} \)N peaks with frequencies of 24.5 MHz and higher. For example, although spectra a and b in Fig. 3 have a shoulder at about 25 MHz as the highest frequency feature, this is a proton resonance as demonstrated by its shift with microwave frequency (Fig. 3, c and d). Analogous arguments apply to the histidyl radical.

Employing the calculated \( ^{14} \)N HFS tensors (Table III) in Equation 7 predicts that the \( ^{14} \)N ENDOR signals for the cation radical should be centered in the low-frequency region of the spectrum, for example, at about 4 MHz when the external magnetic field lies along the unique direction. With quadrupole couplings from reference compounds with tricoordinate nitrogen (e.g. pyrrole (45)), Equation 7 indicates that the four-line pattern should have a width of about 4 to 6 MHz. However, the spectra of ES in Fig. 3 show no evidence of such a pattern, and in fact have no peaks at all with frequencies of about 7 MHz or less, which includes most of the range expected for the nitrogen of a tryptophyl cation radical.

Thus, a \( \pi \)-radical site is neither in accord with the \( g \)-values, the proton ENDOR, nor with the absence of observable \( ^{14} \)N hyperfine interactions in the EPR and ENDOR spectra of ES. Last, and not least, recent evidence shows that tryptophanyl radicals are not stable in a protein (46).

**Is \( R \) Associated with a Fragment of the Oxidant?**—Peroxyl radicals have \( g \)-values comparable to those of ES (14), but they are unlikely on chemical grounds. First, we assume that the initial steps in the formation of ES parallel those believed to occur during the formation of Compound I of horseradish peroxidase; thus, the O-O bond would not be left intact in ES (1-3, 15). Moreover, studies employing a variety of oxidants show that the same compound is formed regardless of reagent (6, 7), and thus make it unlikely that a peroxyl fragment is retained in ES. The rich proton ENDOR spectrum of Figs. 3 and 4 also would not be expected for such a site, and the finding that the heme is in an \( S = 1 \) state (11) rules out the particular heme-iron bound radical originally suggested (10).

**Is \( R \) a Sulfur Center?**—We were led to consider sulfur-based radicals (10, 18) by the process of elimination just presented, along with the recognition that sulfur centers normally exhibit considerable \( g \)-anisotropy (47). As cytochrome \( c \) peroxidase has only one cysteine, which can be modified with \( n \) loss of enzymic activity, the only possible sulfur-based radicals would involve methionine. Thus, the sulfur atom of methionine 171, found 3.6 Å from the proximal side of the heme plane (15) is an obvious candidate for \( R \). As discussed earlier (10, 18), a monomeric thioether cation radical does not appear to provide a good model for the radical center of ES, but a methionyl radical which is nucleophilically stabilized through interaction with a second residue appears to offer a well preceded possibility.

Studies of oxidized thioethers show that the cation radicals are strongly stabilized by interactions with nucleophiles (48-53). Interaction with a hydroxyl or carboxylate group can lower the reduction potential of a dialkyl sulfide by as much as \( \sim 600 \) mV (49), corresponding to a stabilization free energy of almost 14 kcal/mol, and stabilization by nitrogen also occurs (50). The best-characterized model compounds involve interaction with a neutral divalent sulfur atom, leading to a dimeric thioether radical (R(SSR)\( ^{+} \)) (49-54). Such a center, which could be formed by two methionyls which share the loss of one electron, is stabilized by about 5 to 6 kcal/mol, as compared to the monomer cation radical and a free thioether (49, 51). The \( R \) radical of ES exhibits a broad absorption peak at \( \sim 600 \) nm, with \( \epsilon = 3000 \) m\(^{-1} \) cm\(^{-1} \) (6), which compares favorably with the optical absorption of a "strained" dimeric thioether radical (54). Turning to the present ENDOR results, all proton hyperfine couplings for a thioether radical, whether dimeric or monomeric, would be of the \( \beta \)-type exhibited by ES. Estimation of the hyperfine couplings for a monomeric radical with about unit spin-density on sulfur shows they would be roughly 2-fold larger than any observed for ES, and this led us to propose (10, 18) an identification of \( R \) as a cation-radical dimer in which the sulfurs from two methionines share the spin density (\( \rho S < \frac{1}{2} \)) (52, 53).

\* We note that quadrupolar broadening can render \( ^{14} \)N ENDOR unobservable in radicals with small \( g \)-anisotropy. However, the anisotropic spin-lattice relaxation rates for ES suggest that the resonances from \( g \) and \( g \) are sufficiently nonoverlapping that this problem would not occur.

\* C. H. Kang, unpublished.
The crystal structure (15) and sequence (16) studies, which became available after our initial publication, do not appear to provide a nearby methionyl residue which might stabilize an oxidized form of methionine 171. However, spin delocalization onto another type of nucleophile would likely cause a similar reduction in proton hyperfine couplings. For example, the crystal structure shows that the oxidation of methionine 171 might be facilitated by nucleophilic interactions involving the side chains of one or more of the following residues: glutamine 239, histidine 174, and tyrosine 153. The side chains of the first two seem well positioned to form a charge delocalization system. One might even speculate that the methionyl sulfur interacts with the oxygen of the Schiff-base tautomeric form of the glutamine 237 side chain, and that this in turn is stabilized by further interactions. Depending on the residues considered, formation of the free radical center of ES might require either a small or a large change from the resting state ferri enzyme conformation. For example, optimal arrangement of the elements of a charge-stabilization system would require a minimal change, whereas interaction with the side-chain of tyrosyl 153 would apparently require a rather large change, namely, a one-turn loosening of the E a-helix of cytochrome c peroxidase. The stabilization free energies mentioned above could serve as the driving force for structural changes upon hydrogen peroxide oxidation of the resting state enzyme.

Conclusions—In this paper, we have presented detailed analyses of the divers magnetic characteristics of the free radical center of ES. No single feature predicted for either kind of tryptophanyl radical is observed, and detailed examination of each observed feature of ES discloses a contradiction with such an identification. Therefore, although no single point need be considered definitive, we believe that the EPR and ENDOR results as a whole demonstrate convincingly that the second oxidizing equivalent of compound ES does not exist as a tryptophanyl radical, even if one ignores the evidence that tryptophanyl radicals are not stable (46).

Considering the intimate contact between the heme and the indole ring of tryptophan 51 observed by x-ray crystallography (18), our conclusion could be considered surprising. However, Poulos and Kraut (15) proposed a mechanism in which hemo lytic cleavage of H2O2 produces an "Fe(V)" oxene intermediate which oxidizes the adjacent tryptophan 51 to the ferryl-heme/free radical pair which comprises ES. It is equally plausible to propose that O—O bond cleavage is followed by electron transfer from porphyrin to metal to form a ferryl-heme-radical center identical with that of horseradish peroxidase Compound I, and that this subsequently oxidizes a nearby amino acid side chain to form the stable ES intermediate of cytochrome c peroxidase. Since it is generally believed that electron transfer is feasible over distances of 10 Å or more (55), it seems immaterial whether the heme to R distance is 3.6 Å, as is the heme plane to tryptophan 51 indole ring distance or, say, 5.6 Å, as is the heme plane to methionine 171 sulfur distance (15). The final product of cytochrome c peroxidase oxidation by H2O2 must be determined by the relative thermodynamic stabilities of any of its potential forms, and it now appears that this product does not contain either of the oxidized forms of tryptophan 51 which have been proposed.

Our suggestion that R is sulfur-based and involves methionine 171 would appear to offer the best possibility of satisfying the magnetic properties of this center. However, the present results do not appear to admit of the assignment to an isolated methionyl radical, but to require nucleophilic stabilization of the charge and delocalization of the spin. Available models for such a center suggest that the nucleophile might be a second methionyl sulfur, an oxygen, or a nitrogenous base, with the first possibility apparently eliminated by the x-ray structural results. Because this proposal requires one of a limited number of possible arrangements of atoms, this work leads to a new stage in the dialogue between spectroscopy and x-ray crystallography, one which could be substantially advanced by crystallographic analysis of ES itself.

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Note Added in Proof—EPR spectra of Compound ES prepared from 50% 18O-enriched H2O2 are indistinguishable from those presented in Fig. 1. This observation is excellent support for our conclusion that no fragment of the oxidant is part of R.

REFERENCES

EPR and ENDOR of Peroxidase

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