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Interaction of Ferricytochrome *c* with Cardiolipin Multilayers: A Resonance Raman Study

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Abstract: The complex formed by the interaction between cardiolipin and ferricytochrome *c* exhibits the accepted resonance Raman spectroscopic frequency markers, implying that the iron atom is reduced by the association. No obvious reductant exists, however, in the system to induce this change. In comparison to solution spectra of ferricytochrome *c*, the visible absorption spectra of the cardiolipin–ferricytochrome *c* complex show slight shifts of the α, β bands but do not exhibit the characteristic features of the reduced ferrocytochrome *c* species. The C–H stretching mode region of the spontaneous Raman spectrum of the lipid component of the system indicates that the number of gauche conformers along the cardiolipin acyl chains has increased upon interaction with the protein and that the double bond regions (9,10 and 12,13 cis double bond positions) of the acyl chains are involved in the formation of the complex. Interaction of the protein with cardiolipin appears to change both the porphyrin ring conformation and heme coordination to mimic that of reduced cytochrome *c*; however, no electron transfer occurs, and the iron atom remains in the ferric state.

Because of the role in energy transduction of electron-transferring proteins and enzymes in the mitochondrial inner membrane, components of the inner membrane have been the subject of intense study for the last 2 decades.[1,2] Cytochrome *c*, one of the smallest of the proteins associated with the inner membrane, is thought to be a mobile electron-transfer agent that diffuses within the space between the inner and outer mitochrondial membranes and shuttles electrons among the other more firmly bound cytochromes.[3] Probably spanning the lipid bilayer, cytochrome *c* oxidase, a major component in the electron transport chain, forms an integral part of the mitochondrial membrane. An immobilized lipid annulus surrounding the oxidase is involved with the binding of cytochrome *c* and is essential for the oxidation of ferrocytochrome *c*.[4,5] This lipid boundary layer requires at least three to five molecules of cardiolipin per monomer of oxidase in order for the enzyme to show activity.[4]

Cardiolipin also interacts specifically with cytochrome *c*. In a study of mixed lipid systems in which cardiolipin was a constituent, Brown and Wüthrich[6] demonstrated that this lipid binds preferentially to cytochrome *c*, separating the protein from the more fluid lipid matrix. DeKruijff and Cullis[7] showed in ^{31}P NMR studies that cytochrome *c* interacts with cardiolipin, forming a complex which is interpreted as having domains in the hexagonal (H_{II}) lipid phase. These authors suggest that the protein may be oriented within the aqueous channels provided by the H_{II} phase.[7]

Vibrational spectroscopy provides versatile techniques for identifying and clarifying the various structural reorganizations displayed by phospholipid bilayer systems.[8] Specific Raman and infrared frequency shifts, bandwidths, and intensity alterations sensitively reflect conformational and dynamical changes within the discrete head group, glycerol backbone, and acyl chain regions of the phospholipid assembly. Since resonance Raman spectroscopic markers exist for identifying the oxidation state and spin state, as well as conformational changes in heme chromophores,[9-15] spontaneous Raman and resonance Raman techniques are ideal for characterizing the interactions of bilayer lipids with various heme proteins. For example, distinct spectral features in the Raman spectral C–H stretching mode region monitor the interchain and intrachain order–disorder processes of the bilayer acyl

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chains and identify perturbations to unsaturated portions of the lipid chains.[8,16] For heme proteins, resonance Raman lines for the in-plane porphyrin modes in the 1000–1700-cm^{-1} region not only provide sensitive markers for assigning the oxidation and spin states of the central metal but also present the basic vibrational frequency data for performing normal coordinate analyses.[10,17,18] In the present study we investigate a characteristic lipid–protein interaction for the cardiolipin–ferricytochrome *c* complex by using Raman and resonance Raman spectroscopic data. Since for the complex the 514.5-nm Ar^+ laser exciting line overlaps the vibronic β absorption band of ferricytochrome *c*, we are able to monitor conveniently both the resonance-enhanced Raman lines corresponding to the 1000–1700-cm^{-1} heme protein modes and the spontaneous Raman lines originating from the 2800–3100-cm^{-1} acyl chain C–H stretching modes of the lipid matrix. Although the cardiolipin complex is formed with the oxidized cytochrome *c* species, the resonance Raman frequency shifts reflect the reduced, ferrocytochrome *c* state. This suggests structural distortions within the heme system that result in a weakening of the bonds defining the porphyrin ring.

Experimental Section

Raman spectra were recorded with a Ramalog 6 spectrometer (Spex Industries, Metuchen, NJ) equipped with a NIC-1180 data system (Nicolet Inc., Madison, WI) for spectral accumulation. Laser excitation at 514.5 nm was obtained from a Coherent Radiation (Palo Alto, CA) CR-3 argon ion laser. Approximately 200 mW of power was used in recording the spontaneous Raman spectral data, while about 30 mW of power was used in obtaining the resonance Raman data. Raman frequencies, calibrated with Ar^+ lines, are reported to ±2 cm^{-1}. Sample temperatures were kept constant between 10 and 15 °C by using a thermoregulated sample holder. Visible spectra were obtained with a grating spectrometer by using Northern Scientific diode array detection. Electron paramagnetic resonance spectra were obtained with an E-line Series Varian X-band spectrometer.

Ferricytochrome *c* (Sigma VI Horseheart) was purified by chromatography through a CM-cellulose column according to the procedure of Brautigan et al.[19] The integrity of the ferricytochrome *c* was verified by visible spectra. The cardiolipin (bovine heart) was used within 1 week after receipt from Avanti Polar Lipids. Cardiolipin–ferricytochrome *c* complexes will form only with pure starting materials. The solutions were rendered oxygen free by blowing a stream of nitrogen over the protein or lipid solution for several hours while stirring. Oxygen was eliminated from the TRIS,[27] HEPES,[27] and phosphate buffer solutions by bubbling nitrogen through the liquid.

In order both to oxidize and to eliminate possible impurities in cardiolipin that are capable of reducing ferricytochrome *c*, cardiolipin preparations were treated by adding ferricyanide to an aqueous dispersion of the disodium salt of the lipid. A 1 M sodium chloride solution was then added to precipitate the cardiolipin. This precipitate was repeatedly washed by successive treatments with the 1 M NaCl solution and then separated by centrifugation until no further yellow was visually detected.

Ferricytochrome *c* added to a dispersion of cardiolipin in either 0.1 M pH 7 TRIS or HEPES buffer forms a complex manifesting a slightly deeper reddish brown color than that of ferricytochrome *c* alone. This complex was pelleted at the bottom of melting point capillaries by spinning in a hematocrit centrifuge. The Raman spectra were observed directly from these capillary tubes. Samples containing approximately 1 M NaCl exhibited no change in their Raman spectra in comparison to the buffered samples in which no NaCl was added. The cardiolipin–ferricytochrome *c* complexes were formed with mole ratios of lipid to protein ranging from 8:1 to 40:1. No attempt was made to determine the stoichiometries of the complexes. All cytochrome *c* concentrations were greater than the minimum stoichiometric binding ratio given by DeKruijff and Cullis.[7]

Results and Discussion

Differences between the resonance Raman spectra of a solution of ferricytochrome *c* and an aqueous dispersion of the cardiolipin–ferricytochrome *c* complex are dramatic. In particular, the vibrational frequencies commonly used for oxidation state markers

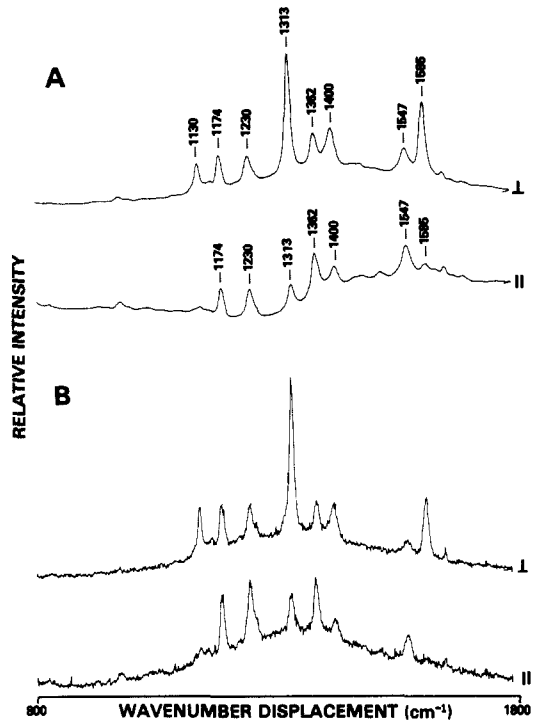

Figure 1. Resonance Raman spectrum of (A) the cardiolipin–ferricytochrome *c* complex and (B) a ferrocytochrome *c* solution in the 800–1800-cm^{-1} region for parallel (∥) and perpendicual (⊥) polarizations. Raman spectra are excited by the 514.5-nm Ar^+ ion laser line.

shift, respectively, from 1375 and 1562 cm^{-1} for the oxidized state to 1362 (ν_4, p) and 1547 cm^{-1} (ν_{11}, dp) as the complex forms.[11,12] A comparison between spectra of the cardiolipin–ferricytochrome *c* complex and solution spectra of the reduced ferrocytochrome *c* species alone (Figure 1) indicates that the resonance Raman spectrum reflecting the heme chromophore of the lipid–protein complex is nearly identical with that of the reduced ferrocytochrome *c* species; that is, the oxidation state markers of 1547 and 1362 cm^{-1} are similar in both systems. In order to confirm the specificity of cardiolipin for inducing the observed spectral changes in ferricytochrome *c*, the resonance Raman spectrum of a solution of ferricytochrome *c* was compared to an aqueous dispersion of a (DPPC) dipalmitoylphosphatidylcholine–ferricytochrome *c* complex. In this case, spectra for both systems exhibited the characteristic Raman frequencies for the oxidized ferricytochrome *c* species. As a further precaution, the cardiolipin used to form the lipid–ferricytochrome *c* complex was washed with a ferricyanide solution in order to oxidize any residual impurities which might reduce the heme protein. In addition, treatment with potassium ferricyanide inhibits possible photoreduction of ferricytochrome *c*.[20] The ferricytochrome *c* complex formed from the treated cardiolipin still exhibited the characteristic resonance Raman lines similar to those of reduced cytochrome *c*. The effect persisted even when residual amounts of ferricyanide remained in the solution containing the lipid–heme protein complex. The preparation and manipulation of the samples prior to obtaining the Raman spectra were conducted both in the dark and in the absence of oxygen for systems involving either the TRIS or HEPES buffer. Further, in order to demonstrate the absence of photoreduction during the resonance Raman measurement, the EPR spectrum of the cardiolipin–ferricytochrome *c* complex was measured at liquid nitrogen temperature. This spectrum was characteristic of the frozen ferricytochrome *c* solution. There was no reduction in the intensity of the EPR Fe(III) signal after irradiating the sample at room temperature with 50 mW of laser power at 514.5 nm for over an hour. This treatment corresponds to a higher excitation power and longer radiation time than that

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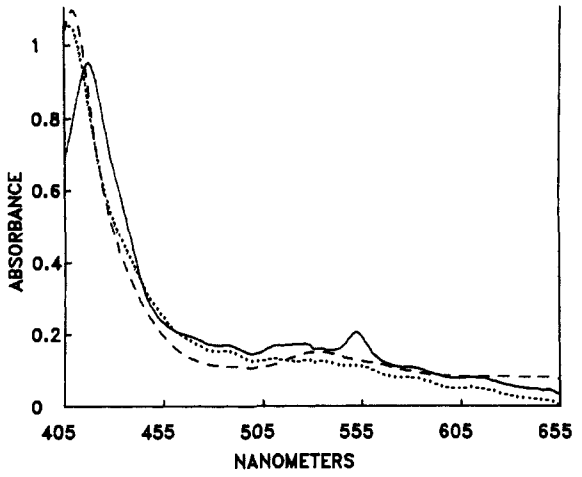

Figure 2. Visible solution absorption spectra of ferricytochrome *c* (---), cardiolipin–ferricytochrome *c* complex (···), and the complex to which some dithionite had been added to reduce the iron (—). A neutral density filter was removed for the spectra of the complex to approximate the apparent intensity of the cytochrome *c* solution and partially compensate for the scattered light upon introduction of the lipid.

used in acquiring the resonance Raman data.

A complex of ferrocytochrome *c* and cardiolipin was formed by reducing an oxygen free solution of ferricytochrome *c* with a small portion of ascorbate to which was added the cardiolipin dispersion at pH 7. The resonance Raman spectrum of the resulting system was also nearly identical with that of ferrocytochrome *c* and the cardiolipin–ferricytochrome *c* complex illustrated in Figure 1 (data not shown). (The α, β band region of the visible absorption spectrum of the cardiolipin–ferrocytochrome *c* complex was also similar to the visible spectrum of ferrocytochrome *c* alone.)

The visible absorption spectrum of the cardiolipin–ferricytochrome *c* complex shown in Figure 2 differs slightly from that of ferricytochrome *c* alone. The α and β bands at approximately 520 nm have shifted and decreased in intensity. The spectrum of the complex, however, is quite different from the visible spectrum observed for the reduced ferrocytochrome *c* species. (Spectra of the reduced species were obtained after adding small amounts of dithionite to the cardiolipin–ferricytochrome *c* complex.) Thus, the electronic spectrum of the cardiolipin–ferricytochrome *c* complex reflects the oxidized species, while the resonance Raman spectrum displays the in-plane porphyrin vibrational modes that are analogous in frequency and intensity to those of the reduced form of cytochrome *c*. Visually, the cardiolipin–ferricytochrome *c* complex does not show the characteristic salmon pink color of ferrocytochrome *c*.

The spontaneous Raman spectrum of the cardiolipin–ferricytochrome *c* complex in the 2800–3100-cm^{-1} lipid acyl chain C–H stretching mode region is shown in Figure 3. A comparison with the spectrum of cardiolipin alone, also displayed in figure 3, indicates that the symmetric methylene stretching mode has shifted significantly to higher frequencies from 2853 to 2857 cm^{-1} in the complex. The features at 2892 and 2979 cm^{-1} are assigned to methyl group C–H symmetrical and asymmetrical stretching modes for the complex. Normalizing to the 2853- and 2857-cm^{-1} lipid features, we note that the Raman band at 3013 cm^{-1}, assigned to C–H stretching modes of the double bond moieties, is considerably reduced in intensity in the complex, compared to its intensity in the pure liposome, and may be shifted. We emphasize that the possible catalytic phospholipid oxidation by cytochrome *c*[21] was precluded by the oxygen free conditions used in sample preparation. (Eighty-seven percent of the cardiolipin chains has cis double bonds in the 9–10 and 12–13 positions of each of the lipid chains.) The intensity of the band at 3053 cm^{-1} increases in direct correlation with the amount of protein in the complex.

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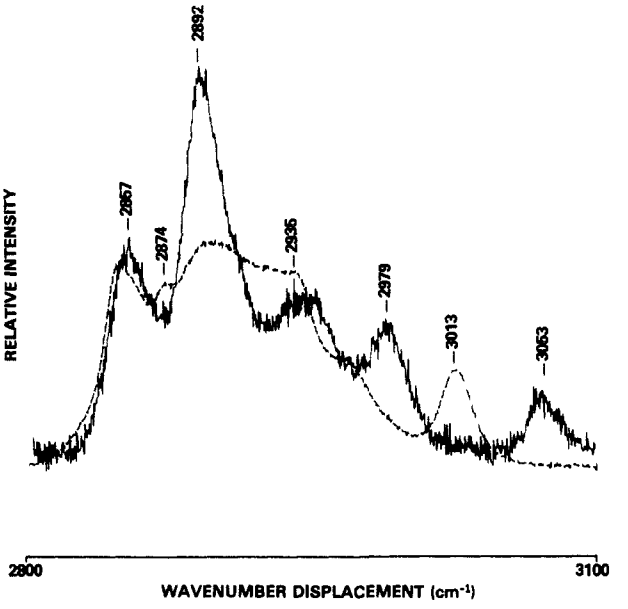

Figure 3. Raman spectra, using 514.5-nm Ar^+ ion laser excitation, of the cardiolipin–ferricytochrome *c* complex (solid line) (~8:1 lipid:protein mole ratio) and the disodium salt of cardiolipin (dashed line) in the 2800–3100-cm^{-1} C–H stretching mode region using 514.5-nm Ar^+ laser excitation.

The resonance Raman response to the complexation of ferricytochrome *c* by cardiolipin is remarkable, even though the visible spectrum of the lipid–protein complex is only moderately perturbed in comparison to the spectrum of ferricytochrome *c* alone. Since the absorption band at 550 nm, which is characteristic of ferrocytochrome *c*, is absent in the cardiolipin–ferricytochrome *c* complex, the conformation of the porphyrin ring structure in the complex must be altered by cardiolipin to reflect the vibrational Raman spectral markers characteristic of a reduced ferrocytochrome *c* species. That is, although formation of the complex alters the geometry of the porphyrin group to mimic the porphyrin skeletal frequencies of the reduced form, the oxidation state of the iron atom is unaffected. The oxidation-state markers at 1362 (A_{1g}, p) and 1547 cm^{-1} (B_{1g}, dp) correspond to normal modes which involve the in-plane coupled C–C stretching vibrations (C_α–C_β, C_β–C_β, C_α–C_N of the pyrrole outer-ring), modes which are sensitive to perturbations of the heme periphery.[17,18,22]

The anomously polarized line at 1585 cm^{-1} (A_{2g}, ap), assigned to a ring distortion involving the C_α–C_M and C_α–C_β stretching modes, does not shift upon complexation of ferricytochrome *c* with cardiolipin.[22] This vibrational frequency is used both as a spin-state marker[12,13] and a measure of the movement of the central metal out of the pyrrole coordination plane.[22,23] The constancy of this mode on forming the complex implies that the iron atom remains in the plane of the heme group.

The resonance Raman spectrum of the cardiolipin–ferricytochrome *c* complex may be suggestive of a slight movement of the heme system toward a hydrophobic microenvironment. For example, the differences in the crystallographic structures between the reduced and oxidized forms of tuna cytochrome *c* are quite small, with the modifications existing primarily in the movement of a buried water and its hydrogen-bonded amino acids near the heme crevice.[24] That is, on reduction, the heme group moves 0.15 Å deeper into the nonpolar crevice, changing slightly the coordination of the iron atom.[24]

The Raman spectra of cardiolipin in the C–H stretching region shows significant differences when complexed with ferricytochrome *c*. The increase in the symmetric methylene stretching frequency

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from 2853 to 2857 cm^{-1} indicates increased numbers of gauche conformers from which a larger amount of conformational disorder of the lipid chains may be inferred.[25] The decrease in the acyl chain double bond C-H stretching mode intensity at 3013 cm^{-1} in the complex demonstrates the involvement and possibly change in the surrounding environment of the cis double bonds at the 9-10 and 11-12 positions of the lipid chains. The participation of these regions of the lipid chains in complex formation strongly suggests that this lipid binding involves substantial conformational changes within the acyl chains and that the binding consists of more than a simple attraction of the negatively charged lipid head groups with the positively charged lysines ringing the heme crevice in the protein.

In summary, complexation of ferricytochrome *c* with cardiolipin modifies the structure of the heme group to mimic the heme conformation of the reduced ferrocytochrome *c* species, while not affecting the iron oxidation state. The reduction potential for the cardiolipin complex of ferricytochrome *c* is altered from 273 mV for the uncomplexed cytochrome *c* to 225 mV for the bound system.[26] The presence of cardiolipin in the inner mitochondrial membrane and the ease with which cardiolipin forms a complex with cytochrome *c* suggest that this specific lipid-protein complex may be important in clarifying the functional roles of the protein. In view of the structural changes observed for cytochrome *c* in these studies, it is important to assign oxidation states of heme or porphyrin systems with extreme care when using resonance Raman spectra as guides.

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Registry No. Cytochrome *c*, 9007-43-6.

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(27) TRIS, tris(hydroxymethyl)aminomethane. HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

Interaction of Ethylene with the Ru(001) Surface†

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Abstract: The interaction of ethylene with the Ru(001) surface has been investigated via high-resolution electron energy loss spectroscopy and thermal desorption mass spectrometry. Following desorption of an ethylene multilayer at 110 K, di-σ-bonded molecular ethylene is present on the surface. Competing desorption of molecular ethylene and dehydrogenation to form adsorbed ethylidyne (CCH_3) and acetylide (CCH) as well as hydrogen adatoms occur between approximately 150 and 260 K. The ethylidyne is stable to approximately 330 K, whereupon it begins to decompose to carbon and hydrogen adatoms. The desorption of hydrogen occurs in a sharp peak centered at 355 K, resulting from simultaneous ethylidyne decomposition and desorption of surface hydrogen. Further annealing of the overlayer to 380 K causes cleavage of the carbon-carbon bond of the acetylide, creating carbon adatoms and adsorbed methylidyne (CH). The methylidyne decomposes above 500 K with accompanying hydrogen desorption, leaving only carbon adatoms on the surface at 700 K.

I. Introduction

The adsorption and reaction of ethylene on single crystalline surfaces of the groups 8-10 transition metals[1-17] have been the subject of intense study both as a prototype for olefin hydrogenation and dehydrogenation reactions[18-21] and to provide a basis for comparing the bonding of olefins to surfaces with the bonding that has been observed to occur in multinuclear organometallic cluster compounds. Spectroscopic studies of ethylene adsorbed on these surfaces have shown that both the nature of the bonding of molecular ethylene to the substrate as well as the thermal decomposition pathway of the adsorbed ethylene vary widely. For example, ethylene rehybridizes to a di-σ-bonded molecular species when adsorbed on Fe(110), Fe(111), Ni(110), Ni(111), Ni[5(111) × (110)], Pt(111), and Pt(100),[1-7] whereas molecularly adsorbed ethylene on Co(001) at 115 K is π-bonded,[8] as is ethylene adsorbed on Pd(111) at 150 K and on Pd(110) at 110 K.[6,9-13,17] A mixed overlayer of π- and di-σ-bonded ethylene forms on Pd(100) at 150 K.[14,15] Ethylene adsorption on the Ru(001) surface has been

† In this paper the periodic group notation in parentheses is in accord with recent actions by IUPAC and ACS nomenclature committees. A and B notation is eliminated because of wide confusion. Groups IA and IIA become groups 1 and 2. The d-transition elements comprise groups 3 through 12, and the p-block elements comprise groups 13 through 18. (Note that the former Roman number designation is preserved in the last digit of the numbering: e.g., III → 3 and 13.)

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