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Linear Electric Field Effect Measurements of Variant Low-Spin Forms of Ferric Cytochrome *c*†

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ABSTRACT: The linear electric field induced *g* shifts in electron paramagnetic resonance have been measured at 4.2°K for two low-spin forms of cytochrome *c*. At pH 6.6, with the magnetic and electric fields aligned approximately normal to the heme plane (C. Mailer and C. P. S. Taylor (1972), *Can. J. Biochem. 50,* 1040), the shift parameter $S = (1/E(\Delta g/g) = 0.95 \times 10^{-9}$ cm V^{-1}. At pH 10.0 the shift parameter for the same field orientations increases to 3.3×10^{-9} cm V^{-1}, verifying a ligand exchange. This result also indicates that the difference in crystal field strength of the axial ligands is considerably greater in the pH 10.0 form than in the pH 6.6 form of the protein.

The electron paramagnetic resonance (epr) spectrum of mammalian cytochrome *c*, which is a low spin ($s = \frac{1}{2}$) ferric heme protein, has been studied both in frozen solution (Salmeen and Palmer, 1968) and in single crystals (Mailer and Taylor, 1972). As in other low-spin ferric heme proteins the results can be described by a *g* tensor with three principal *g* values. At near neutral pH these values are 3.07, 2.23, and 1.21. At elevated pH the spectrum is, however, dominated by another low-spin heme species with *g* values 3.4, 2.07, and 1.5 (Blumberg *et al.*, 1973; Lambeth *et al.*, 1973).

It has been unequivocably demonstrated by X-ray analysis of single crystals (Dickerson *et al.*, 1971; Takano *et al.*, 1973) and in part verified by nuclear magnetic resonance (nmr) spectroscopy of protein solutions (Wüthrich, 1970; Redfield and Gupta, 1971; McDonald and Phillips, 1973) that the axial ligands in the neutral pH form of cytochrome *c* are methionine sulfur and histidine imidazole nitrogen, confirming the earlier suggestion of Harbury *et al.* (1965). No X-ray studies have been made on the high pH form, but since the *g* values lie close to the values obtained for carboxymethyl cytochrome *c* (Schejter and Aviram, 1970) where nmr evidence (Gupta and Koenig, 1971) indicates that an amine from an endogenous lysine nitrogen atom has been substituted for methionine sulfur, it is provisionally concluded that the ligands in the high pH form of cytochrome *c* are amine nitrogen and imidazole nitrogen. Comparison of the *g* values to those of similar heme compounds would tend to reinforce this conclusion (Seamonds *et al.*, 1972; Peisach *et al.*, 1973a). It cannot be definitely inferred, however, whether the imidazole ligands in each case are in the neutral form, or in an ionized form with a proton removed at the N-3 position (Peisach *et al.*, 1973a).

For both forms of ferric cytochrome *c* the iron site is noncentrosymmetric and we may, therefore, expect to observe a linear shift in *g* values when an electric field is applied to the sample.

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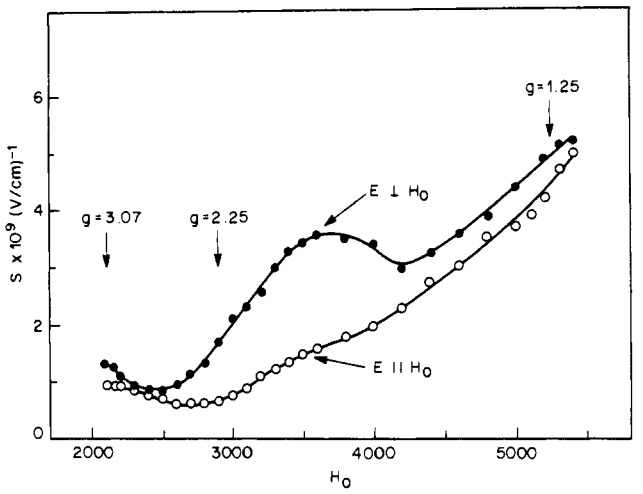

FIGURE 1: Linear electric field effect for horse heart cytochrome *c* at pH 6.6. The applied electric field is aligned parallel (O) to the magnetic field ($E \| H_0$); or perpendicular (●) to the magnetic field ($E \perp H_0$). The principal *g* values for cytochrome *c* are indicated on the figure. At $E \| H_0$, $g = 3.07$, the electric field is aligned in the direction of the axial ligands; at $E \| H_0$ $g = 1.25$, the electric field is aligned perpendicular to the axial ligands and in the plane of the proximal imidazole (see Figure 4).

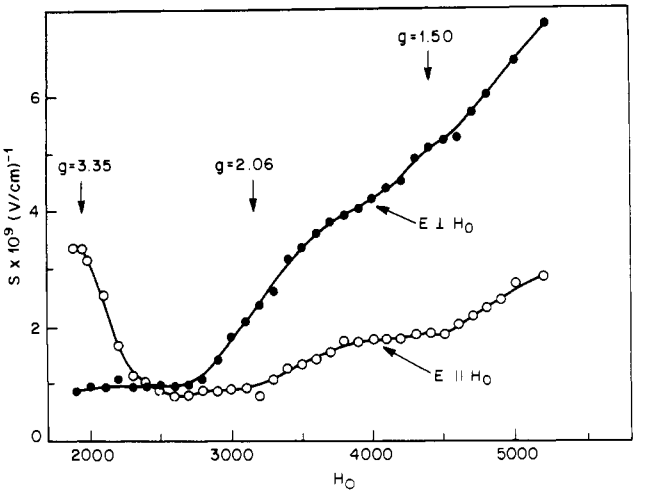

FIGURE 2: Linear electric field effect for horse heart cytochrome *c* at pH 10.0. The applied electric field is aligned parallel (O) to the magnetic field ($E \| H_0$); or perpendicular (●) to the magnetic field ($E \perp H_0$).

A linear electric field effect (LEFE) of this kind was observed in hepatic cytochrome P-450 and in other heme mercaptide complexes (Peisach and Mims, 1973). In this study it was suggested that the *g* shift obtained when the electric and magnetic fields are parallel to one another and perpendicular to the plane of the porphyrin, could be used as a measure of the differences in the d electron bonding of the two axial ligands. In the present communication we report LEFE measurements for pH 6.6 and pH 10.0 samples of cytochrome *c* studied in glassy unoriented samples and attempt to correlate the results with the two models for the heme center.

Materials and Methods

The low pH sample was prepared by dissolving horse heart cytochrome *c* (Sigma, Type VI) in water to a final concentration of ≈2mM and adding 1M KH_2PO_4 to adjust the pH to 6.6. A value somewhat below pH 7 was chosen in order to minimize the presence of the high pH form (Theorell and Åkesson, 1941; Margoliash and Schejter, 1966; Morton and Bohan, 1971; Margalit and Schejter, 1973). The high pH sample was prepared in a similar way, using 1M K_3PO_4 to raise the pH to 10.0.

The LEFE was measured at 4.2°K and at a frequency ≈9.1 GHz by the electron spin echo method (Mims, 1964). The procedure adopted was to set the time τ between the two microwave pulses to some convenient value and to adjust the size of the voltage pulse until its application caused a reduction to half-amplitude in the spin echo signal. It is perhaps interesting to note that we were able to apply fields of up to 70 kV/cm to the sample immersed in liquid helium at 4.2°K without causing electrical breakdown.

The LEFE is characterized by a shift parameter

$$S = (1/E)(\overline{\Delta g}/g) = \overline{\Delta f}/Ef = d/[6f(\tau V)_{1/2}] \quad (1)$$

where E is the applied electric field in V/cm, f is the microwave frequency in Hz, d is the sample thickness in cm, and $(\tau V)_{1/2}$ is the product of time, τ, in seconds, and V, in volts, required to halve the echo signal. The quantity $\overline{\Delta f}$ is the mean shift in Larmor frequency corresponding to the mean shift $\overline{\Delta g}$ in *g* value brought about by the electric field. The mean is taken over the number of different orientations of cytochrome *c* molecules in the unoriented samples which contribute to the epr spectral line at each chosen value of the Zeeman field H_0. (For a more detailed description of the application of the electron spin echo method to samples of this type, see Peisach and Mims (1973).)

Results and Discussion

As might be expected, electron spin echo signals were observed throughout the range of magnetic fields, H_0, corresponding to the interval between the maximum (g_{max}) and minimum (g_{min}) values of *g*. Thus, for cytochrome *c* at pH 6.6 signals were seen from approximately 2100 to 5200 G, corresponding to *g* values from $g = 3.1$ to 1.2. The cut off of the signal at the low-field end of the spectrum was fairly abrupt, but at the high-field end signals of ever diminishing intensity could still be observed well beyond the magnetic field corresponding to g_{min}. We assume that these high-field signals resulted from the very considerably broadened nature of the resonance line at g_{min} (Mailer and Taylor, 1972). There was nothing in our observations (*e.g.*, discontinuous changes in the LEFE) to suggest that they were due to an impurity species.

The results of these measurements as a function of H_0 are shown in Figures 1 and 2 for cytochrome *c* at two different values of pH and for two orientations of the magnetic field in relation to the electric field. In addition, the shifts at the magnetic field setting corresponding to g_{max} are shown in Figure 3 as a function of the angle between E and H_0. The interpretation of these data is made easier by the single crystal epr studies of cytochrome *c* by Mailer and Taylor (1972), who show that the g_{max} principal axis is within 5% of the heme normal, while the g_{min} principal axis lies approximately along the intersection of the heme plane and the plane of the proximal imidazole. We shall tentatively assume here that principal *g* axes lie in the same direction for the high pH form of cytochrome *c* also. Thus, at the g_{max} end of the epr spectrum with $E \| H_0$ the electric field lies roughly along the line joining the axial ligands to the heme, and at the g_{min} end with $E \| H_0$ the electric field is perpendicular to this line and in the plane of the imidazole. The situation with $E \perp H_0$ is less well-defined. At the g_{max} setting, the electric field is distributed over all orientations in the heme plane, some almost in the imidazole plane and some almost

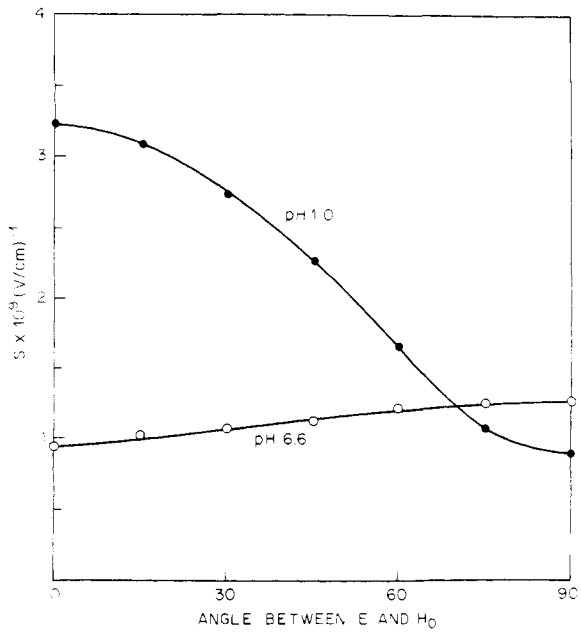

FIGURE 3: Angular dependence of linear electric field effect at g_{max} for horse heart cytochrome *c* at pH 6.6 (○) and 10.0 (●).

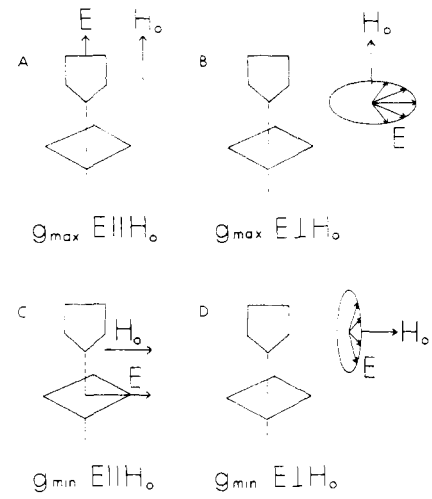

FIGURE 4: Diagram showing the relative orientation of magnetic field, H_0, electric field, E, the plane of the porphyrin, and the approximate orientations of the proximal imidazole ligand of cytochrome *c*. In A, the electric field E is aligned parallel to the magnetic field, H_0, at the g_{max} setting and the electric field is therefore aligned approximately in the direction of the axial ligands to the heme. When the electric field is aligned perpendicular to the magnetic field, at the g_{max} setting, as in B, the electric field is aligned approximately in the plane of the porphyrin and perpendicular to the axial ligands. In C, the electric field is aligned parallel to the magnetic field at the g_{min} setting and both fields are therefore approximately in the plane of the porphyrin and in the plane of the proximal imidazole ligand. In D, E and H_0 are perpendicular to one another at the g_{min} setting. The electric field therefore lies in a plane perpendicular to the planes of the porphyrin and of the proximal imidazole. The assumptions regarding the orientations of H_0 at the g_{max} and g_{min} settings in relation to the planes of the porphyrin and the proximal imidazole are derived from single crystal studies by Mailer and Taylor (1972).

perpendicular to it. By setting H_0 at the low-field end of the spectrum we select those cytochrome *c* molecules which have their heme normal along H_0, but we are not able to specify how the molecules are oriented about this axis. Likewise at the g_{min} setting with $E \perp H_0$ the E field is distributed in a plane perpendicular to the g_{min} axis, some molecules seeing electric fields along the line joining the axial ligands, some seeing electric fields perperdicular to the imidazole plane, and the majority seeing electric fields with intermediate orientations (Figure 4). At the setting of H_0 corresponding to the middle g value (g_{mid}) we observe those cytochrome *c* molecules whose g_{mid} principal axes lie along or close to H_0 but other orientations also contribute to the spectrum here (see Poole and Farach (1972)) and will have some influence on the measured shift parameter. For those molecules with the g_{mid} principal axis along H_0, the electric field is in the heme plane when E is parallel to H_0 and is spread over a range of directions in the imidazole plane when E is perpendicular to H_0.

It is difficult to interpret all the results obtained here since very little is at present known concerning the action of applied electric fields on proteins and on paramagnetic centers of this kind. Nevertheless, we shall attempt to suggest explanations where possible bearing in mind these explanations may appear excessively naive in the light of future knowledge. Let us first consider the case of $H_0 \| E$ at g_{max} which we can conveniently describe as the "axial electric effect." For the pH 6.6 sample $S = 1.3 \times 10^{-9}$ $(V/cm)^{-1}$ and for the pH 10.0 sample $S = 3.4 \times 10^{-9}$ $(V/cm)^{-1}$, a factor of 2.6 larger. As was suggested earlier (Peisach and Mims, 1973), the magnitude of the axial electric effect can be interpreted as arising from differences between the crystal field contributions of the axial ligands. Expressing this result somewhat crudely, and assuming that the axial ligands are the ones indicated earlier for these proteins, we can say that

$$(L_{imid} - L_{amine}) = 2.6(L_{imid} - L_{meth})$$

where L_{imid}, L_{amine}, and L_{meth} stand for the axial crystal field contributions of imidazole, amine, and methionine sulfur ligands. A comparison of crystal field strengths in a number of low-spin heme iron compounds has demonstrated that $L_{imid} > L_{meth} > L_{amine}$ (Peisach *et al.*, 1973b). It should be noted, however, that the LEFE gives a measure of the odd crystal field potentials arising from the *difference* in crystal field contributions of two oppositely situated ligands, whereas the g values give a measure of even crystal field potentials which depend on the *average* of the contributions of two such ligands. The LEFE is therefore considerably more sensitive to changes in crystal field contributions due to one particular ligand than the g values, especially when, as in the case of methionine sulfur and imidazole nitrogen, the pair of ligands is nearly matched.

The interpretation of the LEFE at other orientations of E and H_0 and at other positions in the spectrum is harder to make. The results themselves are in marked contrast with those obtained earlier in heme mercaptide complexes which tended to show large effects at g_{max} with $E \| H_0$ (large axial effects) and small effects everywhere else. The large effects observed for cytochrome *c*, pH 6.6 at g_{min}, $E \| H_0$ ($S \approx 5 \times 10^{-9}$ $(V/cm)^{-1}$) indicate that there is a very marked asymmetry in the ligand field in a direction along the g_{min} principal axis, *i.e.*, in the plane of the proximal imidazole (Figure 4C). We might perhaps attribute this to the asymmetry of the π-electron distribution in the proximal imidazole since this ligand is bonded covalently to the Fe^{III}. The small effects seen for $E \| H_0$ in the vicinity of the g_{mid} setting could then be attributed to the fact that the π-electron charge density is symmetrical perpendicular to the plane of the imidazole ligand. But it is not clear why the g_{min}, $E \| H_0$ effect should be so much smaller ($S \approx 2.7 \times 10^{-9}$) for the pH 10 sample than it is for the pH 6.6 sample. The effects seen in some other instances seem to depend on the odd component of the axial field. Thus, the large effect ($S \approx 6.5 \times 10^{-9}$ $(V/cm)^{-1}$) at g_{min}, $E \perp H_0$, in the pH 10 sample is probably associated with the fact that the electric field can

have a component along the heme normal (Figure 4D). The corresponding effect for the pH 6.6 sample is smaller ($S \approx 5 \times 10^{-9}$ (V/cm)$^{-1}$).

In conclusion, we should like to remark on an interesting relationship which appears to exist between the LEFE shift parameters and the line widths observed by Mailer and Taylor (1972) in single crystal studies. Essentially the epr line is broad where the LEFE is large. This is a phenomenon which has already been observed in a number of inorganic crystals doped with paramagnetic ions (Mims and Gillen, 1966; Bugai *et al.*, 1967; Jones *et al.*, 1968) where it is interpreted as line broadening due to the presence of internal randomly oriented electric fields in the sample. In the case of ionic crystals the fields originate from point defects in the crystal lattice. Their mean value can be deduced from the line width and the LEFE parameters, and is generally of the order 10^4–10^5 V/cm. If we interpret the line broadening in cytochrome *c* in the same way, we arrive at electric fields of approximately 10^7 V/cm. This seems a relatively large value but is not an implausible one if we assume that the charged groups on the cytochrome *c* molecule are neutralized by ions and water dipoles from the surrounding medium in a somewhat random fashion. (An entirely regular scheme of charge neutralization would displace but not broaden the line.) No definite conclusions can be based on this one limited set of experimental observations but it will be interesting to see if the same pattern of behavior is found in other cases.

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