Electron-Transfer Properties of Cytochrome c Langmuir−Blodgett Films and Interactions of Cytochrome c with Lipids

ARTICLE *in* LANGMUIR · OCTOBER 1998

Impact Factor: 4.46 · DOI: 10.1021/la980319l

5 AUTHORS, INCLUDING:

Rafael Arechabaleta
Nova Southeastern University
4 PUBLICATIONS **207** CITATIONS
SEE PROFILE

Nongjian Tao
Arizona State University
223 PUBLICATIONS **12,796** CITATIONS
SEE PROFILE

Electron-Transfer Properties of Cytochrome *c* Langmuir−Blodgett Films and Interactions of Cytochrome *c* with Lipids

S. Boussaad,† L. Dziri,‡ R. Arechabaleta,† N. J. Tao,*,† and R. M. Leblanc‡

Department of Physics, Florida International University, Miami, Florida 33199, and Department of Chemistry, University of Miami, Coral Gables, Florida 33124

Received March 20, 1998. In Final Form: July 20, 1998

The structural and electron-transfer properties of cytochrome *c* (Cyt *c*) Langmuir−Blodgett (LB) films have been studied on graphite electrode with tapping mode atomic force microscopy and cyclic voltammetry (CV). Cyt *c* in the LB films forms an ordered monolayer in which the individual proteins pack into a quasi-hexagonal structure. The monolayer undergoes a reversible electron-transfer reaction in phosphate buffer. The interactions of Cyt *c* with cardiolipin (CL) and phosphatidylcholine (PC) LB films have been studied. The LB films of CL and PC are both ordered on graphite, but their interactions with Cyt *c* are quite different. On a CL monolayer, Cyt *c* adsorbs spontaneously and the adsorbed protein preserves the electron-transfer reaction. However, on a PC monolayer, Cyt *c* does not adsorb.

Introduction

Immobilizing redox proteins with intact conformations on electrode surfaces is an important task in the development of biosensors and in the study of the proteins using surface analytical techniques such as scanning probe microscopy.[1] Metalloproteins undergo reversible electron-transfer reactions[2] that play important roles in many biological processes and are attractive for biosensor applications.[3,4] However, many metalloproteins, such as cytochrome *c* (Cyt *c*), often denature when they adsorb on a solid electrode. One way to eliminate the problem is to coat the electrode with an organic monolayer.[5,6] Hill et al. have shown that a gold electrode coated with an appropriate organic monolyer can indeed allow Cyt *c* to be immobilized on the modified electrode while preserving the protein native conformation.[5] The organic molecules chosen for the monolayer have one end (e.g., S and N) that can strongly bind to the electrode and the opposite end that attracts Cyt *c*. The attraction between the protein and the organic monolayer also allows the redox center of Cyt *c* to align toward the electrode surface which is important for fast electron exchange to take place between the redox center and the electrode. Recently, we have shown that an adsorbed phosphate anion layer on a graphite electrode may also serve the role of the organic monolayer.[7] These studies show that the appropriate orientation of Cyt *c* is essential for fast electron transfer between electrodes and the adsorbed protein.

In the present study, we show that Cyt *c* preoriented at the air/water interface forms a well-ordered monolayer and exhibits a reversible electron-transfer reaction after transferring onto a graphite surface using the Langmuir−Blodgett (L−B) technique. We have also extended the study to the adsorption of Cyt *c* on two phospholipid monolayers, cardiolipin (CL) and phosphatidylcholine (PC), and the electron-transfer properties of the adsorbed Cyt *c* using tapping mode AFM and cyclic voltammetry.

CL and PC are important components of the mitochondria membrane and their compositions in the membrane are 16−20% and 38−45%, respectively.[8] Both lipids are present in the inner membrane of mitochondria where Cyt *c* shuttles electrons between two membrane-bound enzymes, Cyt *c* oxidase and Cyt *c* reductase.[9] CL is the major anionic component of the inner membrane and believed to be functionally relevant for the energy-transduction process.[9] For instance, it has been observed that CL is required for the function of cytochrome *c* oxidase.[10,11] The origin of this requirement is still unclear, although there is evidence that CL facilitates the binding of Cyt *c* to Cyt *c* oxidase. A better understanding of the interactions between Cyt *c* and the lipids is important for a full understanding of the electron-transfer process.

Experimental Section

Cyt *c*, CL, and PC were purchased from Fluka and used without any further purification. The protein is dissolved in a phosphate buffer solution (100 mM, pH = 8.0) to a concentration of 0.33 mg/mL using Nanopure water (18.3 MΩ·cm of resistivity) from a Bioresearch grade purification system (Barnstread Co.) fed with campus-distilled water. The same buffer solution was used as a subphase for preparing the Langmuir films. To spread Cyt

* To whom correspondence should be addressed. E-mail: taon@fiu.edu.

† Florida International University.

‡ University of Miami.

(1) Chen, X.; Davies, M. C.; Roberts, C. J.; Tendler, S. J. B.; Williams, P. M.; Davies, J.; Dawkes, A. C.; Edwards, J. C. *Langmuir* **1997**, *13*, 4106.

(2) Salamon, Z.; Hazzard, J. T.; Tollin, G. *Proc. Natl. Acad. Sci.* **1993**, *90*, 6420.

(3) Ryder, K. S.; Morris, D. G.; Cooper, J. M. *Langmuir* **1996**, *12*, 5681.

(4) Boncheva, M.; Vogel, H. *Biophys. J.* **1997**, *73*, 1056.

(5) Eddowes, M. J.; Hill, H. A. O. *J. Chem. Soc., Chem. Commun.* **1977**, 771. Albery, W. J.; Eddowes, M. J.; Hill, H. A. O.; Hillman, A. R. *J. Am. Chem. Soc.* **1981**, *103*, 3904.

(6) Taniguchi, I.; Toyosawa, K.; Yamaguchi, H.; Yasukouch, K. *J. Chem. Soc., Chem. Commun.* **1982**, 1032. Rusling, J.; Nassar, A.−E. F. *J. Am. Chem. Soc.* **1993**, *115*, 11891. Cullison, J. K.; Hawkridge, F. M.; Nakashima, N.; Yoshikawa, S. *Langmuir* **1994**, *10*, 877.

(7) Boussaad, S.; Tao, N. J.; Arechabaleta, R. *Chem. Phys. Lett.* **1997**, *280*, 397.

(8) Daum, G. *Biochim. Biophys. Acta* **1985**, *822*, 1.

(9) Bosshard, H. R. In *Cytochrome c: A Multidisciplinary Approach*; Scott, R. A., Mauk, G. A., Eds.; University Science Books: California, 1995; p 373.

(10) Robinson, N. C.; Strey, F.; Talbert, L. *Biochemistry* **1980**, *19*, 3656.

(11) Marsh, D.; Powell, G. L. *Bioelectrochem. Bioenerg.* **1988**, *20*, 73.

S0743-7463(98)00319-9 CCC: $15.00
Published on Web 09/19/1998

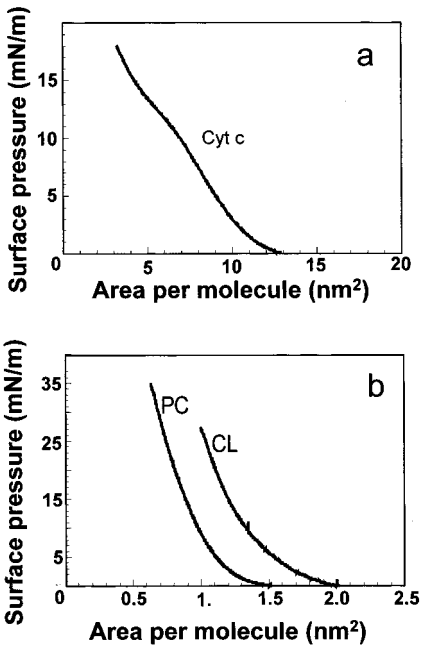

Figure 1. Surface pressure vs molecular area isotherms of Cyt *c* (a), CL (b), and PC (b) monolayers at the air/water interface.

c at the air/water interface, the Cyt *c* solution is mixed with ethanol (Sigma Co., HPLC grade) to a ratio of 1/1 (v/v) before spreading. The lipids were dissolved in chloroform (Sigma Co., HPLC grade) to a concentration of 1 mg/mL. The monolayers of Cyt *c*, CL, and PC were prepared in a homemade double trough ($55 \times 15 \times 0.6$ cm^3) at constant temperature (20 ± 1 °C) and humidity (50 ± 1%). The monolayers were transferred from the air/water interface onto graphite at a rate of 2 mm/min. The surface pressures at which the monolayers were transferred were 10, 25, and 28 mN/m, for Cyt *c*, CL, and PC, respectively. At these surface pressures the protein and lipid monolayers are expected to be compact and uniform (Figure 1).

The monolayers are scanned in air and then in phosphate buffer solution with a Nanoscope III-a multimode AFM (Digital Instrument Inc.). Commercial-sharpened Si_3N_4 tips attached to rectangular beams were used in air whereas those attached to triangular cantilevers were used in liquids. The set point was adjusted to minimize the force between the tip and the sample.

We used Pt and Ag wires as counter- and quasireference electrodes in order to control the potential of the graphite electrode. The Ag quasireference electrode was calibrated against a Ag/AgCl electrode (3.0 M KCl), and all the potentials in this paper are quoted versus the Ag/AgCl reference electrode.

Results and Discussion

a. Cyt c Monolayers. Figure 2a is a typical AFM image of Cyt *c* LB film that shows a uniform layer decorated with islands and bare graphite areas (indicated in the figure). We believe that the uniform Cyt *c* layer is a monolayer because of the presence of bare graphite areas and the surface pressure used for deposition. The monolayer consists of domains of parallel stripes as revealed more clearly in the phase image (Figure 2b,c). The orientations of the stripes in different domains can differ by 120° (Figure 2b) which reflects the symmetry of the underlying graphite substrate, indicating the role of the protein−substrate interaction in the monolayer deposition. The inset of Figure 2c is an image of the ordered monolayer after removing high-frequency noise which reveals a two-dimensional lattice with $a = 4.4 \pm 0.2$ nm, $b = 5.3 \pm 0.2$ nm, and $\gamma = 71 \pm 3°$. If each blob is interpreted as a Cyt *c* molecule, then the dimensions are

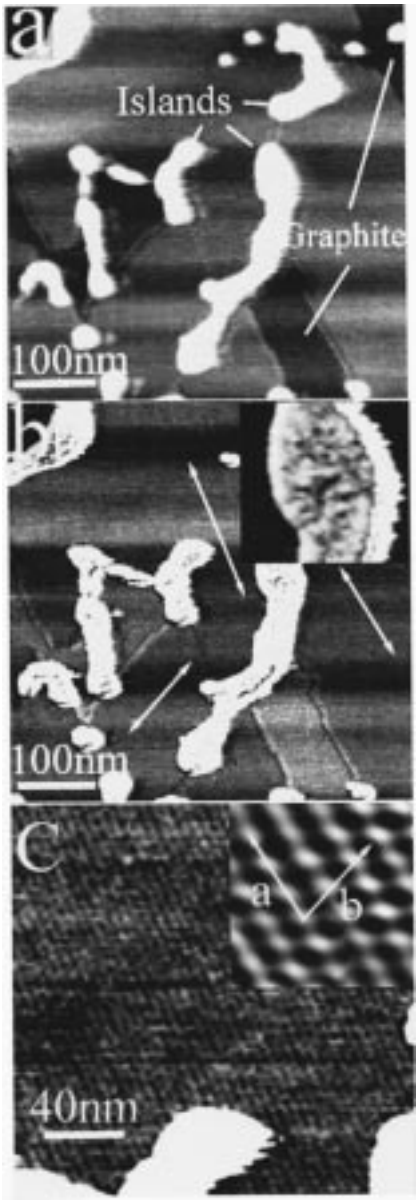

Figure 2. AFM images of the Cyt *c* L−B monolayer deposited onto graphite at a surface pressure of 10 mN/m in air. Images (a) (height) and (b) (phase) are recorded simultaneously. The inset in (b) is a high-magnification image of the islands, and the arrows in (b) point to the domain directions of the monolayer. Image (c) is a higher magnification image that shows the ordered packing of Cyt *c* in the monolayer more clearly. The inset in (c) shows the individual Cyt *c* after removing high-frequency noise with a Fourier filter.

in good agreement with the reported X-ray data.[12] The corresponding area per protein is about 20 nm^2 which is much greater than the limiting molecular area of 7.0 nm^2, extrapolated from the surface pressure versus the molecular area isotherm (Figure 1). This apparent discrepancy may be attributed partially to the loss of proteins in the subphase during the transfer of the monolayer from the air/water interface onto the graphite substrate, and to the formation of protein aggregates in the islands which we will discuss next. The islands protrude out of the monolayer by 1.8 nm and appear to consist of large blobs with an average diameter of 8 nm. In contrast to the ordered packing in the monolayer, the blobs are randomly

(12) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585.

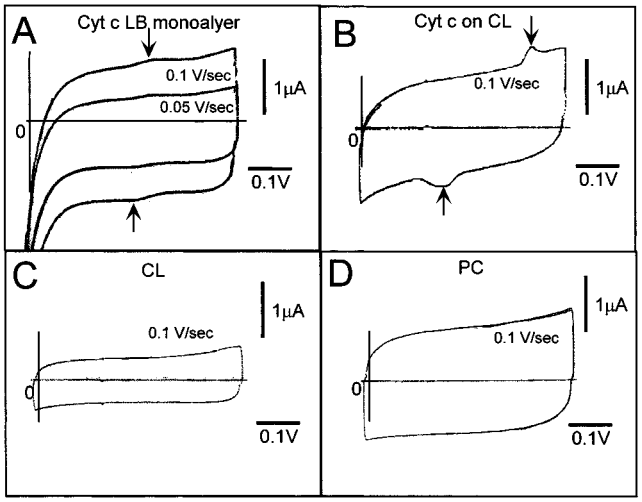

Figure 3. Cyclic voltammograms of a Cyt *c* L−B monolayer (a), Cyt *c* adsorbed onto CL L−B film (b), CL L−B film (c), and PC L−B film (d) in 0.1 M phosphate buffer.

distributed in the islands. These blobs could be Cyt *c* aggregates or modified Cyt *c* due to the presence of ethanol in the preparation of the monolayer.

We have studied the electron-transfer properties of the Cyt *c* monolayer with cyclic voltammetry (CV). A pair of peaks, corresponding to the reversible electron transfer of $Fe^{2+} \leftrightarrow Fe^{3+}$ of the heme group of Cyt *c*, appears in the CV plot (Figure 3a). This is in sharp contrast to the Cyt *c* monolayer spontaneously adsorbed on graphite which exhibits a reversible electron transfer only *after* cycling the potential to a large positive value.[7] At positive potentials, a stable phosphate anion layer is formed which attracts the positively charged groups of Cyt *c* and orients the heme group closer to the electrode surface to allow the electron transfer to take place. In the present case, Cyt *c* is preoriented at the air/water interface before transferring onto graphite, thus it requires no reorientation by cycling the potential to positive values. The redox peaks of the Cyt *c* monolayer are about 0.08 V more positive than those of the spontaneously adsorbed Cyt *c*[7] (after reorientation). The electron-transfer rate as extracted from the separation between the oxidation and reduction peaks as a function of the swept rate is 20−30 s^{-1}, which is smaller than 60−80 s^{-1}, the value for the spontaneously adsorbed monolayer. The difference in the redox peak positions and electron-transfer rate between the ordered LB monolayer and the disordered adsorbed monolayer probably reflects the difference in the local environment of the protein in the two situations.

b. Lipid Monolayers. To study the interaction of Cyt *c* with lipids, we have characterized the LB films of CL and PC on graphite. Figure 4a is a large-scale AFM image of a CL LB film which shows a uniform monolayer with patches of bilayers. In some areas, edges of the upper and lower layers and graphite are observed which confirms the interpretation of bilayer patches (Figure 4b). The thicknesses of the upper and lower layers are about 0.8 and 1.6 nm, respectively. The thickness of each layer is in good agreement with the molecular structure of CL, but the value of the upper layer may reflect an orientation which is different. Higher resolution images (Figure 4c) show parallel stripes with a spacing of ∼4.6 nm on both the upper and lower layers. A similar lattice structure has been recently observed in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)[13] supported bilayers and dimyristoylphosphatidylcholine (DMPC) LB films.[14] The Fourier-filtered image reveals ordered packing, with lattice

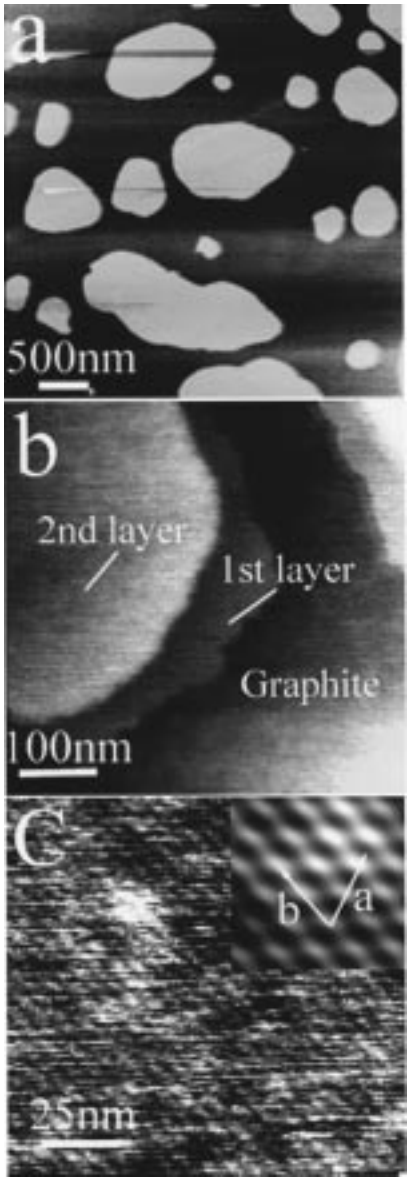

Figure 4. AFM images of the CL monolayers deposited onto graphite at a surface pressure of 25 mN/m in air. High-frequency noise in the inset of (c) has been removed with a Fourier filter.

constants, $a = 5.0 \pm 0.2$ nm, $b = 6.0 \pm 0.2$ nm, and $\gamma = 60 \pm 3°$. The lattice constants are much greater than the intermolecular distance of a closely packed CL layer, which indicates that we cannot simply interpret each blob in the filtered image as a single CL molecule. This conclusion is supported by the fact that the surface area,[15] determined from the surface pressure versus molecular area isotherms (Figure 1), is an order of magnitude smaller than the area of a unit cell in the AFM image. The periodic structure with such large lattice constants must be due to the fact that the individual CL molecules organize themselves into a higher order structure which, unfortunately, cannot be determined based on the present images.

The PC monolayer appears to be less compact and consists of a lot more defects than the CL film (Figure 5a). At higher resolution image of the PC monolayer also reveals a parallel-stripe feature (Figure 5b), and the

(13) Stephens, S. M.; Dluhy, R. A. *Thin Solid Films* **1996**, *284−285*, 381.
(14) Yuan, C.; Yang, X.; Lu, Z.; Liu, J. *Surf. Sci.* **1996**, *355*, L381.
(15) Houle, A.; Techy, F.; Aghion, J.; Leblanc, R. M. *J. Lipid Res.* **1982**, *23*, 496.

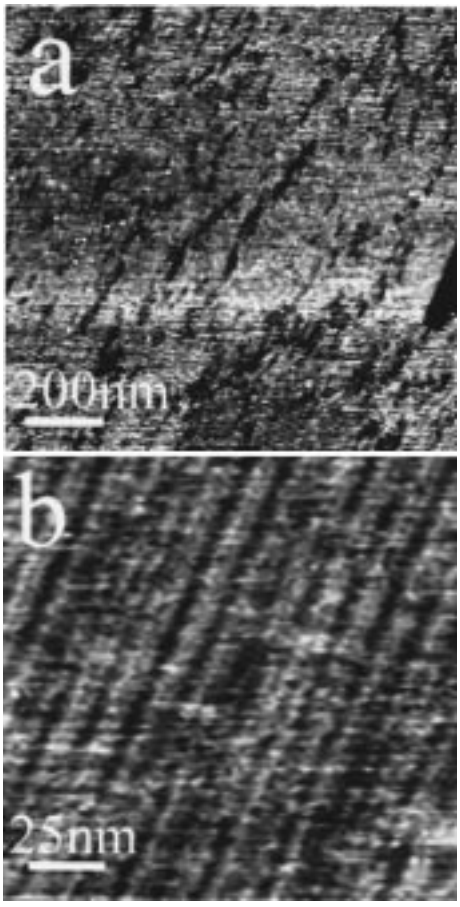

Figure 5. AFM images of the PC monolayers deposited onto graphite at a surface pressure of 28 mN/m in 0.1 M phosphate buffer.

separation between two adjacent stripes is about 10.5 nm which is much greater than the spacing in the CL monolayer. However, it is also rather difficult to determine exactly how the individual molecules arrange themselves in the lipid monolayers based on the images.

We have studied the surface structure of CL and PC LB films in air and in a phosphate buffer. For CL, identical results are obtained, but for PC the monolayer in the buffer solution is much more uniform and compact than in air. This observation shows the importance of hydration in the packing of PC.

The cyclic voltammograms of the CL and PC monolayers in the buffer solution have been obtained (Figure 3c,d). The CVs show no sign of electron-transfer reactions in the potential window of interest. The overall shapes of the CVs are similar to that of the bare graphite electrode, except the polarization current is much smaller when CL and PC monolayers are present on the graphite electrode. This is understandable since the presence of the lipid monolayers decreases the interfacial capacitance, thus the polarization current.

c. Cyt *c* Adsorption on CL and PC Monolayers. Because the interaction of Cyt *c* with CL and PC monolayers may mimic the in vivo interaction between the protein and the inner membrane of mitochondria, we have performed an in situ study of the Cyt *c* adsorption onto CL and PC monolayers supported by graphite. We started out the experiment by imaging a CL or PC LB film in the buffer solution. After a clear image of the film was obtained, we then introduced a drop of Cyt *c* solution into the AFM cell and monitored the entire adsorption process. Figure 6 shows a few snapshots of the adsorption process

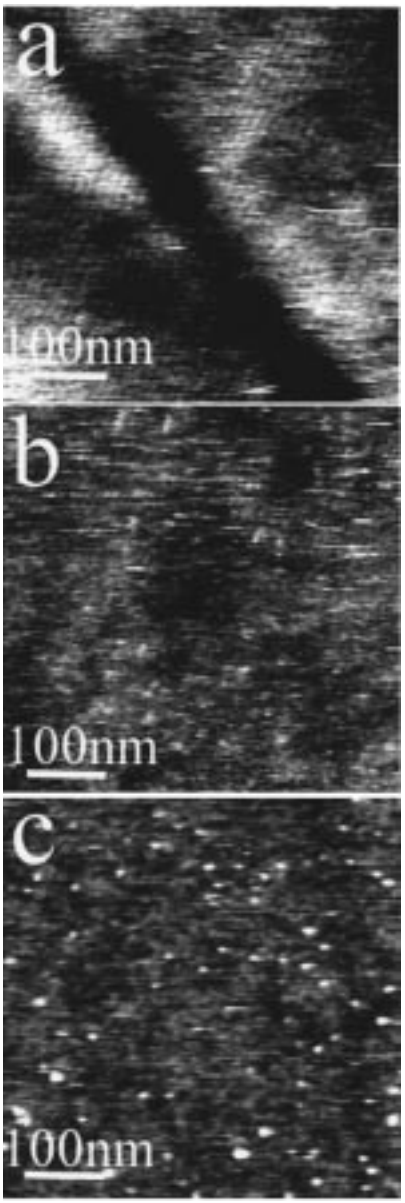

Figure 6. Time-sequence AFM images captured before (a), during (b), and after Cyt *c* adsorption onto CL monolayers deposited on graphite at 25 mN/m in 0.1 M phosphate buffer.

on the CL monolayer. Before introducing Cyt *c* into the cell, the surface of the CL monolayer is uniform with patches of bilayers (Figure 6a). Immediately after 45 μL of 100 μM Cyt *c* is added into the cell, blob-like features appear on the surface, indicating the beginning of the adsorption (Figure 6b). As Cyt *c* covers the surface, the parallel-stripe feature of the CL monolayer fades away and the coverage of Cyt *c* reaches a maximum after about 10 min. Comparing to the Cyt *c* adsorption on bare graphite,[7] the distribution of Cyt *c* on CL appears to be more uniform which is probably due to a specific lipid−protein interaction. We have studied the adsorption of Cyt *c* on PC monolayer in a similar way, but observed no protein layer on the PC monolayer except a few isolated proteins located at defect sites or step edges. This observation indicates a weaker interaction between Cyt *c* and PC than that between Cyt *c* and CL, which is consistent with several previous studies by other techniques.[16−19]

(16) Heimburg, T.; Hildebrandt, P.; Marsh, D. *Biochemistry* **1991**, *30*, 9084.

The electron-transfer properties of Cyt *c* adsorbed on CL and PC monolayers have been examined by cyclic voltammetry in order to evaluate the impact of the lipid−protein interaction on the charge-transfer reaction and the conformation of the protein. In the case of Cyt *c* on CL, a pair of well-defined peaks corresponding to the reversible electron-transfer reaction of Cyt *c* is observed (Figure 3b). The peak separation is much greater than that of spontaneously formed and LB films of Cyt *c* on graphite. The large peak separation is due to the fact that the electron-transfer rate of Cyt *c* on CL is 5−10 s^{-1} which is about 4 times smaller than that of Cyt *c* LB films and 10 times smaller than the rate of Cyt *c* adsorbed on graphite. At a first glance, the decrease in the electron-transfer rate could be attributed to the increased distance between Cyt *c* and the electrode in the presence of the CL layer. However, our AFM image shows that the packing of CL molecules is seriously disrupted as Cyt *c* "penetrates" into the monolayer. For example, the domain boundary which crosses the image (Figure 6a) diagonally before the adsorption disappears after the formation of the Cyt *c* layer (Figure 6c). The disruption of the CL monolayer is supported by the observation of increased capacitance (polarization current in the cyclic voltammograms in Figure 3) when Cyt *c* adsorbs onto the surface, and by previous NMR, Raman, and surface plasmon resonance spectroscopy.[16−19] It is likely that the strong Cyt *c*−CL interaction leads to a change in the Cyt *c* conformation or environment. Several recent studies[20,21] have shown that distortion of the heme group conformation may take place during complexation of a protein and lipid.[22]

The CV of the PC monolayer in the presence of Cyt *c* is similar to that of PC in the absence of Cyt *c*. This observation is also consistent with the AFM images that show no Cyt *c* adsorption onto PC nor any sign of Cyt *c*−PC interaction.

Conclusions

We have studied the structural and electron-transfer properties of LB films of a redox protein, Cyt *c*. The protein in the monolayer is packed into an ordered two-dimensional lattice, with lattice constants, $a = 4.4 \pm 0.2$ nm, $b = 5.3 \pm 0.2$ nm and $\gamma = 71 \pm 3°$, which are consistent with Cyt *c* dimensions determined by X-ray crystallography. The ordered proteins exhibit a reversible electron transfer on a graphite substrate. Since Cyt *c* spontaneously adsorbed on graphite does not undergo the electron transfer, preorientation of the proteins at the air/water interface is essential for the electron transfer. We have also studied Cyt *c* on LB films of phospholipids, CL, and PC. CL forms a uniform and compact ordered monolayer decorated with bilayer domains. The ordered layers form a hexagonal structure rather than the ordered array at the air/water interface. Cyt *c* adsorbs onto both the monolayer and bilayer domains and exhibits a slower electron-transfer reaction than the LB film. Also, the adsorption disrupts seriously the ordered structure of CL, indicating a strong Cyt *c*−CL interaction. PC forms a less compact monolayer and consists of more defects than CL, but it is also ordered as the AFM reveals a parallel-stripe feature that is similar to that of CL. Cyt *c* does not adsorb onto PC. The observation that Cyt *c* interacts with CL more strongly than with PC is consistent with other studies and shows CL as a more important element in the membrane.

Acknowledgment. Financial support is acknowledged through grants from the NIH (GM-08205), Cottrell College Science Award (CC3608), and AFSOR (F49620-96-1-0346).

LA980319L

(17) Muga, A.; Mantsch, H. H.; Surewicz, W. K. *Biochemistry* **1991**, *30*, 7219.
(18) Brown, L. R.; Wuthrich, K. *Biochim. Biophys. Acta* **1977**, *468*, 389.
(19) Salamon, Z.; Tollin, G. *Biophys. J.* **1996**, *71*, 848.
(20) Spooner, P. J. R.; Watts, A. *Biochemistry* **1991**, *30*, 3871.
(21) Spooner, P. J. R.; Watts, A. *Biochemistry* **1991**, *30*, 3880.
(22) Vincent, J. S.; Kon, H.; Levin, I. W. *Biochemistry* **1987**, *26*, 2312.