Spin-Lattice Relaxation Times of Phospholipid Aminoxyl Spin Labels in Cardiolipin–Cytochrome *c* Bilayers: a Pulse Saturation–Recovery EPR Study

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A pulsed EPR saturation-recovery method has been used to measure spin-lattice relaxation times (T_1) for aminoxyl spin labels in cardiolipin bilayers, with and without cytochrome *c*. The relaxation time for each spin label was determined at various positions in the bilayer, which included the bilayer surface and three positions within the hydrophobic interior in the membrane, as well as a position close to the glycerol backbone of phospholipids in the bilayer. A dynamic profile for the hydrocarbon chains in bilayers was found in agreement with results from other techniques. On addition of cytochrome *c* to cardiolipin bilayers, changes in the spin-lattice relaxation time and order parameter were observed around the unsaturated region in the cardiolipin acyl chains. Supported by complementary lineshape analysis, these effects were interpreted as changes in the molecular dynamics around the double bonds in the acyl chains of cardiolipin bilayers, induced upon the binding of cytochrome *c*.

Aminoxyl† spin labels have been used extensively to probe the structure and dynamics of synthetic and biological membranes through the evaluation of line positions and linewidths of the continuous wave (CW) electron paramagnetic resonance (EPR) spectra of spin labels. The development of pulsed EPR permits the measurement of spin-lattice (T_1) relaxation times which are not readily obtained by CW EPR.[1] The experimental difficulty in determining relaxation parameters for aminoxyl in membrane preparations lies partly in the high dielectric loss due to water and partly in the fast relaxation rates, requiring submicrosecond microwave pulses. To date T_1 measurements of spin labels in bilayers have been made mainly on fatty acid spin labels, and only a few measurements have been made on spin labelled phospholipids, in which oxygen transport in membranes was measured.[1,2] Here, we report a systematic evaluation of spin-lattice relaxation times for aminoxyl spin labels located at defined positions across the bilayer.

Cytochrome *c*, a highly basic protein involved in the electron transfer chain on the inner mitochondrial membrane, interacts strongly with negatively charged phospholipids,[3–5] and particularly with cardiolipin bilayers.[6–11] There is some evidence that this binding not only involves electrostatic interactions with surface lysines in the protein, but that cytochrome *c* can penetrate, at least partially, into bilayers containing cardiolipin to interact hydrophobically with the membrane interior.[12,13] More recently this interaction has been examined by solid state NMR studies on cardiolipin-cytochrome *c* complexes.[9–11] These studies focused on the effects on the structure and dynamics of the protein, and on the haem spin configuration, upon binding of cytochrome *c* to cardiolipin bilayers. While the haem in cytochrome *c* in its native structure is in a low-spin state,[14] the complexation with cardiolipin induces conformational changes in the protein. In particular, it induces changes on the conformation in and around the haem group in the cytochrome *c* that lead to a high-spin configuration of the haem iron.[10]

The mechanism by which those conformational changes in the protein occur seems to be driven by strong interactions with the lipid bilayer. In addition to the electrostatic interaction with the charged bilayer surface, cytochrome *c* penetrates partially into cardiolipin bilayers, cardiolipin being the dominant anionic phospholipid in the inner mitochondrial membrane. Analysis of the depth of penetration of cytochrome *c*, or the haem group itself in cytochrome *c*, into cardiolipin bilayers have been attempted by NMR methods.[12] Our approach employs the use of spin-labelled phospholipids to probe the perturbation of cardiolipin bilayers by the protein. In these experiments the spin-label moiety is at a well-defined position in the bilayer and using pulsed EPR methods, the spin-lattice (T_1) relaxation times of spin labels have been determined from saturation-recovery experiments to evaluate more precisely the degree of membrane penetration of cytochrome *c*. Through spectral lineshape analysis a more quantitative description of the dynamics at particular sites in the hydrocarbon chain is obtained.

Materials and Methods

Cardiolipin from beef heart (Sigma Chemical Co., St Louis) was used without further purification. Cytochrome *c* from horse heart, type VI (Sigma Chemical Co., St. Louis) was purified by ion exchange chromatography on Whatman CM-32 and, after complete oxidation with ferricyanide of any ferrocytochrome *c*, the protein in the oxidised form, ferricytochrome *c*, was eluted with 65 mmol dm^{-3} phosphate buffer, pH 7.0.[15] Protein was quantified spectrophotometrically using a molar absorptivity of 2.95×10^4 at pH 7.0 for the protein when reduced with dithionite.[16]

The spin-labelled stearic acids, 5- and 9-SASL, and cholestane spin-label, ChSL, were obtained from Sigma Chemical Co., St Louis. Stearic acid labelled on the 14th carbon, 14-SASL, was prepared according to the procedure described in Marsh & Watts.[17] Spin-labelled phosphatidylcholines, 5-, 9- and 14-PCSL, which were synthesized from 5-, 9- and 14-SASL and egg lyso-phosphatidylcholine, were produced as described previously.[17] The spin label 2,2,6,6-tetramethyl-1-piperidinyloxyl–dipalmitoyl-phosphatidic ester (TEMPO-DPPA) was synthesized from phosphatidic acid and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO),[18] both from Sigma Chemical Co., St. Louis.

† IUPAC recommends the use of aminoxyl rather than nitroxide in describing spin labels.

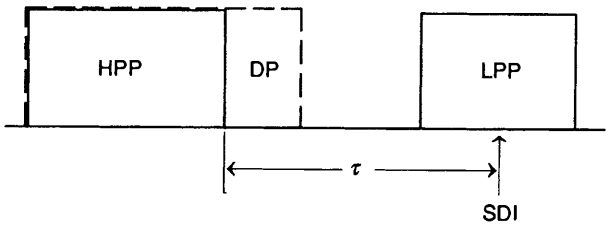

Fig. 1 Saturation-recovery pulse sequence used for spin-lattice relaxation time (T_1) measurements: HPP, high power pulse; DP, defence pulse; LPP, low power pulse, and τ is the variable delay time between the saturating pulse, HPP, and the observing pulse, LPP, at the position of the sampling digitizer, SDI

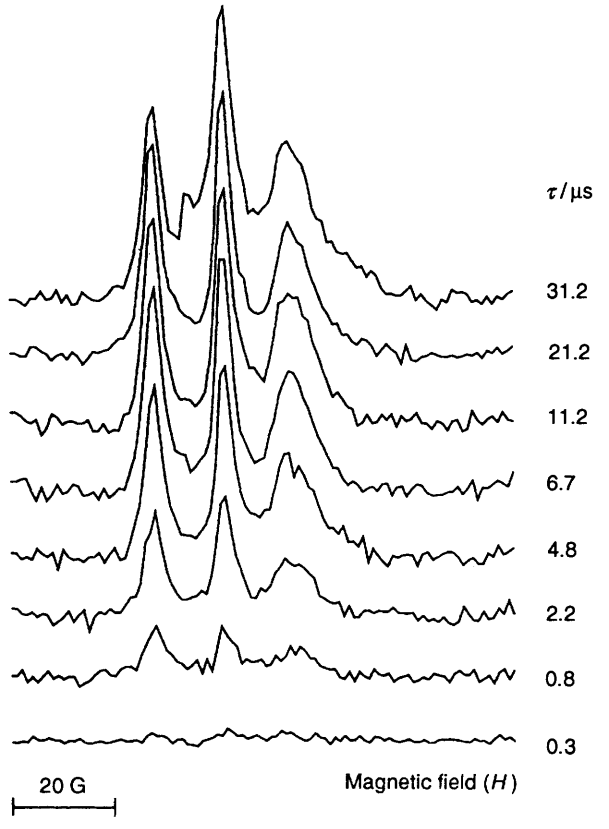

Fig. 2 Absorption EPR spectra in a typical saturation-recovery experiment used for calculation of the spin-lattice relaxation time (T_1) for 14-PCSL in cardiolipin bilayers (see text for details of preparation) recorded at various delay times, τ, used between HPP and LPP (see Fig. 1)

Sample Preparation.—A dry film of 25 mg of cardiolipin, containing 2.5 mol % of spin label, was prepared by rotary evaporation from stock solutions in ethanol. The dry lipid was left under a high vacuum of 10^{-2} Torr for a minimum of 8 h. Multilamellar cardiolipin liposomes were formed by hydration with excess buffer, 10 mmol dm^{-3} Tris, 0.2 mmol dm^{-3} ethylene diamine tetracetic acid (EDTA), 100 mmol dm^{-3} NaCl, pH 7.0. The binding of cytochrome *c* to hydrated cardiolipin bilayers was performed by the addition of the desired weight of lyophilised protein, followed by several freeze–thaw cycles. Identical binding levels were obtained when the lipid film was hydrated with buffer containing the protein, which shows that cytochrome *c* is able to penetrate the inner bilayers of multilamellar liposomes, in agreement with previous studies.[3,5,7] The buffer was previously deoxygenated with oxygen-free grade nitrogen gas (99.98%), and all steps of sample preparation were carried out under an atmosphere of oxygen-free nitrogen (99.98%). Liposomes were then centrifuged at 75000 × *g* for 30 min for lipid-protein complexes and for 3 h for pure lipid liposomes. The pellets were transferred to the EPR tubes and sealed under vacuum after several freeze–pump–thaw cycles.

Pulsed EPR.—Saturation-recovery EPR experiments were carried out on a Bruker ESP-380 pulse EPR spectrometer. The cavity was a variable *Q*, dielectric type, with a typical resonant frequency of 9.7 GHz (X-band). A pulse former unit generated phase and amplitude variable pulses of a minimum duration of 8 ns for subsequent amplification to *ca.* 1 kW by the travelling wave tube amplifier for microwave power saturation of the spin label EPR spectrum. A low power pulse (LPP) channel was used for the generation of a weak detecting field in the saturation-recovery experiments. The microwave signal was pre-amplified, sent together with a microwave reference signal to a detector-amplifier and sampled with a sampling digitizer (SDI). Saturation-recovery experiments were performed with the pulse sequence shown in Fig. 1. Saturation of the spin system was achieved with a 1 kW high power pulse (HPP) of duration 1 μs, whilst the signal was detected within the 800 ns low power pulse (LPP) (Fig. 1), which is not amplified by the travelling wave tube amplifier. The signal was recorded as a function of field for each delay time, τ, between HPP and SDI position. Typically, 2000 shots (a HPP–τ–LPP sequence) per field point (1 Gauss increment) were needed to obtain a reasonable signal-to-noise ratio. Repetition time between shots was set to 2 ms, making the acquisition time for one spectrum approximately 7 min. Sensitivity was impaired in such pulse experiments by the water content of the samples which tends to lower the *Q* of the cavity. The minimum delay time between saturation and detection was limited by the length of the defence pulse, DP (Fig. 1), which protects the sensitive components of the spectrometer (such as the ultra-low-noise microwave preamplifier) from damage from the HPP and through ringing of the cavity after the HPP. Typical dead times were about 300 ns. Neither length nor power of the saturating pulse affected the measured T_1 values, which indicates that spectral diffusion within the time scale of T_1 was not significant. CW power during the 800 ns detection pulses was 0.6 mW. The average total power at the chosen repetition rate, almost entirely from the saturating pulses, was about 0.5 W.

CW EPR Effective Order Parameter Measurements.—Continuous wave (CW) EPR spectra were recorded on a Bruker ESP-300 EPR spectrometer, operating typically at 9.4 GHz (X-band). The effective order parameter, S_{eff}, was calculated from the spectral anisotropies with a correction factor to account for the medium polarity.[19]

Results and Discussion

Saturation-recovery is the most frequently employed technique for making spin-lattice (T_1) relaxation measurements in EPR.[1,2,20] With long and intense microwave pulses, the spin system approaches a steady state, at which the populations of spins at each energy level tend to be equalised. After the saturating pulse is turned off, the recovery of the system to Boltzmann equilibrium is observed with a low, essentially non-perturbing microwave observing power. The saturation-recovery signal is usually recorded with time at a fixed field position, and an exponential recovery curve fitted $\{y = A[1 - \exp(-x/T_1)]\}$, from which T_1 can be calculated. In the present experiments the recovered signal for each delay time between the high power saturating pulse and the observing pulse was recorded with magnetic field in a way similar to the determination of T_1 in NMR experiments. Pulsed EPR spectra were obtained in the absorption mode and an example of a saturation-recovery experiment for a T_1 measurement is presented in Fig. 2. As shown in the typical case given in Fig. 2,

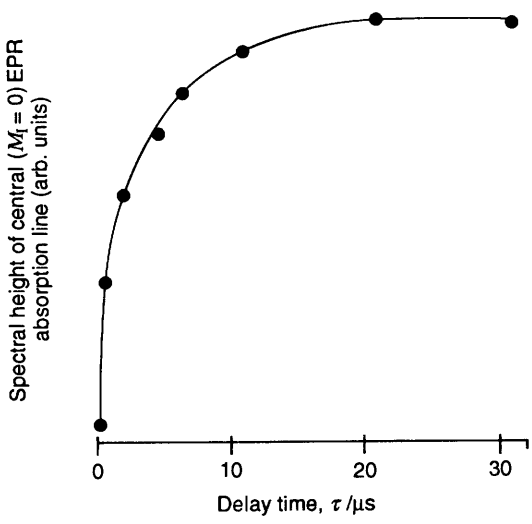

Fig. 3 Typical single exponential fit to the experimental data points of the amplitude of the central EPR absorption line, obtained from the saturation-recovery experiment with TEMPO-DPPA spin label in cardiolipin bilayers with bound cytochrome *c* (lipid : protein mole ratio of 15 : 1; temperature, 22 °C)

Table 1 Spin-lattice (T_1) relaxation times (μs)[a] for the indicated aminoxyl spin labels in cardiolipin bilayers in the absence and presence of cytochrome *c*

Spin label	Cardiolipin bilayers/μs	Cardiolipin-cyt *c* bilayers/μs
TEMPO-DPPA	2.0 ± 0.5	1.3 ± 0.3
ChSL	2.9 ± 0.8	2.5 ± 0.6
5-PCSL	5.6 ± 0.5	5.7 ± 0.6
9-PCSL	10.0 ± 1.0	6.5 ± 0.7
14-PCSL	3.9 ± 0.4	4.2 ± 0.5

[a] The results are averaged values of three or two measurements.

there are no detectable changes in the EPR spectrum as a function of recovery time. Thus anisotropies (*i.e.* different T_1 values for spin labels differently oriented in the magnetic field) or other types of spectral and T_1 heterogenieties appear to be ruled out. Spin-lattice relaxation times were normally determined from the dependence of the signal height of the central ($M_I = 0$) hyperfine line as a function of the delay time τ. The experimental data points were found to fit a single exponential decay with τ, from which T_1 values were determined. A typical example is illustrated in Fig. 3 for the system TEMPO-DPPA spin label in cardiolipin bilayers containing cytochrome *c*.

In Table 1 the spin-lattice (T_1) relaxation times for the various aminoxyl spin labels incorporated in cardiolipin bilayers are given. Measurements of T_1 were made on three different sample preparations of the systems containing the 9-PCSL, and two for all the other systems. Fig. 4 shows the profile of the spin-lattice relaxation times of the spin labels at various positions in cardiolipin bilayers in the absence and presence of cytochrome *c*. In bilayers both with and without cytochrome *c* the spin-lattice relaxation time (T_1) has the smallest value for the polar headgroup region and increases gradually through the glycerol and into the hydrocarbon chain. T_1 reaches a maximum near position carbon-9 (C-9) of the spin label and decreases again towards the end of the hydrocarbon chain. The effect of the binding of cytochrome *c* to cardiolipin bilayers is mainly that the maximum in the relaxation time near position C-9 is lower than in the absence of cytochrome *c*.

Generally, no dramatic effect on the aminoxyl electron spin-lattice relaxation is observed with the addition of cytochrome

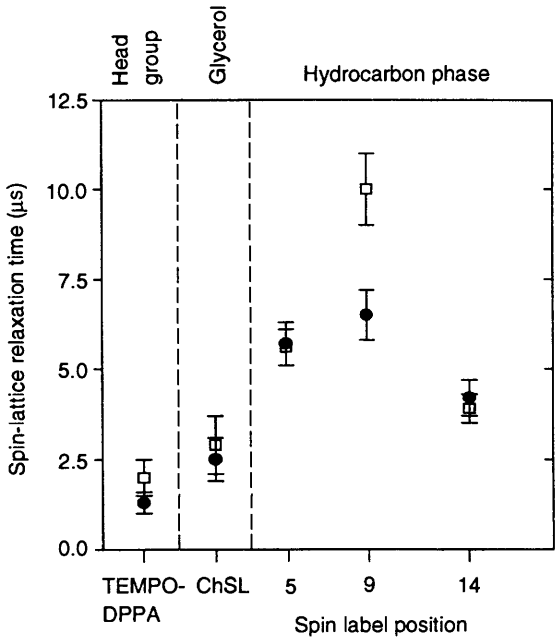

Fig. 4 Spin-lattice relaxation times (T_1) for the aminoxyl spin labels positioned at different depths in bilayers of cardiolipin alone (open symbols), and with bound cytochrome *c* (filled symbols) in the molar ratio range of 15–20 : 1, lipid to protein mole ratio; temperature, 22 °C

c in the molar lipid to protein ratio range of 15–20 : 1. However, an approximately 35 ± 10% enhancement in the electron spin-lattice relaxation for the 9-PCSL was found (Fig. 4). This would be at the region of the unsaturated bonds in cardiolipin bilayers made from bovine heart derived cardiolipin, which contains predominantly linoleoyl (*cis*-18 : 2-$\Delta^{9,12}$) acyl chains.[21] Similarly an enhancement of *ca.* 35% on the spin-lattice relaxation for the nitroxide at the bilayer surface, TEMPO-DPPA, is indicative of some surface perturbation, as observed by others on studies of the selectivity of cytochrome *c* interaction with various anionic phospholipid bilayers.[22,23]

It does not seem surprising in general that the spin-lattice relaxation times are reduced for most spin label positions upon the binding of the *paramagnetic* cytochrome *c* to the spin labelled cardiolipin bilayers. In studies of the phosphorus NMR in cardiolipin bilayers, the phosphorus NMR spin-lattice relaxation time decreased and the phosphorus NMR lines were broadened upon the addition of cytochrome *c*.[10] The results were attributed to the stabilisation of the high-spin form of cytochrome *c* upon binding to cardiolipin so as to cause a rapid interconversion between the low- and high-spin states of the protein. This high-spin form of ferricytochrome *c* has been also reported to occur during haem complexation with small anionic ligands,[24] during complexation in general with anionic surfaces[25–27] including phospholipid bilayers,[28–30] and has been proposed as a labile intermediate involving displacement of the axial ligand methionine 80 to the haem iron.

The interpretation of a paramagnetic effect in the electron spin resonance is not straightforward and it is usually rather complex to analyse.[31] For example, cytochrome P-450 contains a haem iron centre that binds a variety of ligands. Several groups have used spin labelled ligands to examine the spin–spin interaction between the aminoxyl spin label and the iron(III) in the protein.[32,33] These studies are considered a 'case history' which illustrates the increasing sophistication in the interpretation of metal–aminoxyl interactions. While the interactions of cytochrome *c* with the bilayer leads to a 10–100 fold increase in the phosphorus NMR spin-lattice relaxation rates,[10] the effects observed on the electron spin-lattice relaxation in this study are only a few tens of percent changes. Likewise, the interaction of

Table 2 Order parameter S_{eff} for the indicated aminoxyl spin labels in cardiolipin bilayers in the absence and presence of cytochrome *c*. S_{eff} is determined from the anisotropy of the CW EPR spectra

Spin label	Cardiolipin bilayers	Cardiolipin-cyt *c* bilayers
TEMPO-DPPA	~0[a]	~0[a]
ChSL	0.76 ± 0.04	0.76 ± 0.04
5-PCSL	0.59 ± 0.03	0.59 ± 0.03
9-PCSL	0.43 ± 0.02	0.57 ± 0.03
14-PCSL	0.17 ± 0.01	0.17 ± 0.01

[a] Spectrum displays no separate anisotropic features with which an order parameter can be estimated.

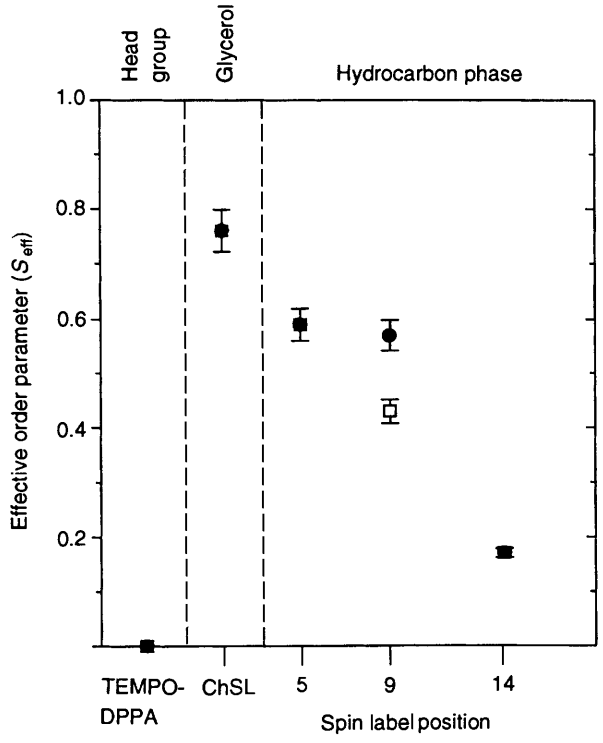

Fig. 5 Effective order parameter (S_{eff}) for the various aminoxyl spin labels at different positions in bilayers of cardiolipin alone (open symbols), and with bound cytochrome *c* (filled symbols) in the molar ratio range of 15–20:1, lipid to protein ratio

dissolved paramagnetic O_2 on spin labels in phosphatidylcholine membranes extrapolated to a comparable concentration would lead to 100–1000 fold increase in the spin-lattice relaxation rates.[2] A possible reason for the lower detection of the paramagnetic effect of cytochrome *c* on the electron spin-lattice relaxation, while strongly observed on the phosphorus NMR T_1 relaxation, may reside in an incompatible time scale sensitivity for the low- to high-spin interconversion process occurring in cytochrome *c* by the former technique. Phosphorus-31 NMR shows correlation times of 0.71 ns in the lipid alone for the process that promotes spin-lattice relaxation of this nucleus.[10] The addition of cytochrome *c* decreases the phosphorus-31 NMR relaxation time and appears to shift the minimum T_1 to higher temperatures (*i.e.*, shorter correlation times). The minimum in X-band EPR spin-lattice relaxation times should be in the neighbourhood of $\tau_c = 1 \times 10^{-11}$ s, whereas in the phosphorus NMR experiments, the T_1 minimum is of the order of nanoseconds.[10] Furthermore, the EPR spectrum of ferricytochrome *c* is ordinarily detected only by reducing the temperature to liquid helium temperatures. Room temperature detection of the EPR signal for the haem in the protein is not possible due to the fast electron spin relaxation of

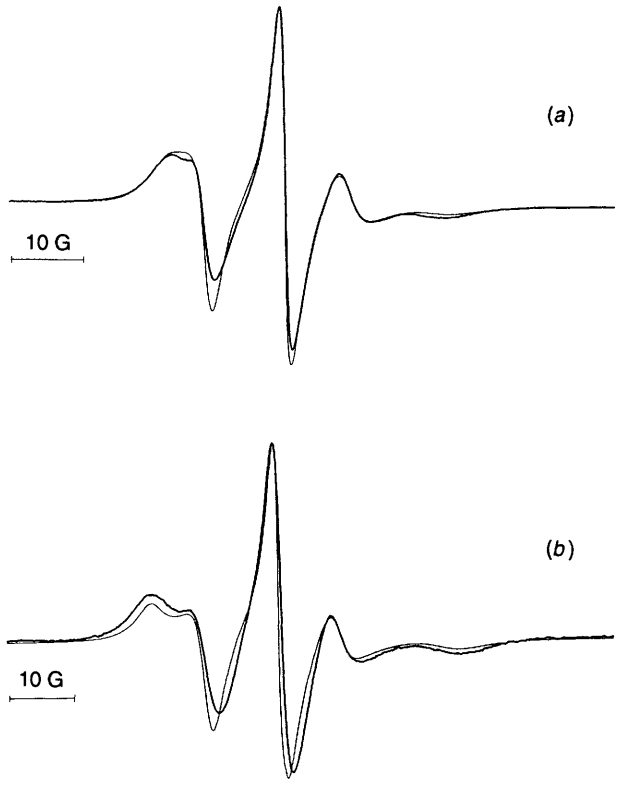

Fig. 6 Experimental (thick line) and calculated (thin line) CW EPR spectra for 9-PCSL in cardiolipin bilayers: (a) in the absence of cytochrome *c*, and (b) in the presence of cytochrome *c*, in the molar ratio 20:1, lipid to protein ratio

iron(III).[31] At those temperatures only a very small amount (~5%) of high-spin Fe^{III} was detected in a cardiolipin–cytochrome *c* complex.[34]

The averaging of the anisotropies of the hyperfine interaction for a series of stearic acid spin labels in lipid bilayers shows that angular amplitudes of motion of the lipid chain segments increase from the headgroup through to the terminal methyl end of the chain.[35] The CW EPR spectra of the same samples in this study show a similar progression both in the presence and absence of cytochrome *c*, as measured by the calculated effective order parameter, S_{eff} (Table 2 and Fig. 5). Thus, the presence of cytochrome *c* does not reorganize the cardiolipin bilayer to the extent that the angular amplitude of motions for the various spin-labelled positions are altered appreciably when compared to the protein-free bilayers. However, the binding of cytochrome *c* to the cardiolipin bilayers does have a specific, measurable effect on the anisotropy of the 9-PCSL spectrum, while it has only a very subtle effect on the other spin labels, as can be seen in Fig. 5. The effect of cytochrome *c* on the 9-PCSL is to *increase* the order parameter by about 10% relative to the 9-PCSL in cardiolipin bilayers alone. Independently, the saturation-recovery pulsed EPR T_1 measurements have shown a decrease in T_1 at this position.

Slow tumbling EPR lineshape analysis using current motional theories[36] indicates an increase in the order parameter (S) from 0.42 to 0.50 on addition of cytochrome *c*, in good agreement with the order parameter calculated from the experimental spectra (S_{eff}). In Fig. 6 are presented the experimental and calculated CW EPR spectra for the 9-PCSL in cardiolipin bilayers with and without cytochrome *c*, and in Table 3 the dynamic parameters determined from those spectral lineshape simulations. The so called 'tilt angle', ψ, which is a measure of the relative inclination between the rotational diffusion axis and the magnetic tensor axis, showed a reduction

Table 3 Dynamic parameters for the 9-PCSL in cardiolipin bilayers in the absence and presence of cytochrome *c*, as determined by the ESR lineshape simulations, assuming the MOMD model [36] and Brownian diffusion

System	$R_{//}/s^{-1}$	$R_{\perp}/s^{-1}$	S	ψ/deg
Cardiolipin bilayers	0.26×10^9	0.20×10^8	0.4193	38
Cardiolipin bilayers plus cyt *c*	0.20×10^9	0.20×10^8	0.4957	34

$R_{//}$ and $R_{\perp}$ are the parallel and perpendicular components of the axially symmetric diffusion tensor; S, the order parameter; and ψ, the diffusion tilt angle.

from 38° to 34° (Table 3) upon binding of the protein. As ψ is an indicator of the molecular amplitude of motions, the observed variation suggests that cytochrome *c* induces a reduction in the amplitude of acyl chains fluctuations at around the unsaturated region in cardiolipin bilayers. As shown in Table 3 the parallel component ($R_{//}$) of the axially symmetric diffusion tensor (the faster component) is reduced in the presence of cytochrome *c*.

Although polyunsaturated lipids are of considerable biological interest, the molecular dynamics around the unsaturated region of acyl chains in lipid–protein complexes is relatively poorly understood. Unsaturated regions of acyl chains have generally been viewed as rigid segments, but recent deuterium NMR studies on a linoeoyl chain incorporated into a hydrated PC bilayer have shown that the polyunsaturated segment undergoes quite unexpected behaviour.[37] Motional fluctuations of large amplitude and an average orientation essentially orthogonal to that expected for methylenes in the unsaturated region of the chain, suggested that the olefinic segment is exchanging rapidly between two conformers. Furthermore, for cytochrome *c* complexes with cardiolipin, the results from carbon-13 NMR cross polarization and relaxation measurements[11] are clearly indicative of an enhanced dynamic state of the olefinic sites upon protein binding.

Specific effects exerted by cytochrome *c* on the fatty acyl chain dynamics in cardiolipin bilayers have been noticed in resonance Raman spectroscopy measurements.[38] It was observed that the protein induces an overall increase in conformational disorder in the lipid chain, with some indication for selective influence on the olefinic chain segment. Our present results are in agreement with the previous studies by various techniques and are a strong indication of localised dynamics in particular sites on the acyl chain in the bilayer. The spin-lattice relaxation times of spin-labels at well defined localised positions in the bilayer have been revealed to be specific for depicting very localised information on segmental dynamics along the aliphatic chain, with particular application for the study of unique modes of lipid–protein interactions.

The changes in the order parameter are indicative of changes in the molecular motional fluctuations of large amplitude, which are in agreement with the observed variation in the tilt angle ψ. Those results suggest that the presence of cytochrome *c* inflicts an overall constraint in the amplitude of chain fluctuations of cardiolipin in the bilayers. On the other hand, the decrease in the electron spin-lattice relaxation time implies faster fluctuations within the acyl chains, which may include a higher extent of *gauche-trans* isomerisation around the olefinic chain segments. This is in agreement with the increase in *conformational disorder* in the lipid chains found by Vincent and Levin (1986) by Raman spectroscopy measurements.[38] We then arrive at this paradoxical conclusion whereby cytochrome *c* restricts the overall amplitude of chain motions in the bilayer, imposing some *macroscopic order*, while simultaneously inducing some *microscopic disorder* by increasing the internal segmental motions within the acyl chains.

Acknowledgements

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