Lipid Specificity in the Interaction of Cytochrome *c* with Anionic Phospholipid Bilayers Revealed by Solid-State ^{31}P NMR†

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ABSTRACT: Phosphorus-31 NMR has been used to investigate the interaction of cytochrome *c* with bilayers of the anionic lipids dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylserine (DOPS), and diacylphosphatidylinositol (diacylPI). All ^{31}P NMR spectra revealed the typical line shapes characteristic of phospholipids in liquid-crystalline bilayers. The effects on the ^{31}P chemical shift anisotropy (CSA) for each system reflect particular modes of phospholipid headgroup interaction with cytochrome *c*. A distinct increase in the CSA for DOPS bilayers was observed upon binding of cytochrome *c*, which is likely to arise from a partial restriction of the amplitude of motion on this phospholipid headgroup. ^{31}P NMR spin–lattice (T_1) relaxation times of the various phospholipid–cytochrome *c* complexes show that conformational changes occur in the protein on binding to anionic phospholipids. These protein conformational changes are observed through paramagnetic enhancement of the measured ^{31}P spin–lattice relaxation times for lipid phosphates. However, the ^{31}P T_1 values for the various complexes with cytochrome *c* show a different temperature dependence for each lipid, revealing different modes of protein interaction for each of the different lipid headgroups. The phosphate of DOPS was most efficiently relaxed by cytochrome *c*, while the relaxation of the phosphate in the PI headgroup was not affected. The relaxation profile for DOPG-bound cytochrome *c* shows a more complex behavior, where the lipid phosphorus relaxation is strongly enhanced above 15 °C, but not significantly affected at lower temperatures. It was found that the enhancement of lipid phosphorus relaxation is a result of the conformational changes in the protein, in which the heme becomes accessible to lipid phosphate upon binding to charged bilayer surfaces. These conclusions from the NMR experiments are supported by differential scanning calorimetry results, which indicate that the binding of cytochrome *c* to the anionic phospholipid bilayers results in a loosening and/or destabilization of the overall protein structure. DOPS- and diacylPI-bound cytochrome *c* shows the larger destabilization of the protein structure as revealed by the poor cooperativity of its thermally induced denaturation, while in the DOPG–cytochrome *c* complexes the protein seems to preserve a more nativelike structure.

Detailed molecular information about the interaction of peripheral proteins with membrane surfaces is scarce. In particular, rather little is known about any detailed structural changes, in either protein or lipid, which may precede or follow the binding of a protein to the membrane surface (Watts, 1987; Watts & van Gorkom, 1991). It is generally assumed that the major stabilizing force in such binding is charge–charge interactions involving charged lipid headgroups at the membrane surface and charges on the protein. For example, cytochrome *c*, a highly basic protein (~12 kDa) involved in the electron-transfer chain at the inner mitochondrial membrane, interacts strongly with negatively charged phospholipids (Rietveld *et al.*, 1983; Devaux *et al.*, 1986; Waltham *et al.*, 1986), particularly with cardiolipin bilayers (Kimbelberg & Lee, 1970; de Kruijff & Cullis, 1980; Demel *et al.*, 1989; Spooner & Watts, 1991a,b, 1992). Cardiolipin is the major negatively charged phospholipid in the inner mitochondrial membrane, and a specific type of interaction with this lipid and cytochrome *c* has been predicted (de Kruijff & Cullis, 1980; Demel *et al.*, 1989). There is evidence not only that the binding of cytochrome *c* to negatively charged phospholipids involves electrostatic interactions with the surface lysines of the protein but also that cytochrome *c* can penetrate, at least partially, into the bilayer to interact hydrophobically with the membrane interior (Brown & Wüthrich, 1977; Szebini & Tollin, 1988; Spooner & Watts, 1991a).

^{31}P nuclear magnetic resonance (NMR)[1] spectroscopy has been used to investigate the structural and dynamic characteristics of hydrated phospholipid bilayers containing bound cytochrome *c* (Rietveld *et al.*, 1983; de Kruijff & Cullis, 1980; Waltham *et al.*, 1986). Resonance Raman spectroscopy on complexes of cytochrome *c* with anionic charged surfaces (Hildebrandt & Stockburger, 1986, 1989a,b), including anionic lipid dispersions (Hildebrandt & Stockburger, 1990; Heimburg *et al.*, 1991), has indicated that a high-spin form of cytochrome *c* can be formed upon complexation. This high-spin form of cytochrome *c* has been monitored by an enhanced ^{31}P spin–lattice (T_1) relaxation of lipid phosphates in cardiolipin–cytochrome *c* complexes (Spooner & Watts, 1991b), which has been interpreted as an indication of conformational changes induced in the protein by the lipid, whereby the high-spin intermediate form of the heme group in cytochrome *c* is formed by disruption of the sixth coordination ligand (Met-80) to the heme iron.

In the present work, we have investigated the lipid specificity in the interaction of cytochrome *c* with anionic phospholipid bilayers using ^{31}P NMR. Through the evaluation of the effects

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[1] Abbreviations: CSA, chemical shift anisotropy; cyt *c*, cytochrome *c*; DMPI, dimyristoylphosphatidylinositol; DOPG, dioleoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; PI, phosphatidylinositol; T_1, spin–lattice relaxation time.

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on phosphorus-31 spin–lattice (T_1) relaxation and chemical shift anisotropy (CSA) of the lipid phosphates in cytochrome *c* complexes with DOPG, DOPS, and diacylPI, information about conformational changes in cytochrome *c* induced upon lipid complexation has been obtained. The extent of lipid-induced perturbations in cytochrome *c* was found to be lipid-dependent and indicative of different modes of lipid–protein interaction for the various lipids studied here. The effects on the structure and dynamics of the phospholipid headgroups on protein complexation have been also analyzed. The results of this study may reveal a general role for lipids in membrane activation of peripheral proteins, such as that found for protein kinase C by diacylglycerols (Nishizuka, 1984, 1986) or for phospholipase A_2 by free fatty acids (Bell & Biltonen, 1989; Jain & Berg, 1989).

MATERIALS AND METHODS

Dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylserine (DOPS) were purchased from Sigma Chemical Co., St. Louis, MO, and used without further purification. Wheat germ diacylphosphatidylinositol (diacylPI) was acquired from Lipid Products, Redhill, Surrey, U.K., its acyl chain composition being 53.9%, 16:0, 37.6%, 18.2: 4.8%, 18:1; and 2.7%, 18:3. Cytochrome *c* from horse heart, type VI, Sigma Chemical Co., containing a variety of deaminated forms of the protein, was purified by ion-exchange chromatography on Whatman CM-32 and eluted with 65 mM phosphate buffer, pH 7.0 (Brautigan *et al.*, 1978). The eluent containing the purified protein was concentrated by ultrafiltration using Amicon YM-5 ultrafiltration membranes, followed by extensive dialysis against cold (4 °C) distilled water to remove phosphate. Aqueous concentrations of cytochrome *c* were measured spectrophotometrically using a molar absorptivity of 2.95×10^4 M^{-1} cm^{-1} at 550 nm at pH 7.0 for the protein reduced with sodium dithionite (Margoliash & Walasek, 1967).

Sample Preparation. A dried film of phospholipid was prepared under rotatory evaporation from chloroform solution and left under high vacuum for a minimum of 8 h to remove all traces of organic solvent. Multilamellar liposomes were formed by hand-shaking the preformed lipid film with excess buffer (10 mM Tris, 0.2 mM EDTA, and 100 mM NaCl, pH 7.0). Lipid–cytochrome *c* complexes were prepared by addition of protein in buffer solution to the previously formed phospholipid bilayers followed with three cycles of freeze–thawing. The lipid–protein complexes were separated by ultracentrifugation (75000*g*; 1 h; 4 °C). The clear supernatant was removed for protein analysis. From the aqueous protein concentrations, it was estimated that less than 5% of total protein content in the pelleted lipid complex was in an unbound state. The buffer was previously deoxygenated with nitrogen gas, and all steps of sample preparation were carried out under a nitrogen atmosphere to prevent the protein from catalyzing oxidation of the lipid unsaturated acyl chains (Spooner & Watts, 1991a).

Phosphorus-31 NMR. ^{31}P-NMR spectra were recorded at 161.98 MHz on a Bruker MSL 400 spectrometer using a Hahn echo pulse sequence, with 90° pulse widths of 5 μs and high-power proton decoupling during acquisition. Phosphorus spin–lattice (T_1) relaxation time were measured with the inversion–recovery pulse sequence 180°–τ–90°, with proton decoupling and a recycle time of typically 5 s, which is at least $5 \times T_1$. NMR measurements were carried out in samples containing 80–100 mg of phospholipid. The lipid–protein complexes contained cytochrome *c* in the molar ratio range (15–20):1, lipid to protein ratio.

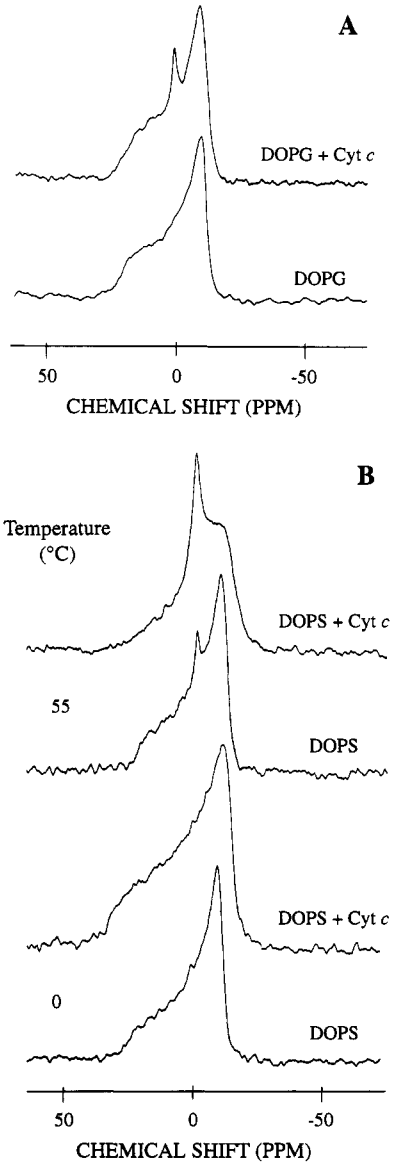

FIGURE 1: Proton-decoupled ^{31}P-NMR spectra at 161.98 MHz for hydrated phospholipid bilayers of (A) DOPG at 0 °C and (B) DOPS at 0 and 55 °C, with and without cytochrome *c*. Lipid–protein complexes in the molar ratio range (15–20):1, lipid to protein mole ratio. Acquisition was recorded over 50-kHz spectral width, using 2-s recycle time, and about 80–124 scans were accumulated.

Differential Scanning Calorimetry. The differential scanning calorimetry experiments were performed with a Perkin-Elmer DSC7 instrument using a scanning rate of 5 °C/min. Typically, pellets of lipid–protein complexes containing 5–10 mg of lipid and bound cytochrome *c* in the lipid to protein molar ratio range of (15–20):1 were used for each scan. Baseline corrections were achieved by filling the reference pan with only buffer, in an estimated equivalent amount of its content in the lipid–protein complex in the sample pan.

RESULTS

Phosphorus-31 Chemical Shift Anisotropy. All lipid dispersions gave the ^{31}P NMR powder pattern spectra determined by an axially symmetric chemical shift tensor, typical of phospholipids in liquid-crystalline bilayers (Seelig, 1978). Some examples of these spectra are shown in Figure 1 for hydrated phospholipid bilayers alone and when complexed

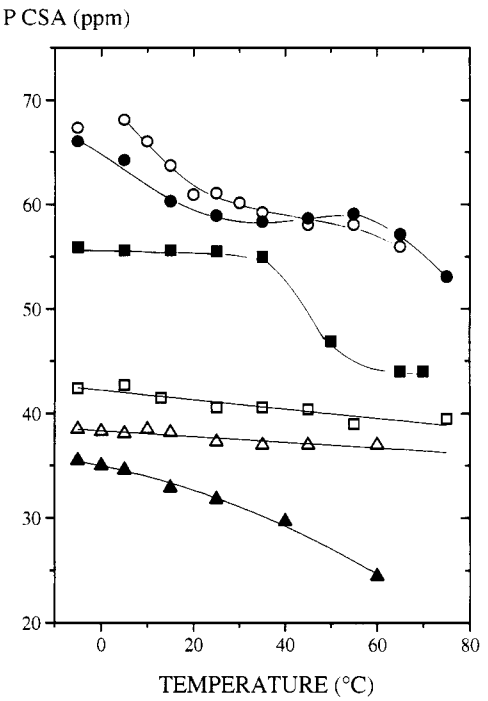

FIGURE 2: Variation of phosphorus-31 chemical shift anisotropy (^{31}P CSA) with temperature for liposomes of DOPG (*triangles*), DOPS (*squares*), and diacylPI (*circles*); open symbols are for the protein-free phospholipid bilayers, and filled symbols are for the lipid–cytochrome *c* complexes in the molar ratio range (15–20):1, lipid to protein mole ratio.

with cytochrome *c*. These line shapes containing an intense high-field peak (at $\sigma_\perp$) and shallow low-field shoulder (extending to $\sigma_\parallel$) are a result on the averaging of the phosphorus chemical shift anisotropy by fast rotation about the bilayer normal (parallel to the long molecular axis) of the whole phospholipid molecule, and additional headgroup wobbling motion (Smith & Ekiel, 1984). The binding of cytochrome *c* is accompanied by the appearance of an additional central spectral component at around the isotropic chemical shift (Figure 1). While this isotropic component appears for the DOPG– and diacylPI–cytochrome *c* complexes even at low temperature (Figure 1A), for the DOPS complexes this component becomes only prominent at higher temperatures (≥55 °C), where some isotropic component also appears in the spectrum of protein-free bilayers (Figure 1B).

The separation of the edges in a ^{31}P NMR spectrum, *i.e.*, the breadth between the lower intense low-field shoulder, $\sigma_\parallel$, and the more intense high-field peak, $\sigma_\perp$, defines the residual chemical shift anisotropy. Figure 2 shows the variation of the phosphorus-31 chemical shift anisotropy (^{31}P CSA) as a function of temperature for phospholipid bilayers alone and with bound cytochrome *c*. ^{31}P CSA was found to be −38 ± 1 ppm for the pure phospholipid bilayers of DOPG, and −40 ± 2 ppm for DOPS bilayers over the temperature range in which spectra were recorded. DiacylPI bilayers show a significant discontinuity in the CSA between 5 and 20 °C. Below 5 °C, the CSA is −68 ± 1 ppm, and above 20 °C, the CSA falls to −58 ± 2 ppm (Figure 2). For DOPG–cytochrome *c* complexes, the presence of protein induces a small decrease (~5%) of the chemical shift anisotropy at lower temperatures, which becomes more pronounced (up to a 30% change) at increased temperatures (Figure 2). In contrast, the CSA for DOPS bilayers is found to increase *ca.* 33% upon binding of cytochrome *c* for temperatures below 35 °C (Figure 2).

The behavior of diacylPI bilayers is quite different from that for the other lipid systems studied here, in that the binding with cytochrome *c* does not affect significantly the trend in the chemical shift anisotropy with temperature (Figure 2). The same temperature profile for the variation of the CSA is observed either for the protein-free bilayers or for lipid–protein complexes. Differential scanning calorimetry on these systems revealed no thermodynamic phase transition for bilayers with or without protein over the same temperature range. A possible explanation may involve a change in the conformation of the inositol moiety at around those temperatures between 5 and 15 °C. Recent NMR studies (Hansbro *et al.*, 1992) on ring-deuterated inositol, DMPI-d_6, have shown that four possible "tilted" orientations may exist for the ring headgroup in DMPI. Furthermore, a similar discontinuity to that seen here by ^{31}P NMR was also observed in the variation of the quadrupole splitting with temperature for DMPI-d_6 in hydrated bilayers, without being related to any thermodynamic lipid-phase transition of the acyl chains (Reid, 1991).

On binding of cytochrome *c*, an additional spectral isotropic component appears at around 0 ppm (Figure 1). Similar narrow components have been observed in phosphorus-31 NMR spectra of cardiolipin–cytochrome *c* complexes (de Kruijff & Cullis, 1980; Rietveld *et al.*, 1983; Spooner & Watts, 1991b), as well in pure lipid systems, particularly of anionic phospholipid bilayers, in which isotropic components have been attributed to vesicular or micellar structures in or on the bilayer, and could be associated with precursors of hexagonal H_{II}-phase formation (Farren & Cullis, 1980). Despite the nonbilayer spectral component being broader from lipid–protein complexes when compared to protein-free bilayers, there is no indication of a well-defined hexagonal H_{II} phase. Moreover, freeze–fracture electron microscopy (data not shown) does not show any evidence for the existence of hexagonal H_{II} structures. The macroscopic organization of the phospholipids is generally the same for protein-free and protein-containing systems; *i.e.*, large multilamellar liposomes are well distributed over all preparations, and smooth fracture faces were observed, which demonstrates the absence of nonlamellar lipid structures. The only detectable difference by electron microscopy for the lipid–protein complexes was the presence of smaller vesicles (diameter ≤500 nm) among the large multilamellar liposomes, which do not appear to be present on the protein-free lipid preparations (Pinheiro, 1993). The number of vesicles observed depended upon the temperature from which the complexes were quenched, with an increasing population of small vesicles at higher temperatures. However, qualitatively the number and hence lipid content of small vesicles is a low proportion of the total lipid. These observations are in agreement with the ^{31}P NMR results, where it was found that the intensity of the isotropic component increased with the temperature, suggesting that the appearance of such an isotropic component may be associated with the formation of small vesicles. Narrow symmetrical ^{31}P NMR spectra have been observed for sonicated phospholipid bilayers as a result of the isotropic motional averaging of the ^{31}P NMR chemical shift tensor by lateral diffusion of phospholipids around the small-diameter vesicles and Brownian tumbling of the entire vesicle (Seelig, 1978; Cullis & de Kruijff, 1979; Cullis, 1976; Burnell *et al.*, 1980).

On the other hand, theoretical investigations by Thayer and Kohler (1981) have shown that different phospholipid headgroup conformations can generate ^{31}P NMR spectra typical of isotropic or hexagonal H_{II} lipid phases, while retaining the bilayer packing arrangement. The rotation of the phospholipid molecules about their long axis in a bilayer structure produces the well-known ^{31}P NMR powder pattern

spectrum dominated by the axially symmetric chemical shift tensor, but further averaging can be produced if more rotational modes are allowed to occur at around the phosphate moiety. For instance, if the phosphate segment can rotate about more than one axis and be allowed to wobble between two or more orientations, a complete averaging of the chemical shift anisotropy can be observed if the combined process of headgroup rotation and wobbling is faster than the frequency of the CSA. The increase in temperature of the bilayers studied here, particularly with bound cytochrome *c*, may provide the required activation energy for those additional rotational modes and, or, wobbling of the phospholipid headgroup, since the averaged isotropic ^{31}P NMR spectra are especially observed at higher temperatures. The isotropic component could therefore also result from additional motional modes around the phosphate moiety, particularly favored in the presence of membrane-bound cytochrome *c*.

Phosphorus-31 Spin–Lattice Relaxation. Spin–lattice (T_1) relaxation times can be related to the correlation time (τ_c) for the molecular motion according to $1/T_1 \propto \tau_c/(1 + \omega_0^2\tau_0^2)$, where ω_0 is the Larmor frequency (Abragam, 1961). If the value of the molecular correlation time, τ_c, is such that $\omega_0\tau_c \cong 1$, then T_1 is most efficient; *i.e.*, the T_1 relaxation time is at a minimum. If the molecular motions are slower or faster than the Larmor frequency, the T_1 relaxation time becomes longer. As the molecular motion depends on the temperature, the variation of T_1 with temperature can exhibit a minimum if at a certain temperature the condition $\omega_0\tau_c \cong 1$ is satisfied. Only a few examples of T_1 minima for biological systems have been reported. In particular with lipid membranes, the observation of a T_1 minimum on ^{31}P NMR of phospholipids depends on the fortunate combination of a few conditions, including a relatively high magnetic field strength, a low lipid-phase transition temperature, to allow sufficient slowing of the motion to occur at lower temperatures without the formation of the gel phase, and additional headgroup–headgroup interactions (Seelig *et al.*, 1981). The scarce examples reported in the literature were observed by ^{31}P NMR measurements on sarcoplasmic reticulum membrane exchanged with POPC (Seelig *et al.*, 1981), on pure POPC bilayers (Tamm & Seelig, 1983), on mixtures of POPC with DOPE or *Escherichia coli* PE (Ghosh, 1988), and on cardiolipin bilayers (Spooner & Watts, 1991b). The importance of finding the T_1 minimum rests on the fact that is not necessary to know the exact relaxation mechanism(s) in order to evaluate τ_c at the temperature of the T_1 minimum. At this point, the relation $\tau_c \sim 1/\omega_0$ holds for all types of T_1 relaxation mechanisms. The T_1 minimum is also of value in providing an unambiguous definition of the motional regime for lipid phosphorus with respect to the NMR observing frequency, and can be helpful to interpret the effect of protein binding on phosphorus relaxation (Spooner & Watts, 1991b).

A typical sequence of spectra obtained in an inversion–recovery experiment for the determination of phosphorus-31 spin–lattice relaxation times is shown in Figure 3 for a complex of DOPG with cytochrome *c*. The broad bilayer component, either for protein-free bilayers or for the protein-containing complexes, relaxes uniformly, which indicates no anisotropic effects on the T_1 for this component (Figure 3). However, the isotropic component in the phospholipid–cytochrome *c* complexes relaxes more rapidly than the bilayer component, as shown in the typical example of Figure 3, revealing either a more mobile fraction of lipid or an efficient relaxation interaction with the protein. Because this isotropic component constitutes a rather small fraction of the total lipid, our analysis

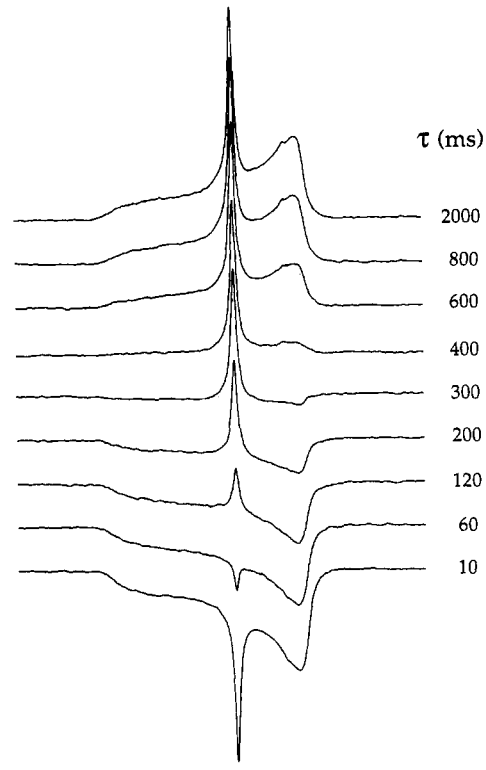

FIGURE 3: Typical inversion–recovery sequence of spectra for the measurement of spin–lattice (T_1) relaxation time on a DOPG–cytochrome *c* complex at 19 °C, showing the various delay times, τ, between 180° and 90° pulses. The faster recovery of the isotropic component can be clearly seen.

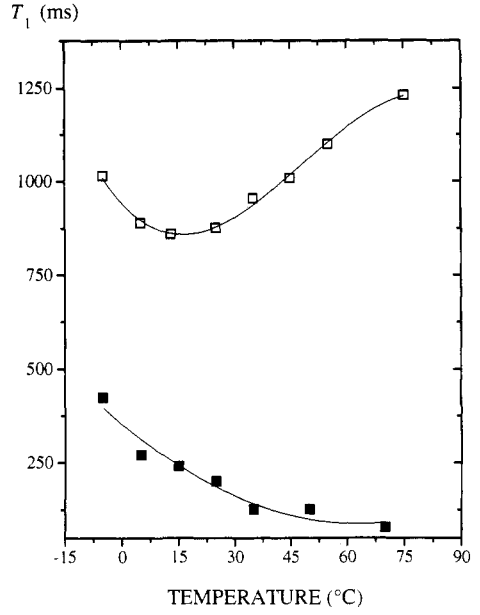

FIGURE 4: Dependence of phosphorus-31 spin–lattice (T_1) relaxation time (at 161.98 MHz) with temperature for DOPS bilayers alone (*open squares*) and with bound cytochrome *c* (*filled squares*) in the molar ratio (15–20):1, lipid to protein ratio.

of the ^{31}P spin–lattice (T_1) relaxation behavior includes only the broad spectral component ("bilayer component").

In Figures 4, 5, and 6 are presented the variation of the phosphorus-31 T_1 relaxation times for the hydrated phospholipid bilayers of DOPS, DOPG, and diacylPI, respectively, and for their corresponding lipid–cytochrome *c* complexes. For the hydrated phospholipid bilayers alone, a T_1 minimum is observed at around 15, 18, and 37 °C for DOPG, DOPS, and diacylPI, respectively. At the ^{31}P resonance frequency

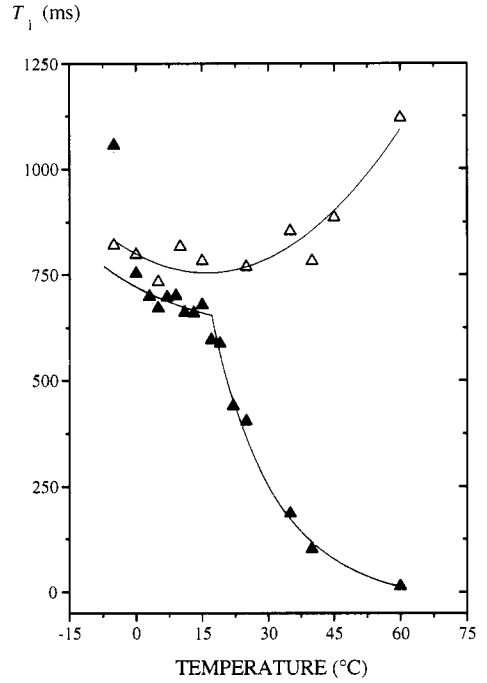

FIGURE 5: Variation of phosphorus-31 spin–lattice (T_1) relaxation time (at 161.98 MHz) with temperature for DOPG bilayers (*open triangles*) and for complexes of DOPG with cytochrome *c* (*filled triangles*) in the molar ratio (15–20):1, lipid to protein ratio.

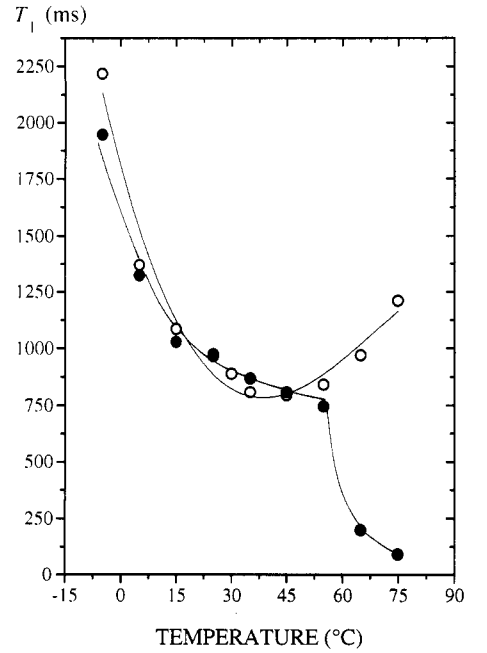

FIGURE 6: Variation of phosphorus spin–lattice (T_1) relaxation time (at 161.98 MHz) with temperature for diacylPI bilayers (*open circles*) and for diacylPI–cytochrome *c* complexes (*filled circles*) in the molar ratio (15–20):1, lipid to protein ratio.

used here ($\omega_0 = 2\pi \times 161.98$ MHz), a correlation time for the molecular motion of the phosphate headgroup in those phospholipid bilayers at the temperature of the T_1 minimum is of the order of 1 ns. As expected, similar values have been reported for the phosphorus correlation times for DOPC bilayers at around 0 °C (Seelig *et al.*, 1981), for POPC at around 15 °C (Tamm & Seelig, 1984), and for cardiolipin bilayers at around 25 °C (Spooner & Watts, 1991b).

The binding of cytochrome *c* to DOPS and DOPG bilayers markedly enhances the spin–lattice relaxation of the phosphorus in the bilayer lipid, with elimination of the T_1 minimum, as shown in Figures 4 and 5. Similar effects have been observed for complexes of cytochrome *c* with cardiolipin bilayers (Spooner & Watts, 1991b), which have been attributed to a strong coupling with an unpaired electron spin existing in the protein heme. It was proposed that upon binding of cytochrome *c* to the charged bilayers, perturbations in the protein structure occur, involving changes in the heme configuration in which a high-spin form of the heme iron is induced. Some direct evidence of this high-spin cytochrome *c* has been reported by ESR in a cardiolipin–cytochrome *c* complex (Vincent *et al.*, 1987), where a small fraction (~5%) of high-spin species was detected close to liquid helium temperatures (at ~10 K). Other studies by resonance Raman spectroscopy on cytochrome *c* bound to charged surfaces (Hildebrandt & Stockburger, 1986, 1989a,b), including anionic lipid dispersions (Hildebrandt *et al.*, 1990; Heimburg *et al.*, 1991), have indicated that two different conformation states, described as states I and II, are induced in cytochrome *c* upon complexation with negatively charged surfaces. While the conformation of state I is very similar to that of the protein in solution, where the native six-coordinated low-spin iron configuration of the heme is preserved, in state II the heme crevice opens, leading to a thermal- and potential-dependent equilibrium between a five-coordinated high-spin and a new six-coordinated low-spin iron configuration. This conformational equilibrium between states I and II, as well as the coordination equilibrium in state II, appeared to be controlled by the electrostatic interaction between the positively charged lysine residues surrounding the heme crevice and the negative charged surface.

The high-spin intermediate (state II) of cytochrome *c* in a phospholipid complex gives rise to paramagnetic enhancement of the lipid ^{31}P spin–lattice relaxation. For DOPS–cytochrome *c* complexes, a strong relaxation enhancement is observed in the entire range of temperatures studied, from –5 to 70 °C, similar to that observed for cardiolipin–cytochrome *c* complexes (Spooner & Watts, 1991b). Therefore, it seems plausible to imply an identical interaction between the phosphate in the DOPS headgroup and the heme in cytochrome *c*, as that found with cardiolipin phosphates. Despite differences in the chemical structures of DOPS and cardiolipin headgroups, the conformation equilibrium between states I and II is promoted over the same temperature interval, and the magnitude of the reduction on the ^{31}P spin–lattice relaxation times is identical.

The complexation of cytochrome *c* to DOPG bilayers seems to be less efficient in promoting the equilibrium between the two conformational states I and II of protein, since the paramagnetic effect in the DOPG phosphorus T_1 is only observed at higher temperature (above 15 °C) (Figure 5), suggesting that a higher thermal energy may be required for the conformational change to take place in cytochrome *c* when complexed to DOPG bilayers than when complexed to DOPS bilayers. On the other hand, it is observed that after the conformational equilibrium been established, the paramagnetic enhancement increases with temperature, which suggests a progressive opening of the heme crevice and thus a consequent increase in the accessibility of the phospholipid phosphate moiety for the interaction with the heme iron. This implies that the paramagnetic effect on the lipid phosphorus T_1 relaxation involves a close proximity between the heme iron in cytochrome *c* and the phosphate in the phospholipid headgroup. This hypothesis is supported by the lack of a paramagnetic effect on the phosphorus T_1 relaxation for diacylPI complexes with cytochrome *c* (Figure 6). The inositol moiety, being a much larger headgroup, cannot be accom-

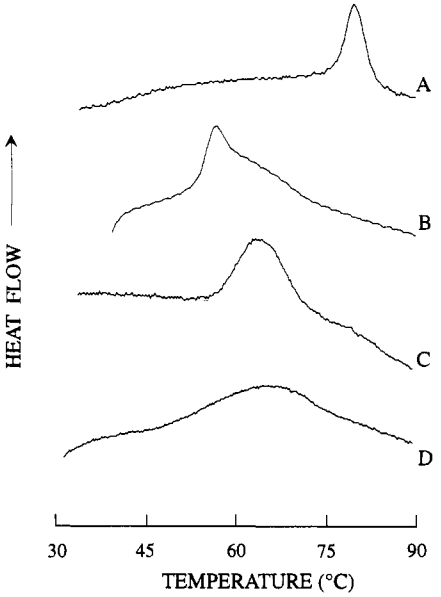

FIGURE 7: Typical differential scanning calorimetry endotherms for the thermal denaturation of cytochrome *c* in buffer solution (A), and when bound to phospholipid bilayers of DOPG (B), DOPS (C), and diacylPI (D) in the molar lipid to protein ratio range of (15–20):1.

modated closely enough to the heme crevice within the limits of maximal aperture before thermal denaturation of the protein takes place (see below).

Cytochrome *c* is a very polar protein, which in the oxidized state at neutral pH carries a net positive charge of +9. The distribution of the positive charges is not uniform, being segregated in well-defined patches on the protein surface (Dickerson *et al.*, 1971). This rather unique charge distribution is likely to play a role in the interaction with its physiological redox partners, or with the surface of the inner mitochondrial membrane. The interaction with the membrane surface may well establish the relationship between the protein structure and its function, which may be modulated by the surface density of charged lipid headgroups.

Differential Scanning Calorimetry. The excess heat flow versus temperature for cytochrome *c* in aqueous buffer solution (pH 7.0) shows an endothermic transition with a maximum at around 80 °C (Figure 7A). This thermal denaturation profile of the protein is essentially similar to the DSC transition of cytochrome *c* at neutral pH reported by Santucci *et al.* (1989). These authors showed that the denaturation of the protein is independent of the scanning rate. However, at slower scanning rates (approximately 1 °C/min), a weaker endotherm centered at approximately 64 °C and preceding the main transition can be resolved by microcalorimetry (Muga *et al.*, 1991). Upon binding of cytochrome *c* to liposomes containing DOPG, the DSC measurements revealed an endotherm with a peak temperature at around 57 °C (Figure 7B). This calorimetric transition represents the thermal denaturation of DOPG-bound cytochrome *c*, which occurs at a temperature approximately 23 °C lower than that of the protein in aqueous buffer solution. For the complexes with DOPS or diacylPI bilayers, the thermal denaturation of cytochrome *c* occurs at around 63 °C, which is about 17 °C lower than the denaturation temperature for the protein in buffer (Table 1). A decrease of about 16 °C for DOPG-bound cytochrome *c* in a lipid:protein molar ratio of 80 and at pH 7.4 has been recently reported (Muga *et al.*, 1991). The thermal denaturation of cytochrome *c* in all lipid complexes here analyzed is an irreversible process.

Table 1: Thermodynamic Parameters for the Thermal Denaturation of Cytochrome *c* in Buffer Solution and When Bound to Phospholipid Bilayers of DOPG, DOPS, and DiacylPI, in the Lipid:Protein Mole Ratio Range (15–20):1[a]

system	peak (°C)	ΔT (°C)	ΔH^{cal} (kcal/mol)
cyt *c* in buffer	80.3	9	98
DOPG/cyt *c*	57.3	24	86
DOPS/cyt *c*	63.7	18	36
diacylPI/cyt *c*	63.5	37	42

[a] "Peak" refers to the temperature at the maximum heat flow in the transition; ΔT reflects the cooperativity of the transition, being measured by the width of the transition at the base line; *i.e.*, $\Delta T = T_f - T_i$, where T_f and T_i are the final and initial temperatures of the transition, respectively.

The large decrease in the denaturation temperature for lipid-bound cytochrome *c* has been taken as evidence of pronounced destabilization of the protein tertiary structure (Muga *et al.*, 1991). However, caution should be exercised when making such interpretations based only on temperature shifts observed in DSC experiments. In fact, a qualitatively similar destabilization of the cytochrome *c* structure could be predicted upon binding to anionic bilayers of either DOPG, DOPS, or diacylPI, as evaluated only from the decrease in the peak temperature of the endotherm. However, a detailed inspection of the DSC traces (Figure 7) reveals instead that subtle differences occur in the interaction of cytochrome *c* with the various phospholipid bilayers. Protein complexes with DOPS and diacylPI bilayers showed a large decrease in the calorimetric enthalpy (ΔH^{cal}) for the denaturation of cytochrome *c* when compared to the value in buffer solution (Table 1). In contrast, the drop in ΔH^{cal} for the thermal denaturation of cytochrome *c* in complexes with DOPG is less pronounced than that for other lipid–protein complexes. The width of the endotherm, measured by ΔT (Table 1), is an indication of the cooperativity of the transition. For all the lipid–protein complexes, there is an appreciable loss in the cooperativity of the thermal denaturation of cytochrome *c* when compared to the protein denaturation in buffer solution. Cytochrome *c* complexes with diacylPI bilayers showed the broadest thermal transition, $\Delta T \cong 37$ °C (Table 1), while DOPS-bound protein has a higher cooperativity, with $\Delta T \cong 18$ °C. Hydrogen–deuterium exchange measurements have shown that upon binding of cytochrome *c* to negatively charged membranes there is a considerable accessibility of protein backbone amide groups to solvent, which suggested a lipid-mediated loosening and/or destabilization of the protein tertiary and secondary structure (Spooner & Watts, 1991a; Muga *et al.*, 1991). The less compact cytochrome *c* structure when bound to a lipid surface seems a plausible explanation for the decrease in the cooperativity of the thermal denaturation for the bound protein when compared with the process in a lipid-free buffer solution.

The thermal denaturation transition profile of DOPG-bound cytochrome *c* seems to be composed of two steps: a highly cooperative transition centered at 57 °C and a less cooperative component centered at around 63 °C (Figure 7B). High cooperativity of the thermal denaturation of bound cytochrome *c* to DMPG bilayers has been suggested to result from strong protein–protein intermolecular interactions at the membrane surface (Sturtevant, 1987). Our results suggest a more plausible alternative explanation, which involves a more compact state of cytochrome *c* structure when bound to DOPG bilayers. In spite of the large decrease in the peak temperature transition for this system, the high cooperativity of the endothermic component centered at around 57 °C and the small reduction in ΔH^{cal} compared to the value for the

denaturation of cytochrome c in buffer solution (Table 1), combined with the absence of a paramagnetic enhancement of the ^{31}P relaxation at low temperatures, strongly suggest a structure for the DOPG-bound cytochrome c which more closely resembles the native structure of the protein in buffer solution. Despite the fact that an identical decrease in the peak transition temperature is observed for the various lipid–protein complexes, the actual state of the protein structure when bound to a lipid bilayer seems to be different for each lipid system, possibly controlled by a subtle relationship between the protein acidic residues and the membrane surface charge density.

DISCUSSION

The results of this study demonstrate that the binding of cytochrome c to anionic phospholipid bilayers induces an overall destabilization of the protein structure. The extent of this structural perturbation varies with lipid and is induced by the close proximity between the positively charged protein residues and the charge density at the bilayer surface, which is dependent on the lipid headgroup chemical structure and geometric factors. The protein destabilization is revealed by (i) the paramagnetic enhancement of ^{31}P spin–lattice relaxation of the lipid phosphorus, which is a result of the formation of a high-spin state of cytochrome c induced by the disruption of the axial ligand Met-80 to the heme iron in the protein, and (ii) the decrease of thermal stability of the lipid-bound cytochrome c as revealed by the thermodynamic measurements with differential scanning calorimetry. While the paramagnetic effect is directly associated with the immediate environment of the heme iron and its accessibility to the lipid phosphate, the destabilization observed by DSC reveals a more extensive structural perturbation of the entire protein molecule.

The electrostatic interaction between cytochrome c and the charged lipid bilayer seems to disrupt the internal cohesive forces within the protein, leading to an overall loosening and destabilization of the protein tertiary structure. This process may start at the weak structural points, such as the axial ligation through Met-80, known to be a very strained bond (Williams, 1989). The disruption of this axial coordination leads to the opening of the heme crevice, allowing the interaction with the lipid headgroup phosphate, as detected by the ^{31}P NMR spin–lattice relaxation. The absence of the paramagnetic effect on the relaxation of the phosphorus in the diacylPI headgroup cannot, therefore, be interpreted as an absence of destabilization of the protein structure, since the DSC results clearly indicate a relatively loose structure, as evaluated by the low cooperativity of the thermal denaturation process and the appreciable reduction of the calorimetric enthalpy. Most likely, the formation of the high-spin form of the heme iron does occur in this complex, but due to the rather large inositol moiety the phosphate itself cannot approach the heme crevice close enough to be able to sense the paramagnetic effect.

The DSC data for the thermally induced denaturation of cytochrome c bound to DOPG bilayers (Figure 7) combined with the temperature profile of the lipid ^{31}P spin–lattice relaxation (Figure 5) suggest a different interaction between cytochrome c and this lipid. While for the DOPS and diacylPI bilayers a broad, less cooperative, thermal transition for the denaturation of bound cytochrome c is indicative of an extensive overall destabilization of the protein structure, which also is revealed by the effects on the relaxation profile for the DOPS–protein complexes over the entire temperature range studied here, for the DOPG-bound cytochrome c a lower temperature high-cooperative component in the DSC trace is present, and the ^{31}P spin–lattice relaxation times were not significantly affected at lower temperatures on protein binding. These combined effects strongly suggest that in the DOPG–cytochrome c complexes the protein structure is not as extensively destabilized as in the DOPS- or diacylPI–protein complexes, and it is postulated that the overall conformation of the protein in DOPG complexes is more like the one in its native solution state.

Despite the fact that the initial driving force for the binding of cytochrome c to anionic phospholipid bilayers is electrostatic, the final state of the bound protein and the extent of lipid-induced perturbations in its structure show some significant differences for the chemically distinct phosphoheadgroup moieties analyzed here (DOPS, DOPG, and diacylPI). The extent of those effects seems to be lipid-dependent, involving very subtle modes of lipid–protein interaction which may constitute the key role of modulating protein structure and function in membranes. The present findings suggest that if a lipid–cytochrome c intermediate does occur in the inner mitochondrial membrane prior to complexation with its redox partners, those conformational changes in cytochrome c would facilitate electron transfer through the overall loosening of the protein structure with concomitant exposure/accessibility of the heme group. Although difficult to discriminate, some specific modes on the lipid headgroup–protein interaction are different for the different lipid types, and they may well play an important role in modulating the function of a peripheral protein through lipid-induced conformational and/or dynamic changes.

Finally, our results may be of greater generality toward our understanding of peripheral proteins. It is probable that in natural membranes comprising a heterogeneous lipid composition, the different lipid–protein interactions may induce a conformational equilibrium in the membrane-associated protein, which provides a switching mechanism between different states of activity or function. Reciprocal conformational and dynamic effects are expected to occur in both lipid and protein, leading to a general mechanism for the activation of membrane-associated processes mediated by lipid–protein interaction.

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