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Investigation of Secondary and Tertiary Structural Changes of Cytochrome *c* in Complexes with Anionic Lipids Using Amide Hydrogen Exchange Measurements: An FTIR Study

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ABSTRACT The structure of cytochrome *c* bound to anionic lipid membranes composed of dimyristoyl, dipalmitoyl, or dioleoyl phosphatidylglycerols, or of bovine heart cardiolipin, has been investigated by Fourier transform infrared spectroscopy. Only small changes in secondary structure, as registered by the amide I band of cytochrome *c*, were observed upon binding at temperatures below that of denaturation of the protein, and these were not coupled to the thermotropic phase transitions of the lipid. The denaturation temperature of the protein decreased by approximately 25–30° upon binding, in a progression which correlated with that of the lipid phase transition temperatures, being approximately 7° lower for complexes with dioleoyl than with dipalmitoyl phosphatidylglycerol. Large changes in the amide proton exchange characteristics, as monitored by the spectral shifts in the amide I band of the protein in D_2O, were observed on binding cytochrome *c* to the lipid membranes. For the slowly exchanging population, the amide deuteration rates of the free protein were nearly independent of temperature, whereas those of the bound protein increased by up to two orders of magnitude over the temperature range from 10 to 40°C. In addition, the extent of exchange differed between the bound and unbound protein. A structural transition in the bound protein was detected as a discontinuous step in Arrhenius plots of the deuterium exchange rates which occurred at a temperature in the region of 22 to 29°C, depending on the lipid, far below that of denaturation. The temperature of this transition was determined by the physical state of the lipid, being 7° lower for the lipids in the fluid state than for those in the gel state, and, for complexes with dimyristoyl phosphatidylglycerol, occurred at an intermediate temperature, being controlled by the lipid chain-melting transition at 27–28°C. These results provide evidence for a coupling of the tertiary structure of the membrane-bound protein with the physical state of the membrane lipids.

INTRODUCTION

Many peripheral proteins and enzymes interact with membranes and their mode of action can depend on the physical properties of the membrane. This has been shown, for example, for different species of phospholipase A_2 (Bell and Biltonen, 1989). It is, therefore, of considerable interest to study the coupling of lipid and protein structural states. This is done in the present work for the interaction of cytochrome *c* with anionic lipids of different acyl chain composition and polar headgroup. To investigate structural changes in cytochrome *c* upon binding, the secondary structure and the amide proton/deuteron exchange rates of the protein have been determined from the Fourier transform infrared (FTIR)[1] spectra of cytochrome *c* in complexes with lipid.

Lipid-dependent conformational changes in membrane-bound cytochrome *c* have been demonstrated previously by resonance Raman spectroscopy (Heimburg et al., 1991). These changes could be correlated with differences in coordination and spin state of the iron and in redox potential of the protein (Hildebrandt and Stockburger, 1989a, b), and therefore can be considered to be potentially of functional significance. However, these observations were, of necessity, confined to the haem prosthetic group and did not provide information on possible structural/conformational changes in the protein backbone that might be driving the transition observed. FTIR studies of structural changes occurring in cytochrome *c* bound to lipids have been performed previously by Muga et al. (1991a), and it was concluded that the secondary structure remained essentially unchanged upon binding, although destabilization of the bound protein was indicated by a large reduction in the denaturation temperature and an increase in the rate of amide proton exchange. Similarly, both solid state ^2H NMR studies of cytochrome *c* bound to cardiolipin (Spooner and Watts, 1991) and high resolution ^1H NMR studies of cytochrome *c* bound to detergent micelles (De Jongh and De Kruijff, 1992) have demonstrated a considerably enhanced rate of amide proton exchange of the bound protein relative to that of the protein free in solution. These and other results were interpreted variously as a reversible unfolding or a highly dynamic folded structure of cytochrome *c* at the lipid-water interface.

In the present paper, the kinetics of hydrogen-deuterium exchange have been investigated in more detail. It is shown that the behavior of a slowly exchanging population of amide protons in cytochrome *c* is very different when the protein is bound to negatively charged lipids from that when it is free in solution. This is attributed to changes in tertiary structure

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[1] *Abbreviations used:* FTIR, Fourier transform infrared spectroscopy; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; BHCL, bovine heart cardiolipin; cyt *c*, horse heart cytochrome *c*; HWHH, half-width at half-height.

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of the protein upon binding. Further, a structural transition in the bound protein is found from the non-Arrhenius behavior of the exchange kinetics. The temperature of this transition lies in the 22–29°C range and depends on the physical state (liquid crystalline or gel) of the lipid; for the protein bound to dimyristoyl phosphatidylglycerol it is controlled by the lipid phase transition which occurs at 27–28°C for the protein complex. These results demonstrate a coupling between the structural states of the protein and of the lipid to which it is bound.

EXPERIMENTAL PROCEDURES

Materials

Cytochrome *c* from horse heart (Type VI) was obtained from Sigma Chemical Co. (St. Louis, MO) and used in the oxidized form without further purification. The fraction of deamidated cytochrome *c* present in commercial preparations (Brautigan et al., 1978) was found not to affect appreciably the binding to negatively charged phospholipids and, because it remains a constant factor in the experiments, also will not affect the difference spectroscopy of the amide I band. Dimyristoyl phosphatidylglycerol (DMPG) and dioleoyl phosphatidylglycerol (DOPG) were obtained from Avanti (Birmingham, AL). Bovine heart cardiolipin (BHCL), dioleoyl phosphatidylcholine, and dipalmitoyl phosphatidylcholine were obtained from Sigma (St. Louis, MO). Dipalmitoyl phosphatidylglycerol (DPPG) was synthesized from dipalmitoyl phosphatidylcholine according to Comfurius and Zwaal (1977). Anionic lipids were used as the sodium salts. D_2O was obtained from from Merck (Darmstadt, Germany).

Sample preparation

Solid cytochrome *c* was added to 50 mM dispersions of the charged lipids (in the case of DMPG, DOPG, and DPPG) or to a 25 mM lipid dispersion (in the case of BHCL) in D_2O buffer (2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pD 7.5). The lipid to protein ratios chosen corresponded to full saturation of the lipid membranes with protein (Waltham et al., 1986; Heimburg, 1989). During mixing of the lipid dispersions and the dry protein, the pD (uncorrected) increased to approximately 9.2. For the time-dependent measurements, the pD was not adjusted further. For the temperature-dependent measurements, the pD was readjusted to 9.0 using a standard pH electrode. The buffering capacity used was only slight in order to keep the ionic strength low so as to ensure complete binding. For experiments with unsaturated lipids (DOPG and BHCL), buffers were saturated with either nitrogen or argon and the lipid samples were kept under nitrogen or argon (or vacuum) at all stages.

FTIR spectroscopy

Fourier transform infrared spectra were recorded on a Bruker IFS25 spectrometer using a CaF_2 cell with a 50-μm Teflon spacer. The spectra were recorded in the range 400–4000 cm^{-1} with a spectral resolution of 2 cm^{-1} and were apodized with a triangular function before Fourier transformation. In time-independent experiments the spectrometer was flushed with dry nitrogen for at least 30 min before starting the measurement to reduce water vapor distortions of the spectra. 100 interferograms per spectrum were recorded. In time-dependent experiments the nitrogen flushing step preceding the experiment was omitted (because the sample had to be prepared outside the spectrometer to initiate deuterium exchange). This gave rise to slight but not very significant water vapor distortions in the first few minutes of spectral accumulation. Ten interferograms per time point were recorded consecutively as rapidly as possible. The temperature was controlled to an accuracy of 0.1°C by circulating thermostatted water through the metal housing of the sample cell. For the time-independent measurements, either the dispersion of the lipid/protein complex or the cytochrome *c* solution (5 mM), respectively, were incubated for several days in D_2O buffer and were subsequently incubated at 40°C for 1 h to maximize the degree of deuteration of the protein amides (see deuteration experiments in Results) and to ensure reversibility of the results in the temperature range between 4 and 40°C. For the time-dependent measurements, the lipid dispersion was added to dry cytochrome *c*, the resulting protein/lipid mixture was vortexed for approximately 10 s and then injected into a preheated CaF_2-cell. The zero time point was taken as the time of addition of the lipid dispersion to the dry protein. Fourier transform self-deconvolution was performed with software using routines provided by the group of H. H. Mantsch in Ottawa, Canada (Kauppinen et al., 1981). Line narrowing of the unsmoothed spectra was performed using a triangular apodization function, assuming an initial bandwidth of HWHH = 17 cm^{-1} with a line narrowing factor of $K = 2$. The components in the amide I region of the spectrum were fitted with Gaussian bands by using the deconvoluted spectrum over the range 1605–1693 cm^{-1} (Byler and Susi, 1986). A constraint on the fitting was that all lines should have comparable widths. The bandshape was first fitted by holding the component widths constant, then with these fixed frequencies the band widths were varied, and finally this parameter set was optimized by releasing both the band frequencies and bandwidths. Difference spectra were obtained from normalized, deconvoluted spectra (integrated area of 100xcm^{-1}). Normalization was performed by defining a linear baseline between the wavenumber limits of the subtraction. The parameter deduced from the difference spectra was the integrated area of the positive region (i.e., above the baseline); the total integral by definition is zero. Spectra are plotted as absorbance with the ordinate units given such that the integrated area of the entire band is normalized to 100xcm^{-1}.

RESULTS

FTIR spectra of cytochrome *c* complexes with anionic lipids

The deconvoluted FTIR spectra of cytochrome *c* in solution and complexed with DMPG at 20°C are compared in Fig. 1. Small but reproducible differences between the spectra, corresponding to changes in the secondary structure, are seen. This is confirmed by the difference spectra presented in the lower panel of Fig. 1.

The changes in the spectrum on binding of cytochrome *c* to DMPG amount to an area above the baseline in the difference spectrum that is approximately 2% of the total area of the band. Five component bands are resolved in the deconvoluted spectra of Fig. 1. The constraint of having comparable component bandwidths requires seven bands to fit the composite bandshape. Of these two extra bands, that at 1660 cm^{-1} is necessary for adequate fitting of the bandshape of the protein in solution and is evident as a slight shoulder in the deconvoluted spectrum given in the central panel of Fig. 1. The two bands at approximately 1671 and 1675 cm^{-1} could, however, be replaced by a single band of greater width. This band fitting analysis of the deconvoluted spectra (see Fig. 1 and Table 1) shows that the secondary structure of the protein is mainly conserved; the main difference is a reduction in α-helical content of approximately 7.5% upon binding of cytochrome *c* to the lipid. The 36% α-helical content is in agreement with previous circular dichroism measurements (Provencher and Glockner, 1981) and x-ray diffraction of the crystalline protein (Takano and Dickerson, 1981; Dickerson et al., 1971).

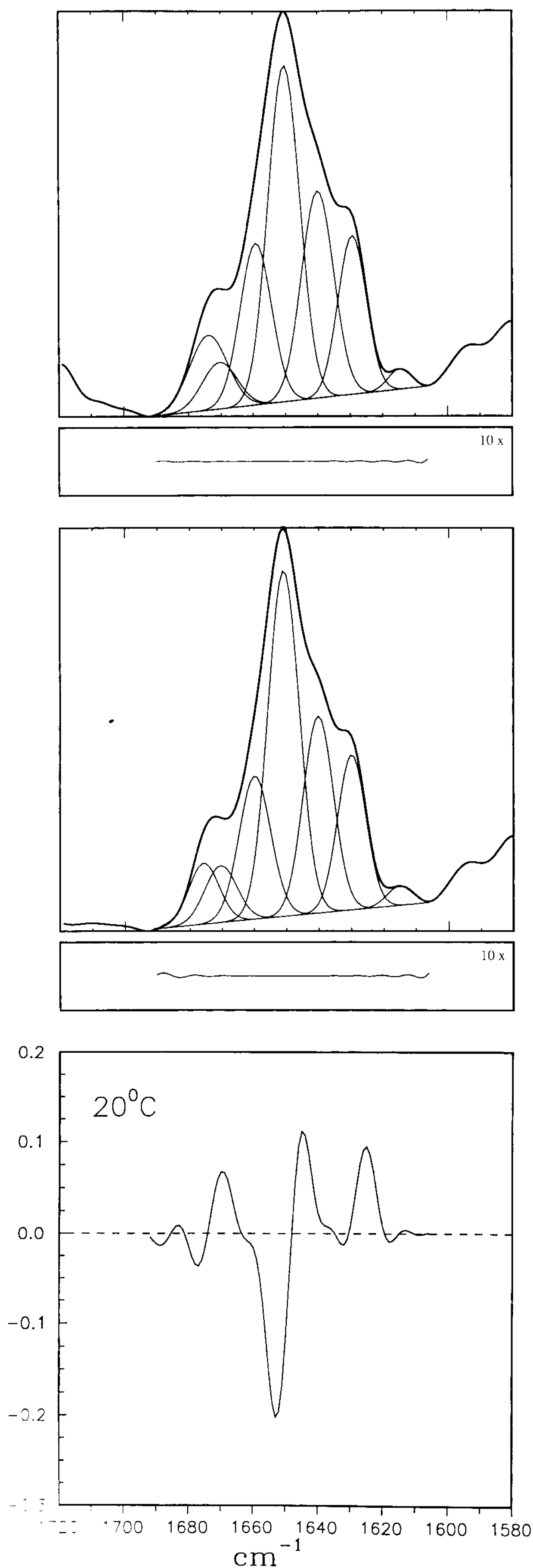


TABLE 1 Parameters obtained by band fitting of the amide I region of the Fourier self-deconvoluted FTIR spectra from cytochrome *c* in solution and in complexes with dimyristoyl phosphatidylglycerol at 20°C (see Fig. 1).

Structure*	cyt *c* in solution		cyt *c* DMPG complex	
	cm^{-1}	% area	cm^{-1}	% area
	1615.3	1.6	1614.8	1.4
Extended chain	1630.1	14.6	1629.5	14.1
Extended chain	1640.5	19.9	1640.2	21.0
α-Helix	1651.2	36.1	1650.7	33.5
β-Turn	1660.2	15.3	1659.5	16.0
Extended chain	1670.9	6.2	1670.8	5.0
Extended chain	1676.2	6.3	1674.3	8.9

* For secondary structural assignments see Byler and Susi (1986).

The temperature dependence of the amide I region in the deconvoluted FTIR spectra of cytochrome *c* bound to DMPG, and the resulting difference spectra obtained by using the spectrum recorded at 4°C as reference, are given in Fig. 2. The difference spectra increase in amplitude with increasing temperature, but the shape remains essentially unaltered until the protein denatures at a temperature around 50°C. The (positive) areas above the baseline in the difference spectra are given as a function of temperature in Fig. 3 for complexes with DMPG, DPPG, DOPG, and BHCL, and for cytochrome *c* in solution. A steady linear increase is found in all cases, followed by a sharp discontinuity at the denaturation temperature. The denaturation temperatures for cytochrome *c* complexed with the different anionic lipids occur at: 49, 51, 54, and 55.5°C for DOPG, BHCL, DMPG, and DPPG, respectively. Although the denaturation occurs at a considerably higher temperature than that of chain melting and the temperature differences are considerably smaller, the order of the denaturation temperatures correlates with that for the chain-melting transition of the complexes with the different phosphatidylglycerols (cf. below). As found previously by Muga et al. (1991a) for complexes with DMPG and DOPG, the denaturation temperature of the protein complexed with lipid is reduced strongly, by approximately 25–30°, relative to that of the protein free in solution. The denaturation of the latter occurs at 82°C, demonstrating a considerable reduction in the protein stability on binding to negatively charged lipids.

The temperature-dependent changes in the difference spectra recorded below the denaturation temperature (Fig. 2) are determined mainly by slight shifts in the individual band positions to higher wavenumbers, resulting from slight increases in the mean hydrogen bond lengths (cf. Tonge and

FIGURE 1 Fourier self-deconvoluted FTIR spectra (amide I region) of cytochrome *c* bound to dimyristoyl phosphatidylglycerol (*upper*) and of cytochrome *c* in solution (*middle*), at 20°C. Band fitting and residuals (×10) obtained by using Gaussian line profiles and the parameters given in Table 1 are shown. The difference between the two upper spectra (with lipid minus without lipid), where the area of each is normalized to $100 \times cm^{-1}$, is given in the lower panel.

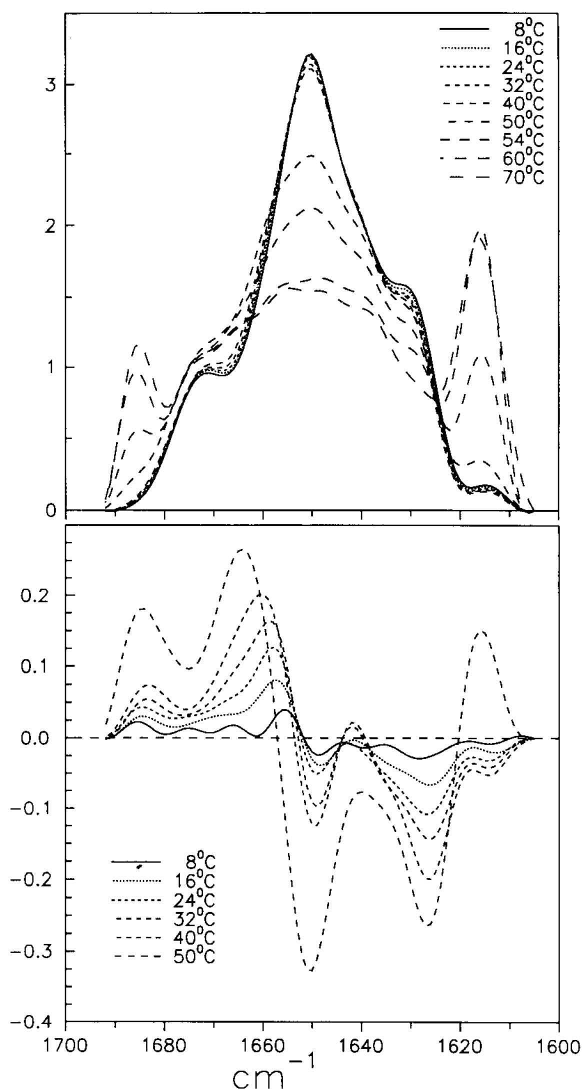


FIGURE 2 Upper: Fourier self-deconvoluted FTIR spectra (amide I region) of cytochrome *c* bound to dimyristoyl phosphatidylglycerol at the temperatures indicated. The area of the spectra is normalized to 100xcm^{-1}. Lower: difference spectra obtained by subtracting the normalized deconvoluted spectrum recorded at 4°C from the spectra given in the upper panel.

Carey, 1992). These occur equally for the bound and unbound protein, because the difference spectra between the two are essentially independent of temperature (data not shown). Upon denaturation, additional bands appear in the FTIR spectrum at 1616 and 1685 cm^{-1} at the outer extremes of the amide I region. The ratio between the areas of these two bands remains constant during denaturation, indicating that both bands arise from the same structural changes in the protein. Muga et al. (1991a) have attributed these changes to an extended chain-like structure forming between different protein molecules during aggregation. Another possibility is that they arise from the non-hydrogen bonded edges of β-sheet structures, as in, e.g., concanavalin A (cf. Byler and Susi, 1986). They also are found in the FTIR spectra of apo-cytochrome *c* upon complexation of the protein with anionic lipids (Muga et al., 1991b).

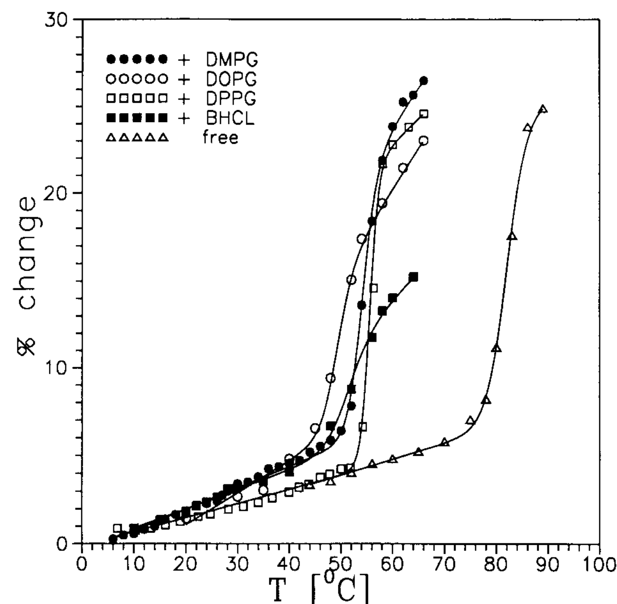


FIGURE 3 Temperature dependence of the total area above the baseline in the FTIR difference spectra (cf. Fig. 2) of the amide I band from cytochrome *c* in solution (△), and from cytochrome *c* complexed with DMPG (●), DOPG (○), DPPG (□), or BHCL (■). Difference spectra were obtained by subtracting a deconvoluted reference spectrum recorded at 4°C from the deconvoluted spectra, with areas normalized to 100xcm^{-1} as in Fig. 2.

The thermotropic (chain-melting) transition of the lipids in the cytochrome *c* complexes has been studied via the temperature-dependent changes in the carbonyl band (1710–1760 cm^{-1}) from the lipid ester bonds. Essentially identical

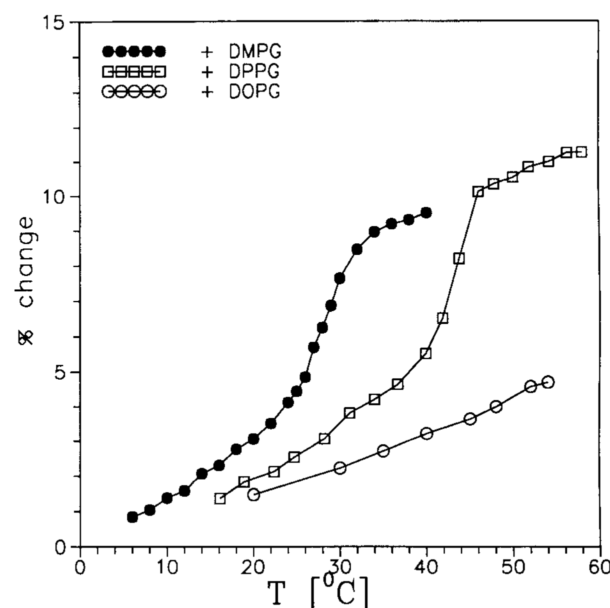


FIGURE 4 Temperature dependence of the total area above the baseline in the FTIR difference spectra of the lipid ester carbonyl band in complexes of cytochrome *c* with DMPG (●), DPPG (□), or DOPG (○). Difference spectra were obtained by subtracting a deconvoluted reference spectrum recorded at 4°C from the deconvoluted FTIR spectra recorded at higher temperatures, with the areas of each normalized to 100xcm^{-1}.

results for the lipid transition were obtained also from analysis of the CH-stretching vibrations at 2750–3050 cm^{-1}. Fig. 4 shows the temperature dependence of the areas above the baseline in difference spectra of the carbonyl band for the various lipid complexes, which were obtained by subtracting the spectrum recorded at 4°C after deconvolution as reference. The temperature dependences given in Fig. 4 can be fitted to an effective two-state transition, assuming a linear temperature dependence above and below the lipid transition. The mid-point temperatures of the transition are: 28 and 44°C, and effective Van't Hoff enthalpies are approximately 100 and 180 kcal/mol for complexes with DMPG and DPPG, respectively. These transitions are significantly higher in temperature and less cooperative than are the chain-melting transitions in the lipid bilayers without bound protein (Watts et al., 1978). Transitions are not observed with DOPG and BHCL, since these are expected to lie below 0°C.

Amide hydrogen-deuterium exchange rates in cytochrome *c*-lipid complexes

The influence of complexation of cytochrome *c* with anionic lipids on the proton-deuteron exchange rates has been investigated in order to obtain information on possible tertiary structural changes in the protein. The FTIR spectra of fully protonated cytochrome *c* (dry film) and of cytochrome *c* that has been deuterated by exchange in D_2O after different periods of incubation at 40°C are compared in Fig. 5. The rates of deuteration of cytochrome *c* in D_2O have been studied from the shifts in the amide I band on deuteration (cf. Fig. 5), by using difference spectroscopy. Data obtained from the

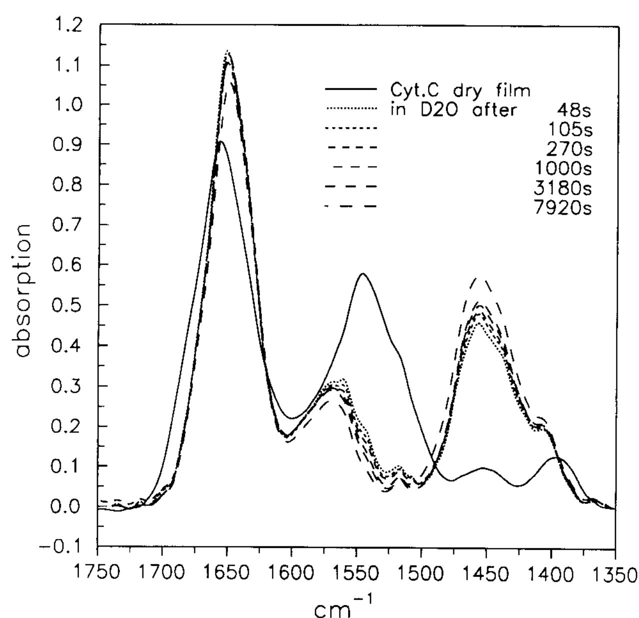
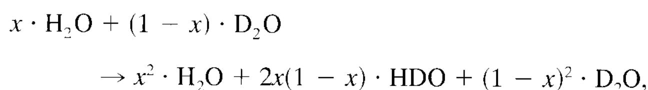


FIGURE 5 FTIR spectra (amide I and amide II regions) recorded at 20°C from a dry film of fully protonated cytochrome *c* (*full line*) and from cytochrome *c* in D_2O after incubation at 40°C for periods from 48 to 7920 s, as indicated (progressively longer *dashed lines*). Both the amide I and amide II bands are shifted to lower wavenumbers on deuteration.

intensity of the amide II band at 1550 cm^{-1} is not presented because of potential overlap from the HDO band at 1450 cm^{-1} arising from exchange and adsorption of H_2O. Redistribution of protons released into the water by exchange, according to the following scheme:



$$x \cdot H_2O + (1 - x) \cdot D_2O \rightarrow x^2 \cdot H_2O + 2x(1 - x) \cdot HDO + (1 - x)^2 \cdot D_2O,$$

indicates that under normal sample preparation conditions the H_2O absorption occurring in the amide I region at 1650 cm^{-1} is much smaller than the HDO absorption and can be neglected ($x \approx 2\text{–}5 \times 10^{-3}$). Additionally, the amide II region is susceptible to possible artefacts from slight leakage of water vapor into the measuring cell at long incubation times. Nevertheless, consistent data is obtained from analysis of the amide II band intensities at shorter incubation times, which further substantiates the method of analysis based on the frequency shifts of the amide I band. Analysis of the amide I band by difference spectroscopy has the additional advantage of allowing spectral resolution in the regions corresponding to the different secondary structures throughout the time course of the amide proton exchange.

For investigating the proton exchange, lipid dispersions in D_2O buffer were mixed with dry cytochrome *c*, resulting in a final concentration of cytochrome *c* of 5 mM. This corresponds to full saturation of the lipid surface with protein. The sample was then transferred immediately into the preheated sample cell, and spectra were recorded sequentially with time. Difference spectra were constructed by subtracting a reference spectrum recorded after long times of incubation. To minimize artefacts from residual water vapor, the spectra were Fourier deconvoluted with a smoothing factor of 0.3 (Kauppinen et al., 1981) but were not resolution enhanced.

The evolution of the FTIR spectra with time after mixing with D_2O are illustrated for a cytochrome *c* complex with DMPG in Fig. 6. The corresponding difference spectra remain unaltered in shape during progress of the deuteration, indicating that the same secondary structural elements are involved throughout the time regime studied. This constancy in shape of the difference spectra also demonstrates that there are no contributions to the kinetic recordings that might arise from possible shifts in band positions attributable to the hydration of the protein. Most secondary structural features seem to be involved in the deuterium exchange; in particular the α-helical band at 1651 cm^{-1} and the extended chain band between 1630–1640 cm^{-1} appear to exchange at comparable rates.

The time course of the deuteration is given in Fig. 7, in terms of the areas of the difference spectra above the baseline normalized with respect to the total area of the amide I band. Data are shown both for cytochrome *c* free in solution and complexed with DMPG. Whereas the rates of deuteration increase strongly with temperature for cytochrome *c* complexed with lipid, there is very little temperature dependence in the deuteration rate of the protein free in solution (at 40°C,

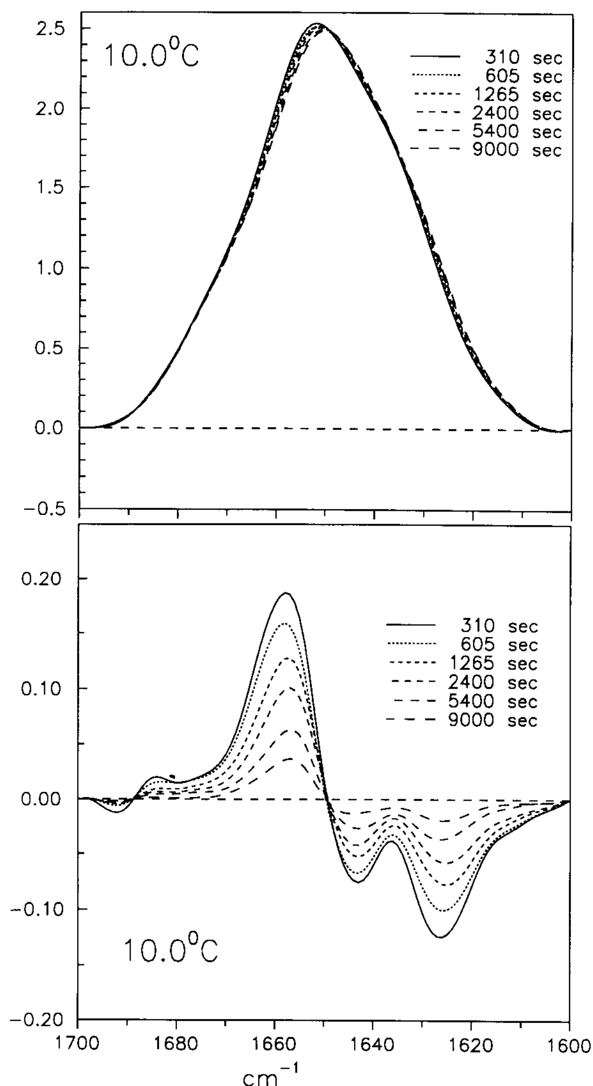


FIGURE 6 Upper: FTIR spectra (amide I region) of cytochrome *c* complexed with DMPG at 10°C recorded at the times indicated after dispersion in D_2O. Lower: difference spectra obtained by subtracting a normalized reference spectrum recorded after long times (i.e., after extensive deuteration) from the normalized spectra in the upper panel.

the rate for the latter is even slightly slower). Additionally, the number of amide groups involved in the exchange process seems to be different for the bound and unbound protein. The area of the difference spectrum for the fully protonated protein obtained from Fig. 5 by subtracting the spectrum of the maximally deuterated protein corresponds to 20% of the total intensity of the amide I band. Over the time period studied, the measurements given in Fig. 7 therefore correspond to the kinetics of approximately 13% of the total amide protons that are exchanging slowly for the protein in water (normalized area of the difference spectrum at zero time = 2.5%) and approximately 20% that are exchanging slowly for the protein bound to DMPG (normalized area of the difference spectrum at zero time = 4%). The other amide protons exchange rapidly and are already deuterated by the time of the first measurement point. These relative amounts exchanged in the two populations remained constant, independent of the temperature. From these data, it is not clear whether the sum of fast and slowly exchanging protons is the same in the free and the bound cytochrome *c*, or whether different amounts of amide groups remain unexchanged at the limiting incubation time point used for constructing the difference spectra. An independent estimate from the changes in the amide II region of the spectrum is not easily possible because of the complicated baseline arising from bands contributed by the protein side chains in this section of the spectrum. Comparison with NMR results suggests, however, that for the protein in solution some protons might remain unexchanged, whereas for the bound protein all protons are likely to be exchanged, on the time scale studied here (Wand et al., 1986; Spooner and Watts, 1991; De Jong et al., 1992).

The residual degree of protonation, $P(t)$, is expected to display a multiexponential decay, corresponding to the different groups, i, of amides that remain protonated:

$$P(t) = \sum_i A_i \exp(-k_i t) \tag{1}$$

where k_i are the rate constants for deuteration of the different groups of amides. A mean rate constant, $\langle k \rangle$, for deuteration can then be defined by:

$$\langle k \rangle^{-1} = \int_0^{\infty} \left[\frac{\sum_i A_i \exp(-k_i t)}{\sum_i A_i} \right] \cdot dt = \frac{\sum_i A_i / k_i}{\sum_i A_i}, \tag{2}$$

which corresponds to the area underneath the time course of each deuteration curve. It was found that the decay curves could all be well approximated by a double exponential time course (*solid lines* in Fig. 7), and the parameters from these fits were used to calculate the values of the mean rate constant, $\langle k \rangle$. The temperature dependences of the mean exchange constants $\langle k \rangle$ for cytochrome *c* free in solution and complexed with the various negatively charged lipids are given as Arrhenius plots in Fig. 8. Whereas $\langle k \rangle$ is relatively unaffected by temperature for unbound cytochrome *c*, it is clear that amide proton exchange is an activated process in the lipid/protein complexes. The mean rates of exchange $\langle k \rangle$ change by up to 2 orders of magnitude between 10° and 40°C (from approximately 10^{-4} s^{-1} to approximately 10^{-2} s^{-1}). Additionally, a transition-like discontinuity is observed in the Arrhenius plots of all lipid/protein complexes, which is not seen for the protein free in solution. The temperature of this transition depends on the particular lipid in the complex, in a manner which seems to correlate with whether this is in a liquid crystalline or gel state (cf. Fig. 4). This interesting feature will be dealt with further in the Discussion.

DISCUSSION

In this paper, structural changes occurring in cytochrome *c* bound to negatively charged lipid membranes have been studied both with respect to alterations in the secondary structure as registered by the amide I band in the FTIR spectrum of the protein, and with respect to alterations in tertiary

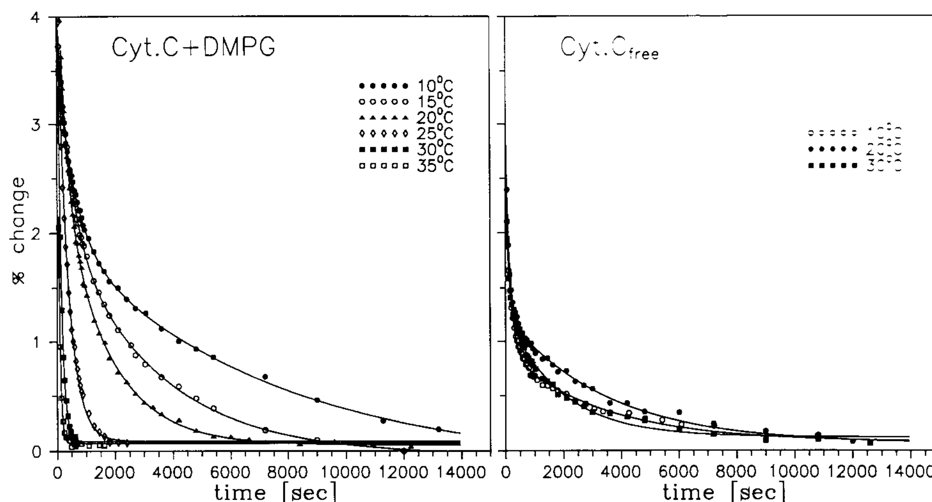


FIGURE 7 Dependence on time after dispersion in D_2O of the area above the baseline in the FTIR difference spectra (amide I region) of cytochrome *c* complexed with DMPG (*left*) and of cytochrome *c* in solution (*right*). Difference spectra were obtained by subtracting the FTIR spectrum recorded after extensive deuteration (see Fig. 6). The time courses of deuteration were recorded at the temperatures indicated.

structure as evidenced by the amide hydrogen exchange rates. The aim of this work was to identify possible couplings between the physical state of the lipid and the conformation of the protein. To this end, complexes of cytochrome *c* with a range of anionic lipids have been investigated over a range of different temperatures. The changes in secondary structure and in tertiary structure of cytochrome *c* on binding to the negatively charged lipid membranes are discussed separately below.

Secondary structural changes in cytochrome *c*/lipid complexes

The results given in Fig. 1 and Table 1 indicate that changes in secondary structure of cytochrome *c* on binding to negatively charged lipids are small. A similar conclusion was reached by Muga et al. (1991a), although no quantitation was presented. The lack of any appreciable temperature dependence in the amide I region of the FTIR difference spectra between free and bound protein at temperatures below that of denaturation further confirms this result, which holds true for complexes with all the lipids studied here. The loosening of the structure of cytochrome *c* on binding to cardiolipin that was observed by ^2H NMR (Spooner and Watts, 1991) may therefore correspond to highly dynamic tertiary structural alterations in the protein rather than to appreciable changes in the overall secondary structure. A similar conclusion has been reached recently with respect to the secondary and tertiary structure of cytochrome *c* bound to detergent micelles (De Jongh et al., 1992).

The temperature dependences of the FTIR difference spectra of the amide I band (Figs. 2 and 3) reveal no discontinuities at the gel to liquid crystalline lipid phase transition for the complexes of cytochrome *c* with DMPG and DPPG. This indicates that there is no change in the secondary structure of the protein in response to chain melting of the lipid. However, differential responses depending on the lipid involved are obtained in the temperature or denaturation, demonstrating that the destabilization of cytochrome *c* on binding to lipids observed by Muga et al. (1991a) and confirmed here can be modulated by the lipid type, even when the lipids are fully charged. It is interesting to note also that an increased susceptibility of cytochrome *c* to denaturation by urea has been observed in the presence of detergent micelles (De Jongh et al., 1992). Additionally, the deconvoluted FTIR spectra in the amide I region of denatured cytochrome *c* bound to lipid (Fig. 2) are significantly different from those of the denatured protein in solution (data not shown). They have relatively higher intensity in the region expected for random structures at around 1648 cm^{-1} for the bound than for the unbound denatured protein. This suggests that in the denatured state the lipid-bound protein is more disordered than is the free protein. Further aspects of the lipid modulation of the protein stability are revealed by the temperature dependence of the amide proton exchange rates which is discussed next.

Tertiary structural changes in cytochrome *c*/lipid complexes

Since the analysis of the structure of the amide I band indicates that any secondary structural changes taking place in cytochrome *c* upon binding to the various negatively charged lipids are small, it seems likely that the large differences observed in the amide proton exchange can be attributed to tertiary structural changes of the protein on binding. This is a feature that is shared with the molten globule state of cytochrome *c* at low pH and high ionic strength (Jeng et al., 1990). These changes may also be accompanied by a reduction in helix stability (Spooner and Watts, 1991), although the frequencies of the major bands in the amide I region of the FTIR spectrum (Table 1), which reflect the hydrogen bond stability, are hardly changed upon binding the protein to lipid. The amide protons corresponding to the majority of the secondary structural elements in cytochrome *c* bound to lipid exchange rapidly in D_2O, suggesting also that

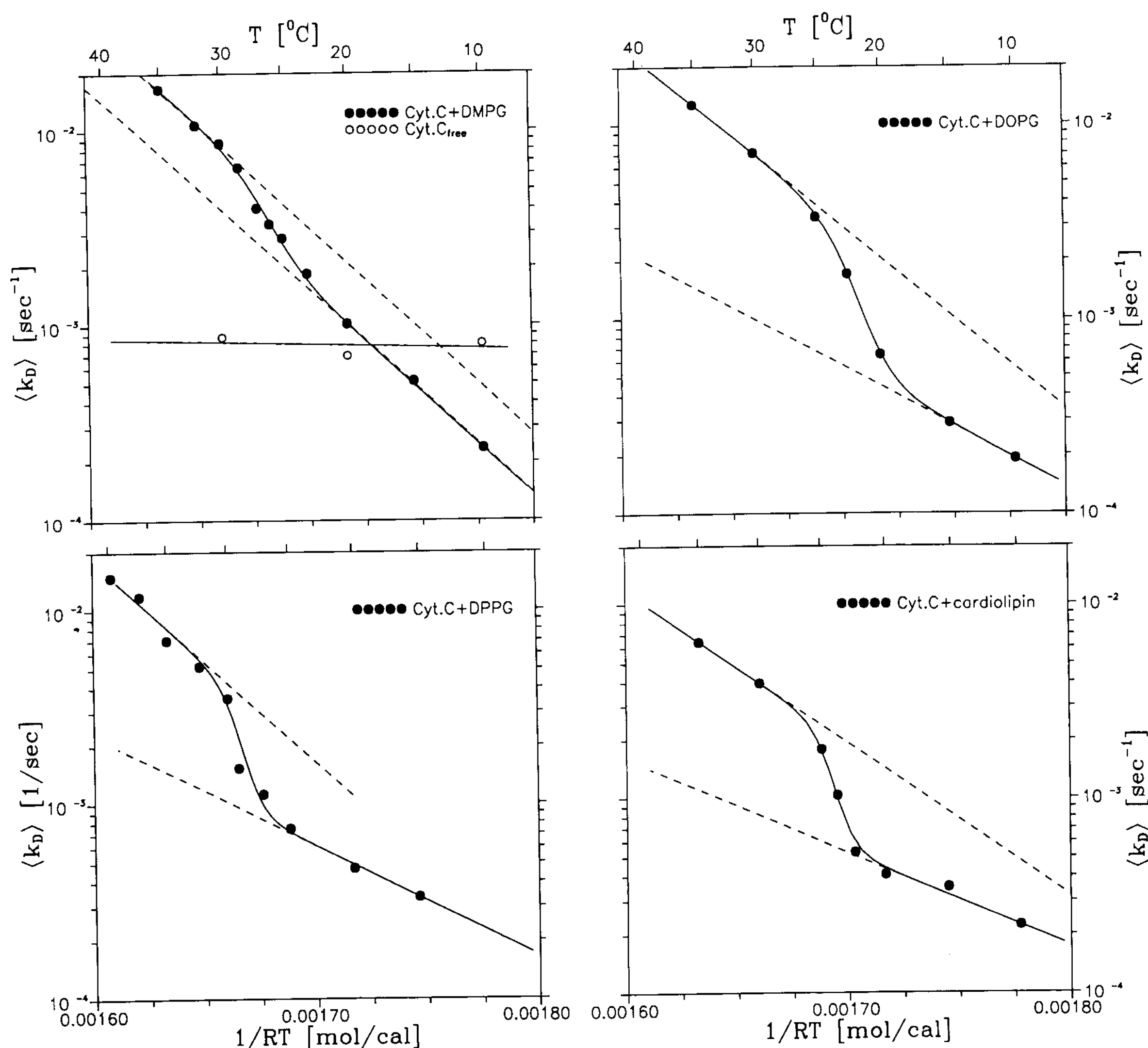
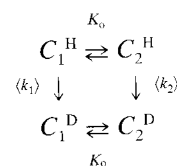


FIGURE 8 Arrhenius plots of the temperature dependence of the mean rate constants, $\langle k \rangle$, for deuteration of cytochrome *c* in solution and bound to lipids. Upper, left-hand panel: cytochrome free in solution (○) and bound to DMPG (●); lower, left-hand panel: cytochrome *c* bound to DPPG; upper, right-hand panel: cytochrome *c* bound to DOPG; lower, right-hand panel: cytochrome *c* bound to BHCL. The solid lines are fits obtained according to Eq. 3 with the parameters given in Table 2.

the slow exchange of the remainder might be due less to the inherent stability of the peptide hydrogen bonds than to the accessibility of these amide groups within the tertiary structure of the protein. In this connection, it is interesting to note that one of the two conformations characterized by resonance Raman spectroscopy for cytochrome *c* bound to lipids corresponds to a change in coordination of the iron that is accompanied by an opening of the haem crevice (Heimburg et al., 1991; Hildebrandt and Stockburger, 1989a, b). Enhanced amide proton exchange of cytochrome *c* bound to lipids or detergent micelles has been observed previously by various techniques (Spooner and Watts, 1991; Muga et al., 1991a; De Jongh et al., 1992).

The discontinuities observed in the Arrhenius plots of the amide deuteration rates for the cytochrome *c*/lipid complexes which are shown in Fig. 8 suggest that a temperature-driven transition takes place between two states or conformations, C_1 and C_2, of the protein. With the assumption that the conformational transitions are rapid compared with the deuteration rates, one has the following scheme for the deuteration:



$$\begin{array}{ccc} C_1^{H} & \overset{K_o}{\rightleftarrows} & C_2^{H} \\ \langle k_1 \rangle \downarrow & & \downarrow \langle k_2 \rangle \\ C_1^{D} & \underset{K_o}{\rightleftarrows} & C_2^{D} \end{array}$$

SCHEME 1

where $K_o = [C_2]/[C_1]$ is the equilibrium constant for the transition, and $\langle k_1 \rangle$ and $\langle k_2 \rangle$ are the mean rate constants for amide deuteration in the two states. The overall deuteration rate measured is then given by:

$$\langle k \rangle = (\langle k_1 \rangle + K_o \langle k_2 \rangle)/(1 + K_o). \qquad (3)$$

The temperature dependence of $\langle k \rangle$ is therefore determined by the activation energies, $E_{a,1}$ and $E_{a,2}$, associated with

TABLE 2 Parameters used for fitting the temperature dependences of the rate constants, $\langle k \rangle$, for deuterium exchange in Fig. 8, according to Eq. 3.

Sample	ΔH_o	T_t	$E_{a,1}$	$E_{a,2}$
	kcal/mol	°C	kcal/mol	kcal/mol
cyt *c*	0			
cyt *c*/DMPG	110	26.8	24	24
cyt *c*/DOPG	130	22.5	14	22
cyt *c*/DPPG	210	29.3	14	22
cyt *c*/BHCL	210	24.5	12	18

the rate constants $\langle k_1 \rangle$ and $\langle k_2 \rangle$, the van't Hoff enthalpy, ΔH_o, of the conformational equilibrium and the transition midpoint, T_t. The solid lines shown in Fig. 8 correspond to fits to Eq. 3 with the parameters given in Table 2. The transition temperature is lowest in the two liquid crystalline cytochrome *c*/lipid complexes (i.e., with DOPG and BHCL), whereas it is highest in the gel state complex (i.e., with DPPG). The physical state of the lipid therefore seems to have an influence on the conformational equilibrium between the substates C_1 and C_2 of the membrane-bound cytochrome *c*. This is somewhat similar to the dependence of the denaturation of the bound protein on the lipid type, where the denaturation temperature is also lowest for complexes with lipids of lower transition temperature and highest for those with the higher transition temperature (see Fig. 3).

The complex of cytochrome *c* with DMPG is particularly interesting with respect to the transition occurring in the protein, because it undergoes a chain-melting transition from gel to liquid crystalline states in the same temperature region as that of the conformational transition. Plots of the normalized degree of transition, $K_o/(1 + K_o)$, for complexes with the various lipids are given in the left-hand panel of Fig. 9. These show that not only does the transition in the DMPG complex occur at an intermediate temperature between those for the complexes with the liquid crystalline (DOPG and BHCL) and gel (DPPG) lipids, but also it is considerably broader ($\Delta H_o \approx 100$ kcal mol) than for the complexes with lipids in a single phase ($\Delta H_o \approx 200$ kcal/mol, with the exception of DOPG). This suggests that, in this case, the transition in the protein is driven by the chain-melting phase transition in the DMPG lipid. Further evidence for this comes from the comparison between the protein transition in the complex with DMPG and the lipid transition registered by the ester carbonyl band which is given in the right-hand panel of Fig. 9. To within experimental accuracy, the two transitions are virtually superimposable. The reason for this is clear. Immediately below the lipid chain-melting transition of the cytochrome *c*/DMPG complex, the sample is below the temperature at which the transition takes place in the protein bound to gel-phase lipid, but above that for the protein bound to liquid crystalline lipid. Therefore, chain melting results immediately in the transition of those proteins which are associated with the fraction of the DMPG that has entered the liquid crystalline phase and, in this special case, the protein transition is directly coupled to and completely controlled by the transition in the lipid.

In summary, the stability of the protein, with respect both to denaturation and to the tertiary structural change evidenced by the amide deuteration rates, depends on the physical state of the lipid to which it is bound, and, in the case of complexes with DMPG, the tertiary structural transition actually is controlled by the transition occurring in the lipid. This coupling between the protein and lipid may hold interesting possibilities for the control of membrane-bound enzymes in general.

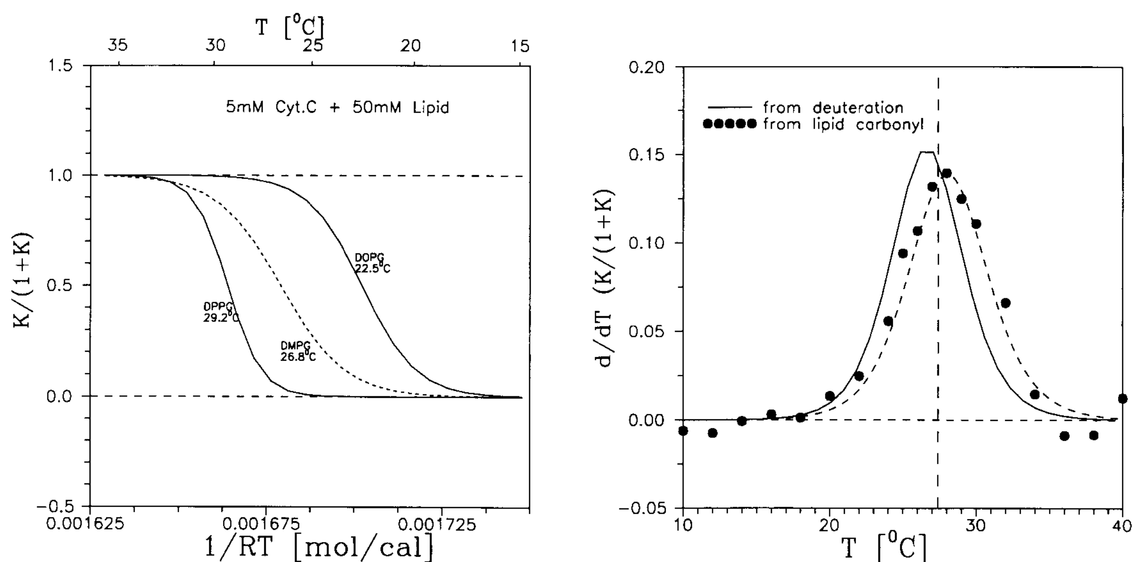


FIGURE 9 Left-hand: temperature dependence of the degree of transition, $K_o/(1 + K_o)$, obtained from the data in Fig. 8 for complexes of cytochrome *c* with (from left to right) DPPG, DMPG and DOPG. Right-hand: derivative transition curves, $d(K_o/(1 + K_o))/dT$, for complexes of cytochrome *c* with DMPG obtained for the protein (*solid line*) from the left-hand panel, and for the lipid (●) from the ester carbonyl band (see Fig. 4).

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