Electron Spin Echo Studies of Cytochrome c Oxidase*

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We have studied the linear electric field effect in pulsed EPR of the "EPR-detectable copper" signal of beef heart cytochrome c oxidase and have compared our results with those for a variety of planar and tetrahedral Cu(II) model compounds and with Cu(II) proteins containing either type 1 or type 2 copper. The electric field induced g shifts (linear electric field effect) for cytochrome oxidase are comparable in magnitude to those for simple Cu(II) complexes and for some copper proteins containing type 2 sites. The shifts are smaller than those for tetrahedral copper complexes and for type 1 copper sites. However, the magnetic field dependence of the linear electric field effect does not resemble that observed for any Cu(II) complex studied nor for type 1 copper. These findings cannot be reconciled with the tetrahedral Cu(II) model proposed by Greenaway, Chan, and Vincow ((1977) Biochim. Biophys. Acta 490, 62-78, 1977) to explain the unusual EPR spectrum of cytochrome oxidase.

An unusual copper binding site that gives rise to an EPR spectrum is found in cytochrome c oxidase. The spectrum for this site is peculiar for a variety of reasons; the nuclear hyperfine structure is poorly resolved at X- and Q-bands, the magnitude of $g_2$ is significantly smaller than for most known Cu(II) compounds, and one principal axis $g$ value is less than 2.00 (1-3). These observations have led Peisach and Blumberg (4) to suggest that the EPR spectrum does not arise from Cu(II) but arises instead from a sulfur radical, similar to that found in irradiated cysteinyl sulfide. If copper is associated with the paramagnetic center, as has been shown in recent ENDOR experiments (5), then the electronic description would, according to this view, be one in which copper is formally cuprous and the unpaired spin resides mainly on RS-, an alkyl sulfurred radical.† On the other hand, the copper is cupric then a different reason must be found to explain the unusual EPR properties for this protein.

One explanation, given by Greenaway et al. (6), is that the copper, as Cu(II), resides in a tetrahedral site and therefore has very weak hyperfine interaction. Failure to observe a well resolved hyperfine interaction at X- and Q-bands is attributed partly to the smallness of the coefficients $A_N$ and partly to line broadening caused by dipolar interaction with a close lying paramagnetic center, in this case most probably low spin ferric cytochrome a. A simulation of the EPR spectrum based on a tetrahedral model is given in the reference.

In order to examine further the validity of these models, we have performed two kinds of pulsed EPR experiments on the paramagnetic centers in cytochrome c oxidase. In the first experiment, we measured the electric field-induced g-shifts, i.e. the linear electric field effect (7-11). This effect is sensitive to the odd component of the ligand field and can be made to yield detailed information about the symmetry of the site. In the second experiment we studied the nuclear modulation patterns in the electron spin echo decay envelope, i.e. the "nuclear modulation effect" (10-12). These patterns, which have been investigated in detail for a number of copper-containing proteins and models (13-18), yield information about the coupling between paramagnetic centers and nearby nuclei.

From the LEFE2 studies it was found that the magnetic field dependence of the electric field shifts for cytochrome c oxidase resemble neither that seen for simple Cu(II) complexes nor that seen for Cu(II) in essentially tetrahedral sites (9). The nuclear modulation data were also unlike those obtained for any copper protein or model hitherto studied (13-17) and therefore constitute yet another property unique to the site in cytochrome c oxidase. Although they yielded little positive evidence as to the nature of the site, they showed clearly that, if indeed it is a Cu(II) site, its ligands must differ from those commonly found in copper proteins.

**MATERIALS AND METHODS**

Bovine cytochrome c oxidase was prepared by the method of Hartzell and Beinert (19) and was concentrated to 0.96 mm Cu(II)-hexaglycine, pH 6.7, was prepared as before (13). The linear electric field effect was measured at X-band and at 1.6K by a method described previously (7, 9, 20). The shift parameter $\alpha$ was obtained by finding the value of electric field required to halve the amplitude of the electron spin echo signal for a particular setting of $\tau$, the time between the two microwave transmitter pulses of the echo-generating sequence. This parameter corresponds approximately to the average fractional g-shift ($\delta g/g$) per unit of applied electric field, the average being taken over those paramagnetic centers which contribute to the electron spin echo signal at the chosen magnetic field setting $B_0$.

The two-pulse electron spin echo decay envelopes were recorded as described by Mims and Peisach (13). Echo envelopes were also

* The abbreviation used is: LEFE, linear electric field effect.

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‡ Although this electronic description is one where the copper is formally reduced, the cysteinyl sulfide is oxidized to a sulfur radical. The oxidation-reduction site would still be able to accept a single electron.

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obtained by the three-pulse method (21) but, since in the case of the copper center the modulation was shallow, the data were accumulated during repetitive sweeps over the time delay $T + \tau$ in a Nicolet 1074 Instrument Computer. The resulting three-pulse envelopes were extrapolated to zero $T + \tau$ and Fourier transformed to yield the superhyperfine frequency spectrum (22).

RESULTS AND DISCUSSION

The EPR spectrum of cytochrome $c$ oxidase arises from cytochrome $c$ and from a second site associated with the so-called EPR-detectable copper, which for brevity we refer to here as the copper site, while reserving judgment as to its exact nature. These two paramagnetic species are present in equal concentrations. However, the cytochrome $c$ signal is observed over a much wider range of $H_0$ settings than the copper signal and is correspondingly weaker. This is illustrated in Fig. 1 where we show the electron spin echo signal for cytochrome $c$ oxidase as a function of $H_0$. Within the range of field settings covered by the EPR signal of the copper site, the contribution due to cytochrome $c$ is relatively small. LEFE and nuclear modulation effect data taken within this range will therefore reflect the properties of the copper center, subject to a small error due to the underlying cytochrome $a$ signal. Some additional discrimination against the cytochrome $a$ signal was achieved by adjusting the microwave transmitter power level and, in the case of the LEFE measurements, by choosing longer times $\tau$ at which the cytochrome $a$ echo signal had undergone a proportionately greater decay.

In the upper curve of Fig. 2 we show the spin echo intensity as a function of magnetic field for the copper site. As can be seen, the amplitude of the spin echo increases with increasing magnetic field so as to be maximal near $g_c$. The apparent peaks in the curve are not due to hyperfine structure, but appear in the spectrum because of the nuclear modulation effect (12). By using a different value of $\tau$, they can be made to change position. The spectrum of electron spin echo amplitudes is approximately equivalent to the integral of the spectrum obtained in a typical EPR spectrometer with field modulation, but it tends to be less useful, except for purely illustrative purposes, because of intervention by this effect.

In the lower portion of Fig. 2, we show the effect of electric field on spin echo intensity as a function of magnetic field for the copper resonance, and for two orientations of the electric field with respect to the magnetic field. The magnitude of shifts observed is comparable to that seen for simple copper complexes (9) and for some copper proteins having type 2 copper sites (18). However, the shifts are smaller than those seen for tetrahedral Cu(II) complexes (9) and the form of the curves is not the same as that formed in any previously studied Cu(II) complex or copper protein (9, 14, 18). Cu(II) complexes and type 2 Cu(II) sites in proteins yield curves which fall off at the low $H_0$ end of the spectrum and dip near $g_c$. The cytochrome $c$ oxidase LEFE data are also quite unlike the LEFE data obtained for type 1 Cu(II) which yield large shift parameters and are characterized by curves in which the $E, H_0$ shifts rise well above the $E_1 H_0$ shifts at $g_1$. We conclude therefore that the ligand field for the copper site in cytochrome $c$ oxidase is not tetrahedral and that it differs radically from the ligand fields associated with type 1 and type 2 Cu(II) in proteins.

Although the signal-to-noise ratio for the spin echo signal arising from cytochrome $a$ was much poorer than that for copper (Fig. 2), we were able to make LEFE measurements over a restricted range of magnetic fields excluding those $H_0$ settings where the copper signal is paramount. The shifts obtained were about an order of magnitude larger than for...
and the data were accumulated during repetitive sweeps. The baseline shown here is fictitious; the actual modulation depth being ≈5 times less than in the figure. The dotted line is an extrapolation of the data to $T + \gamma = 0$ (22).

**Fig. 4 (center).** Three-pulse electron spin echo decay envelope for the EPR-detectable copper signal of cytochrome c oxidase. The data used for the transform are shown in Fig. 4.

Undertake a more detailed study of this shallow component in both cytochrome c oxidase and Cu(II) hexaglycine using three-pulse methods. The three-pulse echo envelope for the former is shown in Fig. 4. Frequencies of 0.9, 1.5, 1.9, and 3.1 MHz are clearly visible in the Fourier transform of these data (Fig. 5) and in the transform for the EPR-detectable copper signal of cytochrome c oxidase. The data for the transform are shown in Fig. 4.

**Fig. 3 (left).** Two-pulse electron spin echo decay envelope for the EPR-detectable copper signal of cytochrome c oxidase. The spectrometer frequency was 9.548 GHz and the magnetic field setting was 3396 G.

**Fig. 5 (right).** Fourier transform of the three-pulse spin echo decay envelope for the EPR-detectable copper signal of cytochrome c oxidase. The data for the transform are shown in Fig. 4.

It should be noted here that the measured LEFE in the region where both cytochrome $a$ and copper signals contribute to the echo is not given by the weighted average of the LEFE’s of the two component species. As may be seen from Figs. 1 and 2 the LEFE shifts for cytochrome $a$ are large compared with the shifts for the copper center. The initial effect of the applied electric field is therefore to eliminate entirely the cytochrome $a$ contribution to the echo signal leaving the remaining signal (in most places >90% of the total amplitude) due solely to the copper center. The rest of the LEFE measurements (i.e., the reduction to 50% echo amplitude) then represents the effect of the applied field on the copper center. In principle it should be possible to resolve the two contributions in cases like this where one shift is much larger than the other. We were unable to do so here because of the weakness of the cytochrome $a$ signal in relation to the copper signal.

It is generally found that the shift seen for the cytochrome $c$ oxidase ferric hemoproteins and models (8). However, the form of the curves was unusual. In low spin heme compounds and hemoproteins it is generally found that the shift seen for the $E_J$-$E_O$ setting is larger than that seen for the $E_J$-$E_O$ setting at $g_{max}$, whereas at $g_{max}$ the opposite is observed. This can be explained by the fact that the $g_{max}$ principal axis and the axis of the two asymmetric bonds responsible for the odd ligand field are both oriented in approximately the same direction along the heme normal. In the one exceptional case of myoglobin hydroxide the LEFE curves suggest that the $g_{max}$ principal axis is perpendicular to the asymmetric bond axis and thus lies, presumably, in the heme plane rather than normal to it. Although the LEFE data for cytochrome $a$ are incomplete, they point to a similar conclusion. The data obtained at the ends of the EPR spectrum, where there is no contribution from the cytochrome $c$ oxidase copper center, show clearly that the shift for the $E_J$-$E_O$ setting is less than that for the $E_J$-$E_O$ setting at $g_{max}$. This result suggests that the $g_{max}$ axis and the odd field asymmetry axis are more or less parallel to one another, thus casting further doubt on the common assumption that the $g_{max}$ axis is normal to the heme plane in all low spin heme proteins.

In Fig. 3, we show the two-pulse echo envelope for the copper signal of cytochrome c oxidase. The major features of the trace consist of 35- and 70-ns periods that arise from weakly coupled protons. There is, in addition, a shallow component with a longer period similar to one which was seen earlier for a Cu(II)-hexaglycine complex and attributed to coupling between the Cu(II) spin and peptide $^{14}N$ nuclei (13). Improvements in technique have made it possible for us to undertake a more detailed study of this shallow component in both cytochrome c oxidase and Cu(II) hexaglycine using three-pulse methods. The three-pulse echo envelope for the former is shown in Fig. 4. Frequencies of 0.9, 1.5, 1.9, and 3.1 MHz are clearly visible in the Fourier transform of these data (Fig. 5) and in the transform for the EPR-detectable copper signal of cytochrome c oxidase. The data for the transform are shown in Fig. 4.
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copper proteins. It does not however exclude imidazole coordination if the unpaired electron is assumed to reside mainly on a sulfur ligand and only to a smaller extent on copper as suggested by Peisach and Blumberg (4). In this case the coupling between the electron spin and the N nucleus would be weakened and might conceivably give rise to modulation effects such as those we have observed.

In conclusion, the pulsed EPR experiments reported here have tended to emphasize rather than to explain away the atypical nature of the EPR detectable copper center in cytochrome c oxidase. The results indicate that the symmetry is not tetrahedral and that the ligation pattern is unlike that found for either type I or type 2 copper in other copper proteins. The smallness of the electric field-induced g-shifts, the unusual symmetry, and the lack of a deep nuclear modulation pattern might indeed all be explained if an otherwise typical Cu(I) center were to receive the donation of an RS· ligand, thus giving a center in which the unpaired electron spin resided mainly on the radical and only to a lesser extent on the copper. Lack of a suitable model makes it impossible, however, to pursue this hypothesis further by the methods employed here.

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