Modulation of cytochrome *c* spin states by lipid acyl chains: a continuous-wave electron paramagnetic resonance (CW-EPR) study of haem iron

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This work is a systematic study, showing a clear correlation between the nature of the lipid acyl chain and the spin states of cytochrome *c* interacting with different types of lipid membranes. According to the lipid acyl chain type, and the head group charge present in the bilayer, three spin states of cytochrome *c* were observed in different proportions: the native cytochrome *c* low spin state with rhombic symmetry (spin 1/2, $g_{\parallel} = 3.07$ and $g_{\perp} = 2.23$), a low spin state with less rhombic symmetry (spin 1/2, $g_1 = 2.902$, $g_2 = 2.225$, and $g_3 = 1.510$) and the high spin state (spin 5/2, $g_{\parallel} = 6.0$ and $g_{\perp} = 2.0$). The proportion of the spin states of cytochrome *c* bound to bilayers was also dependent on the lipid/protein ratio, suggesting the existence of two or more protein sites interacting with the lipids. The lipid-induced alter-

ations in the symmetry and spin states of cytochrome *c* exhibited partial reversibility when the ionic strength was increased, which reinforces the crucial role played by the electrostatic interaction with the lipid bilayer. Different cytochrome *c* spin states exhibited corresponding modifications in the haemprotein UV/visible spectra, particularly in the Q-band associated with loss of the 695 nm band and appearance of a band in the region of 600–650 nm. The observed reactivity of cytochrome *c* with oxidized forms of unsaturated lipids reinforces the possibility of the acyl chain insertion in the haemprotein structure.

Key words: cytochrome *c* conformation, haem iron, liposomes, electron paramagnetic resonance, spin states.

INTRODUCTION

The nature and specificity of the interaction between cytochrome *c* and lipid bilayers has been a major goal in several studies [1–3], but detailed information is still not widely available. In this regard, it is generally accepted that the electrostatic interaction is an important factor in the association of cytochrome *c* with phospholipid membranes; however, the role played by the phospholipid acyl chain is still not well understood. Rytömaa and Kinnunen [4] proposed a model for the interaction between cytochrome *c* and lipid bilayers that has been reinforced by subsequent studies [5]. In this model one phospholipid chain is accommodated in a hydrophobic channel, present in the cytochrome *c* structure in the region of the haem crevice. The authors proposed that this might be a common attachment mechanism for peripheral protein/membranes association.

Recently cytochrome *c* has been the object of great interest, because besides its known role in the respiratory chain, it exhibits the capability to detach from the inner mitochondrial membrane and trigger, in the cytosol, events that promote programmed cell death (apoptosis) [6,7]. These capabilities are related to the topology of cytochrome *c*, since it is the only peripheral protein localized on the external side of the inner mitochondrial membrane [2,8]. The functions exhibited by cytochrome *c* (a haem protein) are related to the redox states of the haem iron, and

involve the crystalline field modulation and changes in the spin states in both Fe(II) and Fe(III) haem iron. We have demonstrated that the association of cytochrome *c* with inner mitochondrial mimetic and negatively charged liposomes, as dicetylphosphate (DCP) bilayers, is modulated by the redox states of the haem protein [9]. Ferrous cytochrome *c* exhibits strong modifications when associated with both membrane types, whereas ferric cytochrome *c* is modified discretely by these liposome types. The association of cytochrome *c* with the negative membranes induces a change in the spin state of the haem iron. The interaction between the charged vesicles and cytochrome *c* leads to structural changes in the protein, pulling out the methionine sulphur atom co-ordinated with the haem iron. The changing of the crystalline field of the haem iron from strong to weak is accompanied by spin state changes from low to high spin, respectively [9]. These facts influenced us to investigate more systematically the nature of the interaction between cytochrome *c* and several different phospholipid chains. The lipid-induced effects in the haem iron crystalline field correlate with the nature of the charged head group and the size and type of the phospholipid chain. This study investigated the lipid-induced changes of haem iron spin states, and symmetry of ferric cytochrome *c*, using continuous-wave electron paramagnetic resonance (CW-EPR) and electronic absorption techniques. The following lipids were used: egg yolk phos-

Abbreviations used: CL, bovine heart cardiolipin; CW-EPR, continuous wave-EPR; DBPC, dibutylphosphatidylcholine; DCP, dicetylphosphate; DMPG, dimyristoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine; DDPC, didecanoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; DSPG, distearoylphosphatidylglycerol; EPR, electron paramagnetic resonance; l/p, lipid/protein; PC, egg yolk phosphatidylcholine; PCPECL, phosphatidylcholine/phosphatidylethanolamine/cardiolipin in molar ratio (2:2:1); PE, phosphatidylethanolamine; t-BuOOH, t-butyl hydroperoxide; UV/VIS, UV/visible.

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phatidylethanolamine (PE), egg yolk phosphatidylcholine (PC), bovine heart cardiolipin (CL), PCPECL (PC, PE and CL in molar ratio 2:2:1), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), dioleoylphosphatidylserine (DOPS), dicetylphosphate (DCP), distearoylphosphatidylglycerol (DSPG), dimyristoylphosphatidylglycerol (DMPG), dibutylphosphatidylcholine (DBPC) and didecanoylphosphatidylcholine (DDPC). For the first time a systematic study shows a clear correlation between the nature of the lipid acyl chain and the cytochrome *c* spin states, corroborating the proposed lipid extended interaction [4,5].

EXPERIMENTAL

Chemicals

Cytochrome *c* (horse heart, type III), Hepes, PE, PC, CL, PCPECL, DPPC, DPPS, DOPS, DCP, DSPG, DMPG, DBPC and DDPC were purchased from Sigma.

Vesicle preparation

In preparation of PE, PC, CL, DPPC, DPPS, DOPS, DSPG, DMPG, DBPC and DDPC liposomes, the lipids were first dissolved in chloroform, and the chloroform evaporated with N_2 gas. Cold Hepes buffer was added to the tube, mixed with a vortex, and sonicated with a microtip-equipped Thornton ultrasonic processor at an output of 60 W on ice in 30 s bursts with 1 min cooling intervals. DCP vesicles were prepared in Hepes buffer according to the method of Mortara et al. [10]. DCP was suspended in Hepes buffer, heated to 70 °C until complete dissolution, and sonicated for 20 min at an output of 60 W.

Electronic absorption spectrometry

Electronic absorption measurements of cytochrome *c* (80 μ M) were conducted in a photodiode spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, U.S.A.), using quartz cuvettes of 1 cm light path and a slit of 0.5 nm.

Electron paramagnetic resonance (EPR) spectrometry

Direct EPR measurements of cytochrome *c* (330 μ M) were carried out using an EPR Bruker (Oxford, U.K.) system ELEXSYS E-580 model, under the following conditions: gain 5×10^3 , modulation amplitude 1.0 mT (for haem iron), microwave power 4 mW, temperature 11 K, time constant 1.28 ms, and conversion time 81.92 ms.

In each medium the cytochrome *c*-containing mixture was quickly introduced into an EPR quartz tube and cooled in liquid nitrogen. The sample was frozen and then introduced into the microwave cavity, at 11 K, before the EPR measurements were taken. The EPR spectra were simulated using the SiMfonia Bruker program and the area of each paramagnetic species was calculated via numerical double integration of the spectra.

RESULTS

Three different haem iron spin states of cytochrome *c* associated with lipid bilayers

We have observed previously that the association of cytochrome *c* with DCP vesicles, a negatively charged phospholipid bilayer, causes a change in the haem iron spin state from the native low spin form with rhombic symmetry (spin 1/2, $g_{\perp} = 3.07$ and

$g_{\parallel} = 2.23$) to a low spin form with less rhombic symmetry ($g_1 = 2.902$, $g_2 = 2.225$ and $g_3 = 1.510$), named herein as 'alternative low spin states', and to a high spin form ($g_{\perp} = 6.0$ and $g_{\parallel} = 2.0$) [9]. The EPR parameters for these three spin states, obtained from the simulation of the EPR spectra, are shown in Table 1.

In order to characterize the influence of the lipid/protein (l/p) ratio in the occurrence of this species, cytochrome *c* was titred with DCP vesicles. Figure 1 shows the EPR spectra of cytochrome *c* in the presence of DCP ($C_{16:0}$) liposomes in several l/p ratios. In the lower l/p ratio, the 'alternative low spin species' was predominant. This species was gradually substituted with the high spin states by increasing the l/p ratio.

The effect of DCP vesicles on the haem iron spin states of cytochrome *c* was compared with that produced by another negatively charged bilayer, CL liposomes. Figure 2 shows the EPR spectra from the titration of cytochrome *c* with CL vesicles in several l/p ratios. In this case, the increase of the l/p ratio was accompanied predominantly by the conversion of the native cytochrome *c* (spin = 1/2) to the 'alternative low spin species'. In the magnetic field region at 110 mT ($g \sim 6$) a very small amount of high spin form was also detected. In higher l/p ratios, simultaneous with the appearance of a lipid-derived radical at approx. 330 mT, a small amount of high spin form with rhombic symmetry ($g = 4.3$, magnetic field at 155 mT) could be detected. This cytochrome *c* high spin rhombic form was previously detected during the reaction of cytochrome *c* with t-BuOOH (t-butyl hydroperoxide), both in homogeneous medium and in the presence of different liposomes [11]. The cytochrome *c* high spin form ($g = 4.3$) resulted from the attack of CL-derived free radicals to the haem structure leading to the concomitant Soret band bleaching [11]. The upper panel in Figure 2 shows the CL-derived free radical spectrum and its simulation with $g_1 = 2.004$, $g_2 = 2.008$, $g_3 = 2.014$ [12,13]. This free radical spectrum is different from that detected during the reaction of t-BuOOH with cytochrome *c* [11]. The results obtained in the presence of CL liposomes are in agreement with the proposed lipid acyl chain insertion in the cytochrome *c* structure attaining the region of the haem crevice. The accessibility of the lipid acyl chain to the haem crevice was tested through the reaction of cytochrome *c* with a lipid acyl chain-derived peroxide (CL-derived peroxide), depicted in Figure 3. This Figure shows that, both in the course of the reaction with t-BuOOH and CL-derived peroxide, the same cytochrome *c* high spin rhombic form ($g = 4.3$) was detected, but different free radicals (high field region) were produced.

Effect of different lipid bilayers and l/p ratios on the proportion of the spin states of cytochrome *c* haem iron

The simulation of the cytochrome *c* EPR spectra, considering the contribution of a Lorentzian and/or a Gaussian line shape, was generated, quantifying the different proportions of cytochrome *c* spin states induced by different lipids (DCP and CL bilayers). Each spin state was quantified by double integration (see Experimental procedures). Figures 4(A) and 4(B) show the gradual conversion of the cytochrome *c* native low spin states to the others species, due the association of the protein with DCP and CL bilayers, in different l/p ratios. Figure 4(A) displays the proportion of cytochrome *c* spin states at each DCP/protein ratio, according to the area obtained from the simulated spectrum of the different species. When the l/p is 12 the predominant cytochrome *c* species present was the native low spin form and the main modified species produced by the association was the 'alternative low spin species'. The low spin species of cytochrome *c* decreased and the high spin form became predominant

Table 1 EPR parameters obtained from the spectral simulation for different l/p samples

The following parameters are shown: g_x , g_y , and g_z ; linewidths, ΔH_x , ΔH_y , and ΔH_z ; and the line shape, Gaussian (G), or Lorentzian (L).

Lipid	Spin states	Line shape	g_x	g_y	g_z	ΔH_x	ΔH_y	ΔH_z
DPPC	5/2	L	5.900	5.900	2.002	95	95	10
	5/2	G	6.500	6.500	2.002	90	90	10
DMPG	5/2	L	5.920	5.920	2.002	64	64	10
	5/2	G	6.600	5.400	2.002	110	110	10
DPPS	5/2	L	5.900	5.900	2.002	65	65	10
	5/2	G	6.550	5.400	2.002	130	130	10
DSPG	5/2	L	5.900	5.900	2.002	65	65	10
	5/2	G	6.550	5.400	2.002	130	130	10
DCP	5/2	L	6.050	5.780	2.000	45	45	10
CL	5/2	L	6.000	5.950	2.000	70	70	10
PS	5/2	L	6.050	5.830	2.000	21	25	10
PCPECL	5/2	L	6.000	5.950	2.000	90	90	90
Alternative Low spin species	1/2	L/G = 0.5	2.968	2.275	1.510	73	73	200
Free cytochrome <i>c</i>	1/2	G	3.085	2.234	1.250	110	150	400

Figure 1 EPR spectra of cytochrome *c* in the presence of DCP liposomes in several l/p ratios

Spectrometer conditions were: gain, 50 db; modulation amplitude, 1.0 mT; microwave power, 16 mW; microwave frequency, 9.48 GHz; time constant, 1.28 ms; conversion time, 81.92 ms; temperature, 11 K. l/p ratios were as indicated above the line traces.

upon increasing the l/p ratio. In the presence of CL liposomes, increasing the l/p ratio led to a predominance of the 'alternative low spin state' (Figure 4B). These results indicate that, besides the lipid charge, the head-group structure and the acyl-chain type are also important factors in the lipid-induced cytochrome *c*

structural modifications. At this point, it was important to characterize the effect of variations on the lipid acyl chain. Figures 4(C) and 4(D) show, respectively, the proportions of cytochrome *c* spin states detected in the presence of DMPG ($C_{14:0}$) and DSPG ($C_{18:0}$) vesicles at several l/p ratios. In both conditions, the same species were detected, but with opposite proportions for the high and the 'alternative low spin species', suggesting that increasing the lipid acyl chain favours the former modified cytochrome *c* species. The effect of unsaturated lipid acyl chains was determined through the proportions of cytochrome *c* spin species obtained in the presence of DPPS ($C_{16:0}$) and DOPS ($C_{18:1}$) at different l/p ratios (Figures 4E and 4F). Similar to the results obtained with cardiolipin (80% linoleic acid content), unsaturated PS (DOPS) favoured the appearance of the 'alternative low spin species' which, in this case, was the only modified species detected.

Rytömma and Kinnunen [4] and Tuominen et al. [5] proposed two sites in cytochrome *c* able to interact with the phospholipid head groups. In the so-called 'A-site', electrostatic interactions occur with Lys-73 and Lys-72 residues while the 'C-site' involves the formation of hydrogen-bonds with the Asn-52 residue. This model suggests that the previous electrostatic interaction is an important factor to modulate the subsequent hydrophobic interaction. In order to check this possibility, we tested the effect of three zwitterionic long-chain lipids: DPPC ($C_{16:0}$), unsaturated egg yolk PC with 16–20-C and egg yolk PE with 16–20-C chains. The experiments were extended to two zwitterionic short chain lipids: dibutyl PC ($C_{4:0}$) and didecanoyl PC ($C_{10:0}$). In all these cases, no modified cytochrome *c* spin state was detected by EPR (spectra not shown). Table 2 summarizes the different lipid-induced cytochrome *c* spin states induced by different charged head groups and acyl chain types.

The reversibility of the lipid-induced cytochrome *c* spin states

Figures 5(A), 5(B) and 5(C) show that the lipid-induced (CL, DCP and DMPG respectively) alternative low and high spin species, were partially reversible in the presence of a large amount of NaCl in the medium. This suggested that one cytochrome *c* site for lipid binding does not encompass electrostatic interaction between the lipid head group and the protein, and, therefore, is not sensitive to the increase in ionic strength.

Figure 2 EPR spectra of cytochrome *c* haem iron in the presence of CL at different l/p ratios

Spectrometer conditions (lower panel) were: gain, 50 db; modulation amplitude, 1.0 mT; microwave power, 4 mW; microwave frequency, 9.48 GHz; time constant, 1.28 ms; conversion time, 81.92 ms; temperature, 11 K. L/p ratios were as indicated above the line traces. The upper panel shows the direct EPR spectrum of a cardiolipin-derived radical formed during the reaction with cytochrome *c*. The solid line is the experimental spectrum and the open circle symbols (○) represents a simulated spectrum with a Gaussian line shape [$g_1 = 2.004$, $g_2 = 2.008$, $g_3 = 2.014$, and line width of 25.0 MHz (≈ 0.9 mT), using a microwave frequency of 9264.08 MHz].

The $g = 4.3$ species detected in the presence of CL liposomes was not reversible, indicating chemical modifications in the protein produced by its reaction with the CL-derived peroxide.

Electronic absorption of different spin states of cytochrome *c* induced by pH and interaction with lipid bilayers

Previously we have observed that the cytochrome *c* spin states conversions were accompanied by alterations in the UV/VIS spectra in the Soret, visible and charge transfer 695 nm band regions [9,11,14]. We compared the cytochrome *c* visible absorbance spectra obtained at different pH levels with those obtained in the presence of DCP and CL liposomes (Figure 6). In the

Figure 3 EPR spectra of cytochrome *c* haem iron in the presence of (a) Hepes buffer, (b) cardiolipin vesicles, (c) oxidized cardiolipin vesicles and (d) t-BuOOH

Spectrometer conditions were: gain, 50 db; modulation amplitude, 1.0 mT; microwave power, 4 mW; microwave frequency, 9.48 GHz; time constant, 1.28 ms; conversion time, 81.92 ms; temperature, 11 K.

visible region (480–580 nm), only at pH 11.0 was the cytochrome *c* spectrum exhibited similar to that obtained at pH 7.4. Interestingly at pH 11.0 and pH 7.4 cytochrome *c* was equally in the native low spin states (results not shown), whereas under all the other conditions cytochrome *c* exhibited changes in the native spin state. Significant differences were observed between these cytochrome *c* spin species in the region from 600 to 750 nm. In all induced different spin states and symmetry of cytochrome *c* (high and 'alternative low spin species'), the loss of the charge transfer band (695 nm) indicates the disruption or weakening of the methionine sulphur–haem iron coordination. At acidic pH, cytochrome *c* exhibited a 620 nm band that was attributed to the loss of the sixth ligand from the protein structure, which was probably replaced by water. In this region a difference can be observed between cytochrome *c* associated to DCP and CL vesicles. Cardiolipin vesicles induced the appearance of a shoulder in the 600–620 nm region, while DCP vesicles led to the appearance of a discrete and red-shifted shoulder. The determination of the spectral characteristics of

Figure 4 Proportion of cytochrome *c* spin states in each DCP/protein ratio, according to the area obtained from the simulated spectrum of the different species

Normalized areas obtained by numerical double integration from the spectral simulation of the EPR spectra of cytochrome *c* associated with different lipid vesicles: (A) DCP, (B) CL, (C) DMPG, (D) DSPG, (E) DPPS, and (F) DOPS as indicated in the Figures, using the SiMfonja Bruker Program. White bars, low spin states; black bars, high spin states; grey bars, alternative low spin state.

Table 2 Cytochrome *c* spin-state species associated with different lipid bilayers

Lipid	Charge of head	Carbons bound	Size of acyl chain	Spin states of EPR spectra
PE	+	unsaturated	C ₁₆ –C ₂₀	low spin*
PC	+	unsaturated	C ₁₆ –C ₂₀	low spin*
CL	–	unsaturated (80% linoleic acid)	C ₁₈ (80%)	low spin and alternative low spin form
PS	+	unsaturated	C ₁₆ –C ₂₀	low spin and high spin
DPPC	+	saturated	C ₁₆	low spin and high spin
DPPS	+	saturated	C ₁₆	low spin and high spin
DOPS	+	saturated	C ₁₈	low spin and alternative low spin form
DCP	–	saturated	C ₁₆	low spin, alternative low spin form and high spin
DSPG	–	saturated	C ₁₈	low spin and high spin
DMPG	–	saturated	C ₁₄	low spin, alternative low spin form and high spin
DDPC	+	saturated	C ₁₀	low spin*
DBPC	+	saturated	C ₄	low spin*

* Low spin is the characteristic spin state of ferric cytochrome *c* Hepes buffer solution.

each cytochrome *c* species, especially the alternative low spin states, is a task that is currently under investigation in our laboratory.

DISCUSSION

The association of cytochrome *c* with lipid bilayers produces reversible alterations in the haem iron spin states which depend of the l/p ratio

The results depicted in Figures 1, 2, and 4 clearly indicate that the association of cytochrome *c* with lipid bilayers is a specific process for the lipid structure, which, in turn, depends on the l/p ratio. This structural specificity is attested by the fact that negatively charged interfaces, obtained with structurally different lipids, do not lead to the same types and/or proportions of cytochrome *c* spin states. In this regard the results obtained in the presence of DCP liposomes are particularly interesting (Figure 4A). In this case two cytochrome *c* modified spin species are produced, the high spin species ($g_{\perp} = 6$ and $g_{\parallel} = 2.0$) and the 'alternative low spin' species ($g_1 = 2.902$, $g_2 = 2.225$ and $g_3 = 1.510$), but the proportion of this species varies according to the l/p ratio. In low l/p ratios the interaction of several cytochrome *c* molecules per DCP vesicles has probably favoured

Figure 5 EPR spectra of cytochrome *c* haem iron in the presence of (A) CL, (B) DCP, and (C) DMPG vesicles

Spectra were obtained in the absence of NaCl (solid line) and in the presence of high ionic strength (NaCl) (dashed line). Spectrometer conditions were: gain, 50 db; modulation amplitude, 1.0 mT; microwave power, 4 mW; microwave frequency, 9.48 GHz; time constant, 1.28 ms; conversion time, 81.92 ms; temperature, 11 K.

the appearance of the alternative low spin species. With the increment of the vesicle number in the medium (high l/p ratios), it is likely that the opposite occurred and one cytochrome *c* molecule could bind to more than one phospholipid vesicle. This phenomenon implies that the hydrophobic interaction occurred in at least two sites of the cytochrome *c* structure. The occurrence of a lipid-induced modified species of cytochrome *c*,

that is still in a low spin form, suggests structural modifications in cytochrome *c* that maintain the haem iron sixth coordination with a strong field ligand. The loss of the haem iron sixth ligand, or its replacement by a weak field ligand, unavoidably leads to the appearance of a haem iron high spin form because it decreases the *d*-orbital splitting. Examples of these phenomena, characterized by EPR techniques, are the classical alkaline transition in ferri-haemoglobins and ferri-myoglobins [15], and microperoxidases, products of the tryptical digestion of cytochrome *c*, which exhibit the haem iron (spin 5/2) without the sixth ligand compared with native cytochrome *c* (spin 1/2). On the other hand, the loss of the coordination with Met-80, that occurs with cytochrome *c* at pH > 9.5, does not lead to the appearance of the high spin form because a lysine or histidine residue is set as the new sixth ligand [16]. So it is reasonable to suppose that two types of DCP interaction with cytochrome *c*, whose difference probably resides in the degree of the named lipid-extend interaction and/or in the protein sites where this interaction takes place [4,5]. In low l/p ratio, the insertion of a DCP lipid chain is probably not enough to completely disrupt the coordination of haem iron with the Met-80, or to promote its replacement by a new strong field ligand, and this results in the appearance of a low spin form with different symmetry. The sixth ligand of cytochrome *c* haem iron in the alternative low spin species has not been identified yet and is under investigation in our laboratory. These events are evidence of the importance of the hydrophobic or lipid extended interaction in the lipid-induced modulation of cytochrome *c* spin states.

The existence of the high spin form (spin 5/2) in vesicle-bound cytochrome *c* has been attributed to protein denaturation [17]. However, we observed that both lipid-induced high (DCP and DMPG) and alternative low spin species (CL, DCP and DMPG) partially reverted to the native low spin form after the addition of high concentrations of NaCl (Figures 5A, 5B, and 5C). The noncomplete reversibility of the spin states of cytochrome *c*, in the presence of high ionic strength, might be due to the existence of hydrophobic interactions that occur independently of the electrostatic ones. Probably, there is more than one population of lipid-bound cytochrome *c* and two or more sites for lipid binding in the protein. This result also suggests that the lipid binding in different sites of the protein can induce changes in the spin states of cytochrome *c*.

The lipid-induced modifications in the symmetry and the spin states of cytochrome *c* are responsive to the type of lipid acyl chain

The fact that different proportions of cytochrome *c* spin states were observed only by varying the lipid acyl chain, more specifically the saturation degree and acyl chain length (Figures 4C, 4D, 4E, and 4F), provided important evidence for the lipid-extended interaction of the phospholipids with cytochrome *c*. Although both saturated and unsaturated acyl chains can be accommodated in the cytochrome *c* hydrophobic channel, different effects are produced by such interactions. The insertion of each different type of lipid acyl chain, favoured by previous structural modifications, might promote different structural effects in both haem iron crevice and geometry. This behaviour results in the different proportions of cytochrome *c* spin states observed after its association with different lipid types. In this case, a good example is provided by the comparison between the spin state proportions exhibited by cytochrome *c* associated with DPPS (C_{16:0}; Figure 4E) and DOPS (C_{18:1}; Figure 4F) vesicles. Both lipid types exhibit the same negative polar head group, but in the presence of DOPS, where unsaturated acyl chain is

present, only the 'alternative low spin species' is observed. In this regard, the profile for the distribution of cytochrome *c* spin states, observed with DSPG ($C_{18:0}$; Figure 4D) vesicles is quite similar to that exhibited with DPPS (Figure 4E). Similar results are obtained by varying the acyl chain length (Figures 4C and 4D), since the enhancement of the acyl chain length increases the content of high spin species of cytochrome *c*, to the detriment of the low spin species content.

The appearance of the cytochrome *c* 'alternative low spin species', because of the presence of unsaturated or shorter acyl chains, suggests that this species is favoured when the lipid insertion in the cytochrome *c* structure is restricted but not totally prevented. In the case of the unsaturated lipids, the reason for the reduction in lipid insertion might be the steric hindrance caused by the 30° rigid bend in the acyl chain that prevents its accommodation in the cytochrome *c* hydrophobic channel.

The previous electrostatic interaction of cytochrome *c* with acidic phospholipids is crucial to the subsequent occurrence of the lipid-extended interaction

The presence of a negative charge in the phospholipid head group promotes structural modifications in the secondary structure of cytochrome *c* with alteration of its α -helix content [9]. These structural modifications probably lead to the opening of the cytochrome *c* hydrophobic channel, making the hydrophobic interaction with both saturated and unsaturated hydrocarbon chains possible. In this regard, the zwitterionic lipids DBPC ($C_{4:0}$), DDPC ($C_{10:0}$), DPPC ($C_{16:0}$), unsaturated egg yolk PC and PE do not cause any changes in the haem iron EPR and electronic absorbance spectra (results not shown). In the presence of zwitterionic long chain lipids the hydrophobic interaction could also be present, but the structural alterations induced in the protein would not be enough to promote alterations in the cytochrome *c* spin states.

These results suggest that the lipid head group net charge and acyl chain type, cooperatively influence the lipid-extended interaction, which modulates the haem iron spin states. Besides the charge, the structure of the head group is another important factor in the lipid-induced cytochrome *c* spin states. This is well exemplified by the comparison of the results obtained with DPPS (Figure 4E) and DCP liposomes (Figure 4A). When compared with DPPS, in high *l/p* ratios, DCP liposomes favour the conversion of cytochrome *c* to the high spin states. Probably the small size of the negative head group, present in DCP bilayers, allows a stronger electrostatic interaction with the lysine residues localized in the opening of the cytochrome *c* hydrophobic channel, as suggested by Rytömaa and Kinnunen [4].

The reactivity of cytochrome *c* with unsaturated lipid acyl chain reinforces the proposed model of the lipid-extended interaction

We have previously demonstrated that conformational and spin state changes, induced in cytochrome *c* by the interaction with negatively charged bilayers, favour the reactivity of cytochrome *c* with carbonyl compounds and peroxides [9,11,14]. This suggests that the electrostatic and hydrophobic interactions lead to the opening of the cytochrome *c* hydrophobic channel and improve the access to the haem group for the carbonyl compounds [14] and peroxides [11]. We have also observed that the association of cytochrome *c* with cardiolipin vesicles containing a fraction of peroxidized lipid is accompanied by the reaction of the haem-protein with the acyl chain-derived peroxide (Figure 3). The $g = 4.3$ species detected after the reaction is the same as that observed in

Figure 6 Absorbance spectra of 80 μ M cytochrome *c* in different media

The spectra were obtained in: 10 mM phosphate buffer at pH 7.4 (thin solid line), 10 mM phosphate buffer at pH 1.5 (thick solid line), 6 mM CL liposomes in Hepes at pH 7.4 (dash-dotted line), 6 mM DCP liposomes in Hepes buffer at pH 7.4 (dashed line), and 10 mM phosphate buffer at pH 11.5 (dotted line).

the reaction of cytochrome *c* with *t*-BuOOH [11]. In the reaction of CL-derived peroxide, the radical species detected is different from the peroxy radical formed in the reaction with *t*-BuOOH, since the average g_n value ($(g_x + g_y + g_z)/3$) is 2.009 instead of 2.014 and the signal is power saturated (results not shown). The possibility that there are several signals, with the peroxy being one component, was discarded because a simulated EPR spectrum exhibits a good fit with the experimental EPR spectrum (Figure 2, top panel). Another possibility is the formation of a cytochrome *c*-centred free radical. In this regard, Barr et al. [18] identified the formation of four radical sites during the reaction of cytochrome *c* with hydrogen peroxide. However, these cytochrome *c*-centred radicals have only been detected with spin trapping 3,5-dibromo-4-nitrosobenzenesulphonic acid and 2-methyl-2-nitrosopropane [18,19] and have not been detected in other pro-oxidant conditions such as during the reaction of cytochrome *c* with peroxides [11], aldehydes (results not shown) and singlet oxygen (results not shown). Therefore this radical is probably derived from the lipid acyl chain and its nature will be investigated in the future.

The alterations in the spin states of cytochrome *c* haem iron are accompanied by alterations in the UV/VIS spectra

The results presented in Figure 6 suggest that both high spin and 'alternative low spin states' lead to concomitant alterations in the UV/VIS cytochrome *c* spectra in the Soret band region. It is important to consider that the UV/VIS measurements were made in different conditions of temperature, total lipid, and protein concentrations, with relative concentrations. In fact there is the possibility that the temperature can influence the spin states equilibrium of the cytochrome *c* haem iron. However it is important to notice that: (i) The UV/VIS spectra of cytochrome *c* obtained at 25 °C and the EPR spectra obtained at 11 K are both indicative of the same spin states at each pH value (results not shown). (ii) 'Alternative low spin species' of cytochrome *c* are produced in distinct percentages by different lipid compositions, as shown by EPR measurements obtained at the same temperature. (iii) The reversibility of the lipid-induced spin states

of cytochrome *c*, previously detected by magnetic circular dichroism measurements [9] was reproduced by EPR measurements (Figure 6). Hence it is possible to propose that the conversion of cytochrome *c* from the native low to the high spin states is accompanied by the described alterations in the UV/VIS spectra.

Conclusions

The results presented here are in accordance with several previous studies that propose two types of interaction of cytochrome *c* with phospholipids: the electrostatic interaction and a specific hydrophobic interaction that involves the insertion of the lipid acyl chain in a hydrophobic channel present in the cytochrome *c* structure. The results are also in accordance with the existence of two or more sites of cytochrome *c* structure that are prone to interact with membranes. The importance of this specific lipid/cytochrome *c* interaction is the possibility of modulating the cytochrome *c* conformation, its spin states and consequently its reactivity, according to the lipid type available to the association. The cytochrome *c* spin states play a role in all known biological functions of this protein, all of them related to its redox potential and association with lipid bilayers [8,11,20]. The existing literature data have suggested that the effectiveness of cytochrome *c* to induce cell apoptosis is dependent on its redox potential and structure, since apocytochrome *c* and other haem proteins are unable to trigger this cell event [6,7]. Equally the association of cytochrome *c* with biological membranes has emerged as another possibly important factor for the efficacy of this protein to trigger programmed cell death. In this regard, it is worthy of note that phosphatidylserine exposure is an early event in the apoptotic pathways [21], following the propagation and amplification of the apoptotic signals, such as cytochrome *c* release by mitochondria [22–24].

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