Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 547-555.
McCarty, R. E., & Moroney, J. E. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) 2nd ed., Vol. 4, pp 383-413, Plenum Press, New York.
Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58-62.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Richter, M. L., Snyder, B., McCarty, R. E., & Hammes, G. G. (1985) *Biochemistry 24*, 5755-5763.
Schinkel, J. E., & Hammes, G. G. (1986) *Biochemistry 25*, 4066-4071.
Schumann, J., & Strotmann, H. (1981) in *Photosynthesis II. Electron Transport and Photophosphorylation* (Akoyunoglou, G., Ed.) pp 881-892, Balaban International Science Services, Philadelphia, PA.
Shahak, Y. (1986) *Eur. J. Biochem. 154*, 179-185.
Strotmann, H., & Bickel-Sandkötter, S. (1977) *Biochim. Biophys. Acta 460*, 126-135.
Wu, D., & Boyer, P. D. (1986) *Biochemistry 25*, 3390-3396.

Low-Temperature Electron Paramagnetic Resonance Study of the Ferricytochrome *c*-Cardiolipin Complex

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ABSTRACT: The electron paramagnetic resonance spectrum of the ferricytochrome *c* complex with cardiolipin was observed at temperatures below 20 K. For the low-spin iron(III) heme system complexed with the negatively charged lipid, the tetragonal and rhombic ligand field parameters ($\Delta/\lambda = 3.58$, $V/\lambda = 1.82$) differ significantly from those ($\Delta/\lambda = 2.53$, $V/\lambda = 1.49$) of the free ferricytochrome *c* sample. The *g* values of the complex ($g_x = 1.54 \pm 0.02$, $g_y = 2.26 \pm 0.01$, $g_z = 3.02 \pm 0.01$) are compared to the values for free ferricytochrome *c* ($g_x = 1.25 \pm 0.02$, $g_y = 2.25 \pm 0.01$, $g_z = 3.04 \pm 0.01$). Spectral alterations are interpreted in terms of the ligand field changes induced within the heme group by association with the negatively charged phosphoglyceride.

Interest in cytochrome *c* interactions with either zwitterionic or charged lipid assemblies stems primarily from attempts to characterize physically the perturbations to membrane structure arising from the binding of extrinsic bilayer proteins (Devaux et al., 1986; DeKruijff et al., 1980; Brown & Wüthrich, 1977). Since cytochrome *c* may interact selectively with cardiolipin in the inner mitochondrial membrane, the properties of this particular model system become important both in assessing putative configurational changes in either the protein or the lipid component of the complex and in assigning functional significance to the structural reorganizations that occur during binding (Vincent & Levin, 1986; DeKruijff & Cullis, 1980). In particular, ferricytochrome *c* in complex form with cardiolipin exhibits considerably different properties from those of the free protein. For example, the redox potential is decreased by approximately 40 mV (Kimelberg & Lee, 1970), and although the visible spectra shift slightly, the spectral patterns remain characteristic of the oxidized species (Vincent & Levin, 1986). In addition, the resonance Raman spectrum of the ferricytochrome *c*-cardiolipin complex reflects the vibrational frequency and intensity markers of the reduced iron species even though the iron oxidation state remains unaffected (Vincent & Levin, 1986). That is, on complexation, the periphery of the porphyrin groups assumes the reduced cytochrome *c* conformation without the presence of a chemical reductant. As part of the latter resonance Raman study, the electron paramagnetic resonance (EPR) spectrum of the ferricytochrome *c* complex was measured at 77 K in order to confirm the existence of the low-spin Fe(III) species (Vincent & Levin, 1986). Since the EPR spectra of the ferricytochrome *c* complex with cardiolipin reflect modifications in the heme structure of uncomplexed cytochrome *c*, we discuss here the perturbed EPR spectra of the associated species, determined at temperatures below 20 K, in terms of the induced ligand field changes.

Experimental Procedures

Ferricytochrome *c*, obtained from Sigma (horse heart VI), was purified by chromatography according to the procedure of Brautigan et al. (1978). The 695-nm transition was clearly observable; the visible spectra of the reduced species were identical before and after treatment with carbon monoxide (Theorell & Akerson, 1941), indicating that the heme group remained fully coordinated with protein ligands (George & Schejter, 1964). Cardiolipin (bovine heart) was obtained from Avanti Polar Lipids and used without further purification.

The EPR spectra were collected with a Varian Century-100 X-band spectrometer. The sample was cooled with helium gas conducted from a liquid helium Dewar by an Air Products and Chemicals Heli-Tran transfer line. The temperature for each experiment was determined by using a germanium resister and maintained during each experiment at a constant temperature between 7 and 20 K. The magnetic field intensity was monitored by counting the frequency of the proton nuclear magnetic resonance; the microwave frequency was calibrated with a cavity wavemeter.

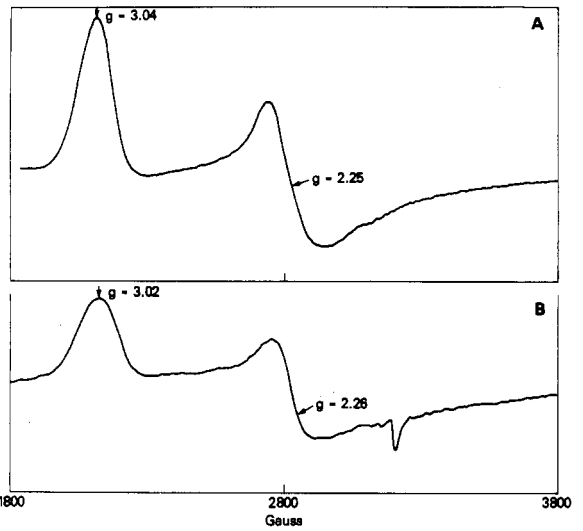

FIGURE 1: Derivatives of the $g = 3$ and $g = 2.25$ EPR spectra for (A) aqueous ferricytochrome c solution and (B) the cardiolipin–ferricytochrome c complex, both at 13 K, are displayed as a function of the magnetic field intensity. The small amount of high-spin iron species is observed at $g = 2.0$. The spectra were recorded at a microwave frequency of 8.984 GHz and a magnetic field modulation amplitude of 10 G.

The ferricytochrome c complex with cardiolipin was prepared in a manner similar to a previously described procedure (Vincent & Levin, 1986). Briefly, water was added to cardiolipin powder under nitrogen, and the system was mechanically agitated. Ferricytochrome c in a 0.085 M phosphate solution, pH 7.0, was added to the cardiolipin and again mechanically mixed under nitrogen. The cardiolipin to cytochrome c mole ratio varied from 8:1 to 12:1 in the samples examined.

Results and Discussion

The EPR spectrum of a 3×10^{-3} M solution of ferricytochrome c, measured at 18 K, is displayed in Figure 1A. The g factors obtained for this low-spin Fe(III) species correspond well with those reported in the literature (Salmeen & Palmer, 1968; Mailer & Taylor, 1972). The EPR spectrum of the cardiolipin–ferricytochrome c complex, shown in Figure 1B, exhibits several departures from the spectra of pure ferricytochrome c. The most noticeable change is the appearance of a small amount (~5%) of high-spin Fe(III) characterized by a sharp, relatively strong, line at $g = 6$ and a much weaker line at $g = 2$. In addition to the high-spin iron, a small amount (2–3%) of a non-heme $g = 4$ species also is observed. These high-spin species are completely absent in the pure ferricytochrome c spectra (Figure 1A) and are presumably the result of partial denaturation of ferricytochrome c complexed with cardiolipin. There are no detectable paramagnetic species in the cardiolipin used for these preparations. The large majority of the complexed species, however, is the low-spin ferricytochrome c whose major components of the g factor appear in Table I. The $g = 3$ peak is slightly lower in value, with its width at half-height being broader, than the corresponding line of the pure cytochome c solution. The high-field line of the complex, illustrated in Figure 2A, has shifted considerably from $g = 1.25$ in pure cytochrome c to $g = 1.54$ in the complex. The intermediate $g = 2.26$ factor is essentially the same in both species.

We have interpreted these spectra using the t_{2g} hole model with the Kramer's doublet wave functions of Taylor (1977). This model presupposes that the product of g factors, $g_x g_y g_z$, is positive, a result confirmed for ferricytochrome c by Huynh et al. (1978). According to Taylor (1977), the g_z direction is nearly perpendicular to the heme plane, while the g_x and g_y directions lie in a plane nearly parallel to the heme plane. The tetragonal distortion $\Delta = 3.58\lambda$ splits the iron d_{xy} orbital from the midpoint of the d_{xz}, d_{yz} single-electron levels, which are in turn split by the rhombic term $V = 1.82\lambda$. (These parameters are determined in units of the spin–orbit interaction λ.) These values may be compared with those of uncomplexed cytochrome c for which $\Delta = 2.56\lambda$ and $V = 1.48\lambda$ (Salmeen & Palmer, 1968; Mailer & Taylor, 1972). The uncertainties in the crystalline field parameters listed in Table I were determined from the uncertainties in the measured values of g_x, g_y, and g_z using an error propagation method similar to that used by Taylor (1977). Thus, the complexation of ferricytochrome c with cardiolipin increases both the tetragonal and rhombic ligand field distortions, thereby increasing the energy differences of the t_{2g} electronic orbitals.

In assessing the derived changes in ligand field parameters on interacting cytochrome c with cardiolipin, we first draw attention to the correlation of the charge transfer transition frequencies $a_{2u}(\pi)$, $a_{1u}(\pi) \rightarrow d_{yz}$ in various ferric low-spin heme systems with sums of the tetragonal and rhombic crystal field energies (Schejter & Eaton, 1984). Specifically, the frequency of the charge transfer bands increases as the orbital splitting parameters become greater. Since the iron d_{yz} orbital receives the electron in the chemical reduction of ferricytochrome c, Schejter and Eaton (1984) suggested a relationship between the spectral frequency of a charge transfer band for a given system and its observed reduction–oxidation potential. Their model proposes that the reduction of the ferric protein proceeds by two hypothetical steps: (1) excitation of an electron by charge transfer from the heme $a_{2u}(\pi)$ orbital to the iron d_{yz} orbital; and (2) addition of an electron to the $a_{2u}(\pi)$ orbital

Table I: Electron Paramagnetic Resonance Parameters for Ferricytochrome c and the Cardiolipin–Ferricytochrome c Complex Observed in Polycrystalline Ice and 0.085 M Phosphate Buffer at 17 K

	ferricytochrome c	cardiolipin–ferricytochrome c complex
g_x	1.25 ± 0.02	1.54 ± 0.02
g_y	2.25 ± 0.01	2.26 ± 0.01
g_z	3.04 ± 0.01	3.02 ± 0.01
line width (g_z) (G)	132 ± 3	154 ± 4
V/λ	1.49 ± 0.01	1.82 ± 0.01
Δ/λ	2.53 ± 0.02	3.58 ± 0.02

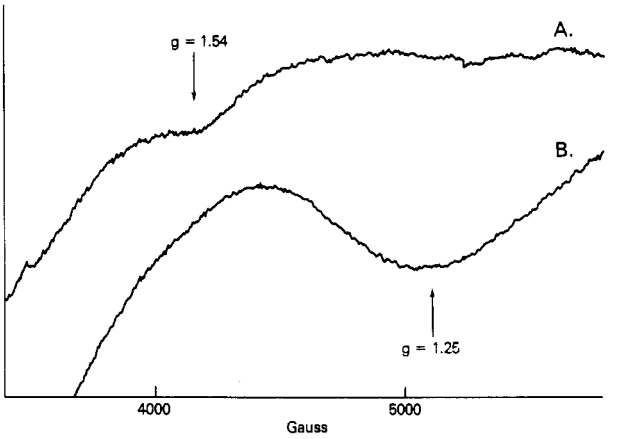

FIGURE 2: Derivatives of the high magnetic field, $g = 1.54$ and 1.25, EPR absorption spectra for (A) the cardiolipin–ferricytochrome c complex and (B) the ferricytochrome c–water solution, both at 18 K, are displayed as a function of magnetic field intensity. The spectra were recorded at a microwave frequency of 8.983 GHz and a magnetic field modulation amplitude of 40 G.

to produce the ground-state ferrous protein. Since the second step depends upon the electron affinity of the excited ferric state, its energy would be sensitive primarily to variations in the electrostatic environment of the entire molecule. Schejter and Eaton (1984) argued that the second step is relatively insensitive to changes in the axial ligands and heme conformation. Therefore, according to their model, the reduction–oxidation potential is dependent primarily upon the nature of the axial ligands, as reflected by changes in the crystal field parameters. Thus, the estimates of changes in the redox potential within various systems are provided by differences in the charge transfer band frequencies, which, in turn, are reflected by g factor anisotropies.

As an example of the correlation proposed by Schejter and Eaton (1984), we note that the redox potential for cytochrome b_5 with an imidazole as an axial ligand is 20 mV (Kawai et al., 1963), while that of normal cytochrome c with a methionine axial ligand is 260 mV (Kimelberg & Lee, 1970). The charge transfer band frequencies (Schejter & Eaton, 1984) for the imidazolocytochrome c are greater (6580 and 7840 cm^{-1}) than those of cytochrome c (5780 and 6825 cm^{-1}), as are the tetragonal and rhombic crystal field splitting parameters derived from the EPR g factors of the imidazole species. This relationship between the g factor anisotropy and redox potential is consistent with the results of the complex formed between cardiolipin and ferricytochrome c. The respective tetragonal and rhombic splitting parameters, Δ/λ and V/λ, for the lipid–protein complex are greater than those of uncomplexed ferricytochrome c, while the redox potential decreases from 260 mV for pure cytochrome c to 220 mV for the complex with cardiolipin (Kimelberg & Lee, 1970). Cardiolipin, therefore, distorts the conformation of the heme group by increasing the crystalline field experienced by the iron. As a consequence, the redox potential decreases by approximately 15%. In addition, as deduced from the resonance Raman data (Vincent & Levin, 1986), conformational changes accompanying the electronic adjustment occur such that the outer portion of the porphyrin ring resembles the conformation of the reduced cytochrome c species.

REFERENCES

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978) *Methods Enzymol. 53*, 128–148.

Brown, L. R., & Wüthrich, K. (1977) *Biochim. Biophys. Acta 468*, 389–410.

De Kruijff, B., & Cullis, P. R. (1980) *Biochim. Biophys. Acta 602*, 477–490.

Devaux, P. F., Hoatson, G. L., Favre, E., Fellmann, P., Farren, B., MacKay, A. L., & Bloom, M. (1986) *Biochemistry 25*, 3804–3812.

George, P., & Schejter, A. (1964) *J. Biol. Chem. 239*, 1504–1508.

Huynh, B. H., Emptage, M. H., & Münck, E. (1978) *Biochim. Biophys. Acta 534*, 295–306.

Kawai, Y., Yoneyama, Y., & Yoshikawa, H. (1963) *Biochim. Biophys. Acta 67*, 522–524.

Kimelberg, H. K., & Lee, C. P. (1970) *J. Membr. Biol. 2*, 252–262.

Mailer, C., & Taylor, C. P. S. (1972) *Can. J. Biochem. 50*, 1048–1055.

Margalit, R., & Schejter, A. (1973) *Eur. J. Biochem. 32*, 4922–4929.

Salmeen, I., & Palmer, G. (1968) *J. Chem. Phys. 48*, 2049–2052.

Schejter, A., & Eaton, W. A. (1984) *Biochemistry 23*, 1081–1084.

Taylor, C. P. S. (1977) *Biochim. Biophys. Acta 491*, 137–149.

Theorell, H., & Akerson, A. (1941) *J. Am. Chem. Soc. 63*, 1812–1818.

Vincent, J. S., & Levin, I. W. (1986) *J. Am. Chem. Soc. 108*, 3551–3554.