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Interaction of cytochrome *c* with cardiolipin: an infrared spectroscopic study ☆

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Abstract

The interactions of cytochrome *c* (cyt *c*) with cardiolipin, a major anionic phospholipid of mitochondrial membranes, and dioleoylphosphatidylglycerol (DOPG), have been compared by infrared (IR) spectroscopy. The Fourier self-deconvoluted IR spectra of the lipid carbonyl groups indicate that both cyt c^{3+} and cyt c^{2+} perturb and/or dehydrate the interfacial region of cardiolipin bilayers. Only a slight perturbation, if any, is observed in the interfacial region of DOPG bilayers. However, the phosphate head region of DOPG is perturbed by cyt c^{3+}, which was not detected in cardiolipin. The results suggest that cytochrome *c* in both redox states can partially penetrate into cardiolipin but not into DOPG bilayers. The interaction of cyt *c* with cardiolipin and DOPG is mainly hydrophobic and electrostatic, respectively. The Fourier self-deconvoluted IR spectra in the amide I region reveal that ca. 10% of the cyt c^{3+} α-helix unfolds to random coil upon binding to cardiolipin bilayers. However, only very slight secondary structural changes, if any, were detected when cyt c^{3+} binds to DOPG bilayers.

Keywords: Protein secondary structure; Phospholipid; Cytochrome *c*; Cardiolipin; IR spectroscopy

The interaction of cytochrome *c* (cyt *c*) and cardiolipin has been extensively studied because cardiolipin accounts for 10 to 20% of the phospholipid in the inner mitochondrial membrane of mammalian cardiac tissue [1], and it is required for optimal cytochrome *c* oxidase activity [2–4]. It has been suggested that cardiolipin promotes binding of cyt *c* at the cytochrome *c* oxidase active site [5] and thereby facilitates the mitochondrial respiratory process. Cardiolipin interacts strongly with cyt *c* [6,7]. RR [8], EPR [9] and ^{31}P-NMR [10] spectroscopic studies indicate that the heme crevice in cyt *c* assumes an open structure accompanied by the five-coordinate, high-spin iron upon binding to cardiolipin. Unbound cyt *c* has a six-coordinate, low-spin iron configuration. The structure of the cyt *c* backbone also changes upon cardiolipin binding. Deuterium NMR spectroscopy indicates that the protein secondary structure is perturbed, such that no stable

Abbreviations: IR, infrared spectroscopy; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; RR, resonance Raman; Card, cardiolipin; DOPG, dioleoylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; Tris, tris[hydroxymethyl]aminomethane hydrochloride

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α-helices can exist in the protein backbone with a lifetime longer than around 10^{-6} s, when cyt c is bound to cardiolipin [11]. A more detailed study using magic-angle spinning NMR has been reported recently [12]. When cyt c binds to cardiolipin the methionine 80 ligand is displaced from the heme, and methionine 65 which is located within a short α-segment is perturbed. The bound cyt c induces new modes of slow motions in the lipid assemblies except for sites within the unsaturated fatty acyl chain regions which are selectively mobilized in the complex with protein.

Many of the above observations are not unique to cardiolipin. Similar results have been observed in other anionic phospholipids such as phosphatidic acid (PA), phosphatidylinositol, phosphatidylglycerol (PG) and phosphatidylserine (PS) [13,14]. An infrared study has shown that the tertiary structure of cytochrome c^{3+} (cyt c^{3+}) is destabilized upon binding to PG [15,16]. ^{2}H-NMR spectra have suggested that there is a small change in the average conformation of the PS head group [14]. Raman spectroscopy has shown that the acyl chain of zwitterionic phosphatidylcholine (PC) is perturbed by cyt c; cyt c penetrates into the hydrophobic region of the bilayer [8]. On the other hand, ^{2}H-NMR indicates that there is no modification in the acyl chains of PS, PG or PC upon binding of cytochrome c [14]. Resonance Raman spectroscopy has shown that the heme crevice of cyt c^{3+} assumes an open structure accompanied by iron in the five-coordinate, high-spin configuration, upon binding to PG [17–20].

With many similarities between cardiolipin and other anionic phospholipids, it is somewhat surprising that cardiolipin is the major single anionic lipid for mitochondrial electron transport. So far only one aspect has distinguished cardiolipin from other anionic phospholipids. Freeze-fracture electron microscopy, ^{31}P-NMR, and X-ray diffraction studies have shown that the basic bilayer structure is retained when cyt c binds to PG or PS, while a non-bilayer phase is induced when cyt c binds to cardiolipin [6,13]. There is no other comparative study which distinguishes cardiolipin from other anionic phospholipids. The degree of penetration of cyt c into cardiolipin bilayer may be different from that into other anionic bilayers. The structural change of the protein upon binding to cardiolipin may be different from that upon binding to other anionic bilayers. Furthermore, the interaction of cyt c with cardiolipin before (cyt c^{2+}) and after (cyt c^{3+}) the electron transfer may be different.

In order to answer these questions, we have investigated the interactions of cyt c^{3+} and cyt c^{2+} with cardiolipin and compared these interactions with another anionic phospholipid, dioleoylphosphatidylglycerol (DOPG), using infrared spectroscopy (IR). DOPG is studied for comparison because it is a precursor of cardiolipin. IR has been successfully utilized in studying membrane and protein secondary structures (see ref. [21] for a review). Due to the strong H_2O absorption at 1640 cm^{-1} most previous IR studies examining the interfacial carbonyl groups (1740 cm^{-1} region) [22,23] or the protein amide I region (1650 cm^{-1}) [15,16] were carried out in D_2O. Since the protein and lipid conformations and their interactions with water and with each other may be different in H_2O and in D_2O, it is important to study the system in H_2O in order to be more biologically relevant.

In this investigation, we have been successful in observing both the carbonyl stretching in the lipid interfacial region and the protein amide I bands by carefully following the methods described by refs. [24–26]. We have used resolution-enhancement to visualize the individual components in the bands and Gaussian curve-fitting to calculate the relative intensities of the components [21,27,28]. Our data clearly indicate that both cyt c^{3+} and cyt c^{2+} perturb the interfacial region of cardiolipin bilayers but not the phosphate head region. However, cyt c^{3+} perturbs the phosphate region of DOPG, but not the interfacial region. A significant amount (ca. 10%) of the cyt c^{3+} α-helix unfolds to random coil or an irregular conformation upon binding to cardiolipin. Only a very slight secondary structural change in cyt c^{3+} was detected upon binding to DOPG bilayers.

1. Materials and methods

Cytochrome c from horse heart (type VI) was purchased from Sigma Chemical Co. (St. Louis, MO). It was purified by a conventional method [10,11]. Briefly, cyt c^{3+} was prepared by addition of

excess $K_3Fe(CN)_6$ to the crude aqueous cyt *c*. The solution was then dialyzed for approximately 8 hours at 4°C against water with frequent changes of water. The protein sample was put into Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.0) by ultradialysis (Amicon YM-3 ultrafiltration membrane and cell). Cyt c^{2+} was obtained by addition of a small amount of sodium dithionite under N_2 [29]. The protein purity and concentration were determined by UV/Vis absorption using a Cary 17 spectrophotometer. Cyt c^{3+} has a Soret maximum absorbance at 409 nm and an α peak at 528 nm ($\epsilon_{528} = 1.12 \times 10^4$ M^{-1} cm^{-1}). Cyt c^{2+} has a Soret maximum absorbance at 416 nm and an α peak at 550 nm ($\epsilon_{550} = 2.77 \times 10^4$ M^{-1} cm^{-1}) [30]. A typical concentration of cyt *c* used for IR was 1 mM.

Lyophilized cardiolipin and dioleoyl-PG (DOPG) were purchased from Avanti Polar Lipids (Birmingham, AL), and were used without further purification. The lipid multibilayers were formed by hydrating the mixtures with Tris buffer solution (50 mM Tris/100 mM NaCl, pH 7.0) and vortexing. To ensure complete dispersion, the lipid multilayers were frozen, thawed and vortexed at least three times. The final lipid concentration of dispersed lipid was in the range of 6.7 mM. Protein/lipid complexes were prepared by vortex-mixing 1 ml of 6.7 mM hydrated lipid in buffer with 10 μl of 7.4 mM protein solution. For cyt c^{2+}/cardiolipin complex, N_2 was blown through the suspension and a small amount of dithionite was added. The suspension was allowed to equilibrate at room temperature and then centrifuged with a Beckman L7 Ultracentrifuge (45000 rpm, 1 h, 4°C). The supernatant containing the unbound protein was decanted and its UV/Vis spectrum was obtained to calculate the amount of bound protein by difference. Typically 90% of the protein was bound, resulting in 1/100, mol/mol, ratio of protein to lipid. The pellet of protein/lipid complex was used for the IR spectra. The integrity of cardiolipin or DOPG before and after cyt c^{3+} or cyt c^{2+} binding was checked by measuring the intensity and band frequency of olefinic C–H stretching mode at 3010 cm^{-1} as well as methylene and methyl C–H bands in the region of 2800 – 3000 cm^{-1}. If the lipid were autoxidized by cyt c^{3+} or reduced by dithionite which was used for making cyt c^{2+}/cardiolipin, the band intensity and band frequency of these acyl chain bands would change. All of our samples remained intact either from oxidation or reduction for the duration of the experiment. The oxidation states of cyt *c* were checked by UV/Vis (cyt c^{3+}: λ_{Soret} = 409 nm, λ_α = 528 nm, cyt c^{2+}: λ_{Soret} = 416 nm, λ_α = 550 nm) before and after the IR experiment.

1.1. Spectra

A flow-through transmission cell with CaF_2 windows and a 6-μm tin spacer (SPECAC, Fairfield, CT) was used for the protein solution. Viscous protein solutions were difficult to load into a 6-mm path-length cell, but by simultaneously pulling a vacuum on one fitting and applying pressure with the sample in a syringe on the other fitting, these cells could be loaded [26]. For samples with lipid, which are paste-like, a demountable transmission cell (Buck Scientific, East Norwalk, CT) was used. The paste-like sample was spread on a CaF_2 window and assembled into a cell with a 6-μm spacer. Cells were placed in a cell mount thermostated by a flow of ethylene glycol/water from a constant-temperature bath ($\pm$0.1°C). The temperature was monitored by two button-shaped Pt thermistors (Omega, Stamford, CT, model ON-909-44004, 2252 ohms at 25°C, resistance measured with a 4.5 digit multimeter) for accurate temperature measurement. One thermistor was taped to the bottom of the back plate of the transmission cell and the other thermistor was taped to the top of the front plate of the transmission cell such that the sample was exactly halfway between the two thermistors. The temperature of the sample was taken as the average of the two temperatures which never differed more than 1°C. Spectra at 2 cm^{-1} resolution were recorded with a Mattson Cygnus 100 Fourier Transform Infrared Spectrometer with a TGS detector. One thousand interferograms were averaged for each spectrum. UV/Vis absorbance spectra of the IR sample in the IR cell were recorded to verify the integrity of the oxidation state of the cyt *c* before and after each IR experiment.

1.2. Analysis of spectra

Difference spectra (water- and water vapor-free) were obtained by following the criteria described in

the literature [25,26]. In the first step, a buffer spectrum was subtracted from the sample spectrum by varying the subtraction factor until a flat base line from 2000 to 1700 cm^{-1} was obtained. In the second step, a water vapor spectrum was subtracted from the difference spectrum obtained in step 1 by interactive subtraction to obtain a flat base line from 2000 to 1700 cm^{-1}. Care was taken not to over-subtract water vapor or not to under-subtract it. When the water vapor is over-subtracted, sharp peaks at 1737, 1731, 1703, 1698, 1656, 1650 and 1536 cm^{-1} appear in the second-derivative difference spectrum of the sample. When the water vapor is under-subtracted, sharp peaks at 1734, 1700, 1695, 1653, 1646 and 1558 cm^{-1} appear in the second-derivative difference spectrum of the sample. The origin of these peaks are discussed by Larrabee and Choi [26]. It should be noted that all spectral features reported in this study were checked for reproducibility using at least 5 separate sample preparations.

Broad featureless, lipid interfacial carbonyl stretching bands and protein amide I bands were resolved by using Fourier self-deconvolution [31]. The optimal parameters were found to be 15 cm^{-1} for the half bandwidth and 2 for K, the resolution enhancement factor. A Lorentzian line-shape function and a Bessel apodization function were used. Curve fitting was performed with an iterative Gaussian fitting program. Deconvoluted spectra were fitted to Gaussian shaped bands, and the intensity of each band was calculated from the area of the final fitted band.

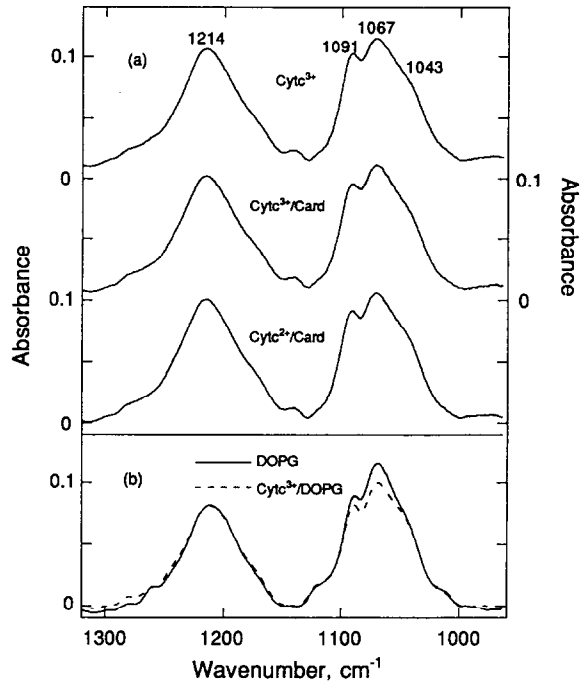

Fig. 1. Difference IR spectra in the PO_2^- stretching (νPO_2^-) region of (a) pure cardiolipin, cyt c^{3+}/cardiolipin (1/100, mol/mol), and cyt c^{2+}/cardiolipin (1/100, mol/mol); (b) pure DOPG, cyt c^{3+}/DOPG (1/100, mol/mol), in Tris buffer (pH 7.0) at 20.5°C.

2. Results and discussion

2.1. Structural properties of membrane phospholipids

Fig. 1a shows the IR spectra of cardiolipin, cyt c^{3+}- and cyt c^{2+}-bound cardiolipin in the PO_2^- stretching region. The three spectra can be superimposed exactly. The band at 1214 cm^{-1} is assigned to an asymmetric PO_2^- stretching mode ($\nu_{as}PO_2^-$) while the three peaks at 1091, 1067 and 1043 cm^{-1} have been related to symmetric PO_2^- stretching modes ($\nu_sPO_2^-$) [22]. The frequency and bandwidth of $\nu_{as}PO_2^-$ reflects the degree of hydration and mobility, respectively, of the phosphate groups. The pattern of $\nu_sPO_2^-$ bands reflects the conformation of torsional angles of the two P–O ester bonds [32,33]. None of these bands shift upon cyt c^{3+} or cyt c^{2+} binding at 20.5°C and at 40.2°C. There seems to be no disturbance of the phosphate region by the protein within the 2 cm^{-1} resolution limit of the IR spectra. Fig. 1b shows the IR spectra of DOPG and cyt c^{3+} bound-DOPG in the PO_2^- stretching region. The two spectra cannot be superimposed exactly. The band frequencies of $\nu_{as}PO_2^-$ and $\nu_sPO_2^-$ are the same, but the $\nu_{as}PO_2^-$ bandwidth slightly broadens and the $\nu_sPO_2^-$ band intensity decreases upon cyt c^{3+} binding. This indicates that the phosphate region of DOPG is slightly mobilized and the conformation of torsional angles of the two P–O ester bonds [32,33] changes upon cyt c^{3+}-binding to DOPG. Since the perturbance of the phosphate region of DOPG by cyt

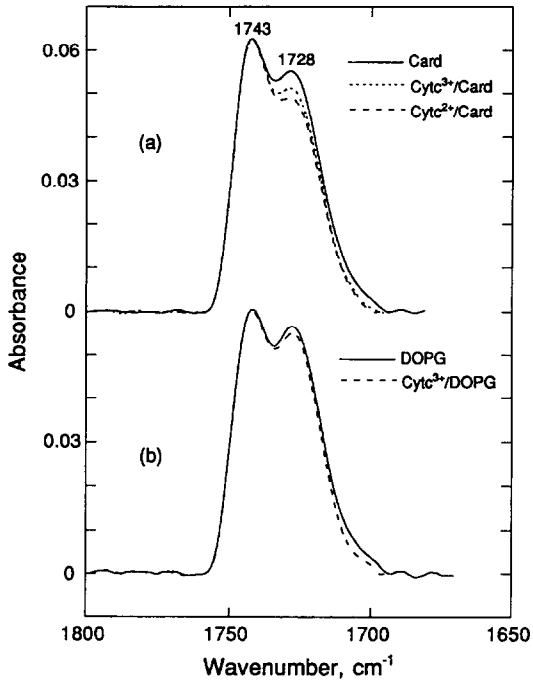

Fig. 2. Fourier self-deconvoluted spectra in the interfacial C=O stretching (νCO) region of (a) pure cardiolipin, cyt c^{3+}/cardiolipin (1/100, mol/mol), and cyt c^{2+}/cardiolipin (1/100, mol/mol); (b) pure DOPG, cyt c^{3+}/DOPG (1/100, mol/mol), in Tris buffer (pH 7.0) at 20.5°C. Deconvolution parameter: half bandwidth = 15 cm^{-1}, resolution enhancement factor = 2.

c^{3+} is detectable within the 2 cm^{-1} resolution of the IR experiment and that of cardiolipin is not detectable, we can conclude that cyt c^{3+} interacts with DOPG more electrostatically than with cardiolipin. Our result supports a previous study on DOPG which also concluded that the interaction of DOPG and cyt c^{3+} is electrostatic [16].

Fig. 2a shows the deconvoluted spectra of the interfacial region of cardiolipin, cyt c^{3+}- and cyt c^{2+}-bound cardiolipin at 20.5°C. It is apparent that the band intensity of 1728 cm^{-1} decreases upon cyt c^{3+} or cyt c^{2+} binding. Initially, these two bands at 1743 and 1728 cm^{-1} were assigned to the carbonyl groups of the $sn-1$ and $sn-2$ chains, respectively [34,35]. However, a more recent report [36] argues that the lower-frequency component is mainly due to carbonyl groups that are hydrogen-bonded to water, while the higher-frequency feature is assigned to non-hydrogen-bonded carbonyl groups. This spectral feature is particularly sensitive to the hydration of the carbonyl groups in calcium-bound anionic phospholipid bilayers [22,23,37]. Whether the bands are due to a different conformation or to differences in hydration, the change of their band intensities can be used as an indicator for changes in the interacial region. The relative integrated intensity of the 1728 cm^{-1} band relative to the intensity of the entire carbonyl envelope calculated from the area of each component generated by Gaussian curve fitting is 74%, 67% and 65% for pure cardiolipin, cyt c^{3+}-bound cardiolipin and cyt c^{2+}-bound cardiolipin, respectively. This indicates that cyt c in both redox states perturbs the interfacial carbonyl region. If Blume et al. [36] are correct in their assignment of the 1743 and 1728 cm^{-1} bands, we can say that the interfacial region of the cardiolipin bilayers is partially dehydrated by cyt c. Our conclusion supports the proposal [38], that cytochrome c may penetrate into the membrane interior, allowing hydrophobic interaction with the bilayer. Although we consistently observed the 2% area difference between cyt c^{3+} and cyt c^{2+} in five different experiments at both 20.5 and 40.2°C, the difference is too small to draw any conclusions. The relative intensity of the 1728 cm^{-1} band in DOPG is changed from 70% to 69% when cyt c^{3+} is bound (Fig. 2b). Again the 1% difference is too small to presume a difference. It is safe to say that hardly any perturbation occurs in the interfacial region of DOPG bilayers due to cyt c^{3+}. It is interesting to note that the interfacial region of dimyristylphosphatidylglycerol (DMPG) was altered by apocytochrome c in D_2O [15].

2.2. Cytochrome c structure

Fig. 3 shows the Gaussian deconvoluted spectra of cyt c^{3+}, cyt c^{3+}/cardiolipin and cyt c^{3+}/DOPG in the amide I region (1700–1600 cm^{-1}). The resolved peaks are located at 1682, 1672, 1656, 1648, 1635 and 1617 cm^{-1}. These values agree within experimental error (± 2 cm^{-1}) with those obtained from the amide I IR second-derivative spectrum by refs. [24,25]. The amide I band arises mostly from the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and CN stretch-

ing modes. The frequency of these vibrations is sensitive to the hydrogen bonding and coupling between transition dipoles [39], which is the origin of a broad amide I band envelope where many C=O stretching bands of amide groups of different geometry are overlapped. These overlapped bands can be partially resolved by resolution enhancement. Although each component has not yet been unambiguously assigned to a specific secondary structure such as α-helix, β-strand, turns, etc. [21,28,40], it is believed that the resolution enhancement technique provides a sensitive diagnostic tool for monitoring, in relative terms, the nature of changes in the conformation of the protein backbone [21]. The relative intensity of each band was obtained by calculating the area of the bands generated from Gaussian curve-fitting. The results are given in Table 1. The relative intensities of the bands of cyt c^{3+} agree well, within experimental error ($\pm 5\%$), with [25] values obtained from the second-derivative method. The most striking difference between the pure cyt c^{3+} and cardiolipin-bound cyt c^{3+} is the relative intensity of 1656 cm^{-1} and 1648 cm^{-1} bands. The relative intensity of the 1656 cm^{-1} band in cyt c^{3+} is decreased from 40% to 30%, while the intensity of the 1648 cm^{-1} is increased from 9% to 24% upon cardiolipin binding.

Table 1
Frequencies (ν, ± 2 cm^{-1}) and relative intensities (I, $\pm 5\%$) of the amide I component bands of Cyt c^{3+}, Cyt c^{3+}/cardiolipin (1/100, mol/mol), and Cyt c^{3+}/DOPG (1/100, mol/mol) obtained from Fig. 3

cyt c^{3+}		cyt c^{3+}/Card		cyt c^{3+}/DOPG	
ν	I	ν	I	ν	I
1682	3	1681	5	1682	5
1672	22	1670	17	1671	16
1656	40	1657	30	1656	35
1648	9	1648	24	1648	14
1635	19	1634	14	1634	19
1619	7	1622	10	1620	11

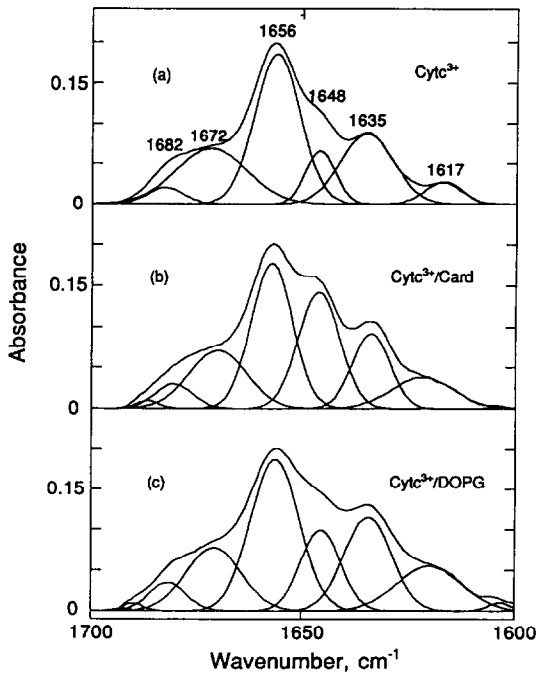

Fig. 3. Fourier self-deconvoluted and individual Gaussian component spectra in the amid I region of (a) free cyt c^{3+}, (b) cyt c^{3+}/cardiolipin (1/100, mol/mol) and (c) cyt c^{3+}/DOPG (1/100, mol/mol) in Tris buffer (pH 7.0) at 20.5°C. Deconvolution parameter: half bandwidth = 15 cm^{-1}, resolution enhancement factor = 2.

Although all of the components of the amide I band have not yet been unambiguously assigned, there is a general consensus that the 1656 and 1648 cm^{-1} bands in cyt c are due to α-helix and random coil, respectively [15,16,24,25,27]. These band assignments are restricted to cyt c [41–47]. Therefore we can say that about 10% of the α-helix in cyt c^{3+} loosens to random coil upon cardiolipin binding. This conclusion is qualitatively consistent with previous ^{2}H-NMR findings that binding of cyt c^{3+} to cardiolipin bilayers eliminates the existence of stable α-helices with a lifetime longer than around 10^{-6} s [10,11]. There are some differences in the relative intensities of other bands before and after cardiolipin-binding, but the differences ($\leq 5\%$) are not significant enough to draw a conclusion.

There is no intensity change of any component in cyt c^{3+} upon DOPG-binding within experimental error ($\pm 5\%$). There may be a very slight change, if any, in the protein secondary structure by DOPG. This is consistent with the previous work where no significant intensity change of the α-helix bands was detected for DOPG-bound cyt c^{3+} in D_2O [16]. The result was interpreted as a lipid-mediated loosening and/or destabilization of the protein tertiary struc-

ture rather than a change of the protein secondary structure.

3. Conclusion

The results reported here strongly suggest that there is a specific interaction of cardiolipin with cytochrome *c* compared with another anionic phospholipid, DOPG. Cyt *c* in both oxidation states penetrates into the cardiolipin membrane interior without perturbing the phosphate head region, allowing hydrophobic interaction with the bilayer. Cyt c^{3+} does not penetrate into the interfacial region of DOPG bilayers, but only interacts with the phosphate head region electrostatically. Cardiolipin uncoils about 10% of α-helix of cyt c^{3+}, while DOPG does not significantly change the protein secondary structure. We could not detect the difference between cyt c^{3+} and cyt c^{2+} in the interaction with cardiolipin.

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