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Adsorption of cytochrome *c* to phospholipid monolayers studied by reflection spectroscopy

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The uptake of cytochrome *c* by charged and neutral lipid monolayers was studied by using reflection spectroscopy. The method was shown to be a very sensitive and useful technique in studies of lipid-protein interactions. It was found that cytochrome *c* is preferentially bound to monolayers of negatively charged monolayers in the solid phase. Polarized light under oblique incidence was used to determine the average orientation of chromophores in cytochrome *c* bound to lipid monolayer. The transition moments of chromophore are oriented parallel to the monolayer plane.

Cytochrome *c*; Phospholipid monolayer; Reflection spectroscopy

1. INTRODUCTION

Proteins adsorb to membrane surfaces and their behaviour at interfaces as well as interactions with lipids are of particular interest in relation to cell membrane organisation and physico-chemical processes in biological systems. Monolayer techniques have been extensively used to investigate lipid-protein interactions since they provide controlled conditions for investigation of ordered biomolecules [1].

Results obtained with phospholipid monolayers under appropriate experimental conditions may be extrapolated to bilayer systems. By studying the phase transitions of phospholipid bilayers and monolayers Blume [2] found that the behaviour of bilayers is very similar to that of the respective monolayers at a lateral pressure of approx. 39 mN/m. Previously it was found that the pressure in the outer monolayers of the erythrocyte membrane is equivalent to a monolayer surface pressure of 30–34.8 mN/m.

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Adsorption of proteins to the solid phase of lipid monolayers cannot be easily followed by conventional monolayer techniques and some other independent technique should be used. We report here the adsorption of cytochrome *c* (cyt.*c*) to phospholipid monolayers at the air/solution interface as studied by reflection spectroscopy. Measurements of reflection with plane-polarised light under inclined incidence have also been carried out in order to provide more information about the average orientation of the cyt.*c* chromophore at the air/solution interface.

2. EXPERIMENTAL

Cyt.*c* (from horse heart, type III) and dioctadecyldimethylammonium bromide (DOMA) (p.a. grade) were purchased from Sigma. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidic acid (DPPA) and dipalmitoylphosphatidylserine (DPPS) were obtained from Larodan Chemicals.

1 mM Tris-HCl solution was used as a buffer and chloroform as the spreading solvent. Deionised water from a Milli-Q was used for preparing the subphase.

The surface pressure was measured at constant area with a 1 cm wide filter paper Wilhelmy balance [4].

The reflection spectrometer for measurement under normal incidence of light [5] and a modified instrument for measure-

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ment of reflection spectra under oblique incidence of linearly polarized light [6] have been described in detail earlier. The reflection is always measured and expressed as the difference ΔR in reflectivity of the solution surface and of the clean water surface.

All measurements were performed at room temperature.

3. RESULTS AND DISCUSSION

We have already reported that the reflection method based on the enhanced reflection due to the presence of a chromophore in a monolayer at an air/water interface, which was developed by Möbius and co-workers [5], introduces a new physico-chemical parameter into in situ studies of protein-lipid interactions at an air/solution interface [7]. Since only chromophores present at the interface contribute to the enhanced reflection without the influence of chromophores from the solution [5], investigation of interaction processes at an interface is made possible. Accumulation of chromophores at an air/solution interface due either to the interaction of solutes from the aqueous solution with a monolayer present on top of the solution or to their adsorption to the surface results in a change in light reflection which depends on the chromophore density and orientation at the interface.

Since cyt.*c* has 8 net positive charges at neutral pH it binds electrostatically to negatively charged phospholipids [8]. The reflection spectra in fig.1 show an enhanced reflection signal with a maximum of the porphyrin Soret band at 410 nm for cyt.*c* interacting with DPPS monolayers at a surface pressure of 10 mN/m (curve 2) and 25 mN/m (curve 3). Curve 1 shows the reflection signal from pure cyt.*c* in aqueous solution which is not greater than the noise level. The enhanced reflection of cyt.*c* upon binding to DPPS monolayers is in agreement with published results concerning the interaction of cyt.*c* with different phospholipid vesicles [9,10], mixed DMPC-DMPS bilayers [11], mixed DPPC-DPPA monolayers [12] and binding of apocytochrome *c* to negatively charged lipid bilayers [13].

The kinetics of cyt.*c* binding to phospholipid monolayers was followed by measuring the dependence with respect to the reflection ΔR at the maximum of the Soret band (410 nm). The results obtained for cyt.*c* interacting with DPPS monolayers are shown in fig.2 for different initial

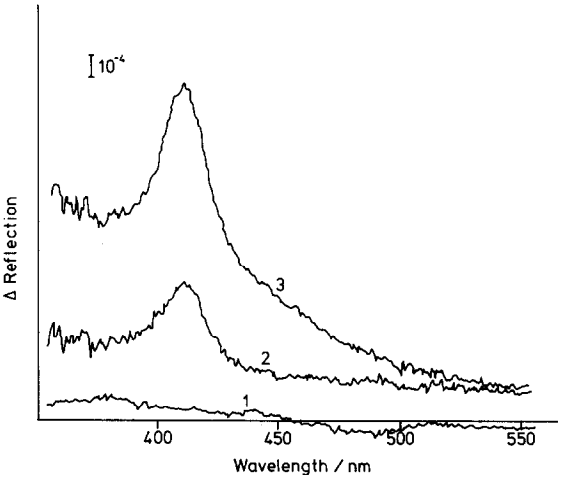

Fig.1. Reflection spectra obtained from cyt.*c* solutions with and without phosphatidylserine monolayers: (1) solution surface; (2) solution with monolayer of DPPS, initial surface pressure 10 mN/m; and (3) solution with monolayer of DPPS, initial surface pressure 25 mN/m. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5.

surface pressures, thus giving the enhanced reflection signal with increasing surface concentration of the lipid. The dependence of the ΔR value upon cyt.*c* concentration in solution for two different initial surface pressures of DPPA monolayers is depicted in fig.3. Values are obtained by following the kinetics of interaction and are measured after equilibrium has been reached. In the case of low initial surface pressure of lipid ($\pi_{initial}$ = 5 mN/m) ΔR is slightly dependent on protein concentration and the curve resembles, to a first approximation, a Langmuir isotherm. A different dependence is obtained for higher surface pressure ($\pi_{initial}$ = 30 mN/m) indicating a different mechanism for the protein interaction with liquid and solid phases of lipid. To ensure that equilibrium is reached within a reasonable time we chose a rather high concentration of cyt.*c* in solution (5 mg/l) and for all systems the measurements of reflection and surface pressure vs time were performed for 1 h, equilibrium being established within this time.

Different patterns for the surface pressure change $\Delta\pi$ and of reflection ΔR, respectively, for the interaction of cyt.*c* with DPPS and DPPA monolayers as a function of lipid surface density were obtained. These results are illustrated in figs 4 and 5. With increasing density of DPPS and

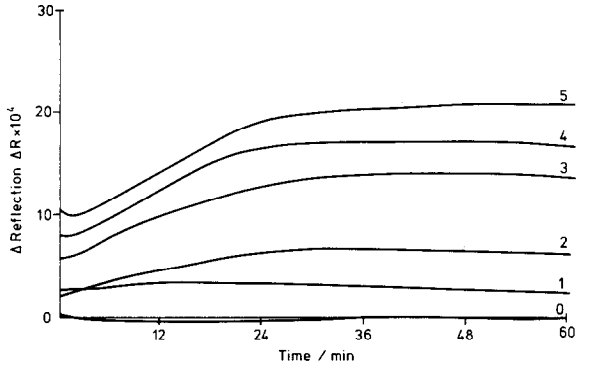

Fig.2. Time dependence of enhanced light reflection ΔR(410 nm) at the maximum of the Soret band for cyt.*c* solution without (0) and with DPPS monolayers at the air/solution interface at different initial surface pressures: 5 (1), 15 (2), 25 (3), 40 (4) and 50 (5) mN/m. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5.

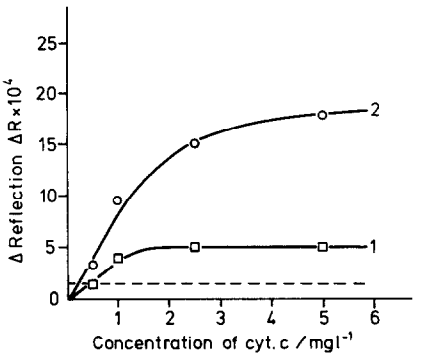

Fig.3. The enhanced light reflection ΔR(410 nm) for the system cyt.*c*-DPPA monolayers vs concentration of cyt.*c* solution at different initial surface pressures of monolayers: (1) π_i = 5 mN/m; (2) π_i = 30 mN/m. (– – –) Reflection from DPPA monolayer on buffer solution. Subphase: cyt.*c* in 1 mM Tris buffer, pH 7.5.

DPPA the surface pressure change, which is usually attributed to the penetration of the protein molecule into the monolayer [1], decreases while the reflection ΔR increases continuously and eventually reaches a constant level. The reflection from a monolayer spread on pure water or a buffer solution without cyt.*c* was found to contribute minimally to the signal obtained from the cyt.*c*-bound monolayer (broken line in the case of DPPA in fig.5).

Different behaviour was observed in the case of cyt.*c* interacting with DPPC and DOMA monolayers. These results are presented in fig.6. The reflection signal increases for neither monolayer over the range of lipid surface density used. The different values lie around the broken lines which represent the ΔR values obtained for these monolayers spread on buffer solution in the absence of cyt.*c*. The change in surface pressure is given for purposes of comparison. From the $\Delta\pi$ data one can observe that cyt.*c* binds to DPPC monolayers (although not detected spectroscopically) in the expanded phase which at this pH value bears no net surface charge. In the case of DOMA monolayers the $\Delta\pi$ data scarcely provide evidence of binding, and spectroscopically no binding is detected. This is reasonable, since due to the positively charged head groups of DOMA, repulsion of cyt.*c* is expected.

Previously it was found that the adsorption of proteins to compressed lipid monolayers, for cases where increases in surface pressure can no longer be detected, can be monitored efficiently with the aid of surface radioactivity measurements using ^{14}C-labelled proteins [14].

According to results obtained through applying the thermodynamic theory of penetration established by Ter-Minassian-Saraga [15] to the penetration of vinblastine sulphate molecules from solution into lecithin monolayers, Panaiotov and co-workers [16] have predicted the possible adsorption of this compound to dense packed monolayers.

Recently, Möhwald and co-workers [17,18] have reported fluorescence microscopic studies on cyt.*c* interactions with phospholipid monolayers and vesicles. They found that cyt.*c* preferentially binds to DMPA monolayers in the liquid phase [17]. They also indicated binding to the solid phase although to a much lesser degree [18]. However, the poor signal-to-noise ratio in these measurements cannot lead to conclusive evidence concerning the interaction of cyt.*c* with the solid phase domain and allows no quantitative analysis. Moreover, fluorescence is a secondary process and consequently the signal obtained may be influenced by changes in absorption (e.g. via altered chromophore orientation) and/or fluorescence quantum yield. Therefore, such studies are insufficient for drawing conclusions regarding the

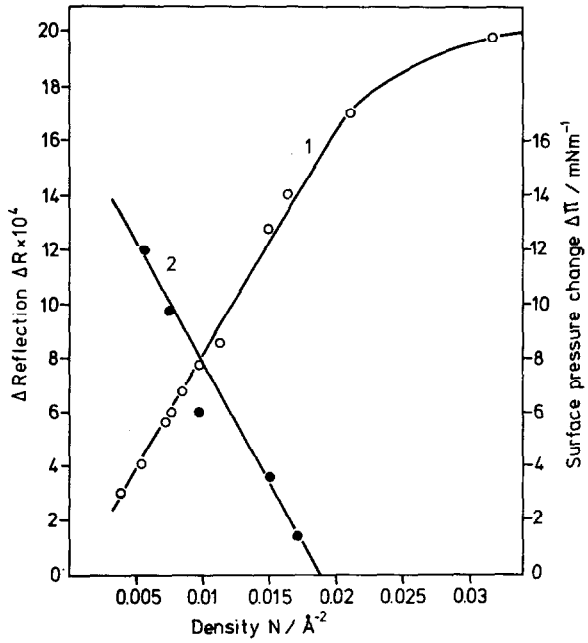

Fig.4. Dependence of reflection ΔR(410 nm) (curve 1) and surface pressure change $\Delta\pi$ (curve 2) on density N of DPPS monolayers at the air/solution interface. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5.

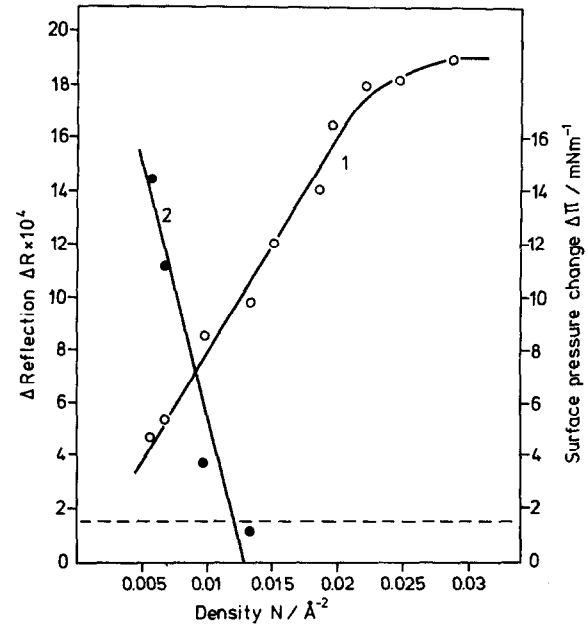

Fig.5. Dependence of reflection ΔR(410 nm) (curve 1) and surface pressure change $\Delta\pi$ (curve 2) on density N of DPPA monolayers at the air/solution interface. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5. (– – –) Reflection of DPPA monolayer on buffer solution.

binding of cyt.*c* to a lipid monolayer in the condensed state.

In contrast, reflection spectroscopy is based on coherent light scattering and no secondary processes are involved, thus being a significant direct method for studying the adsorption of proteins to lipid monolayers. From figs 4 and 5 it is seen that the reflection signal ΔR increases strongly with lipid surface density, providing evidence for strong binding of cyt.*c* to negatively charged phospholipid monolayers in the solid phase.

The average orientation of the chromophores at the interface can be determined by measuring the reflection of polarized light under oblique incidence [6,19]. The spectra shown in fig.7 have been obtained with a DPPA monolayer on a cyt.*c* solution at $\pi_{initial}$ = 30 mN/m for light incident at an angle of α = 45.1° with respect to the normal. The lower curve (1) is for p-polarised light where the electric vector oscillates in the plane of incidence, curve 2 being for s-polarization where the electric vector oscillates perpendicularly to the plane of incidence. It is seen that ΔR is larger for s-polarisation. From the ratio $\Delta R_s/\Delta R_p$ the

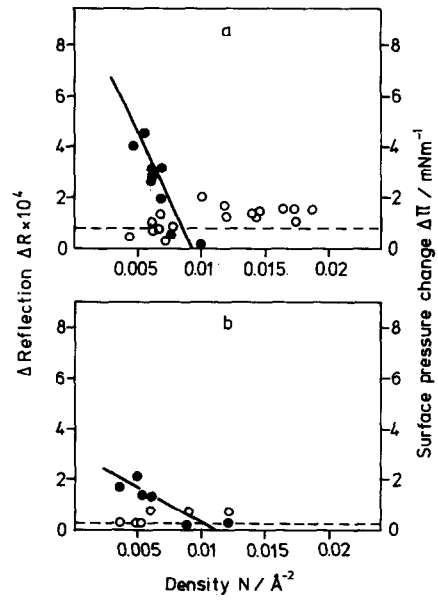

Fig.6. Dependence of reflection ΔR(410 nm) (○) and surface pressure change (●) on density of (a) DPPC and (b) DOMA monolayers at the air/solution interface. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5. (– – –) Reflection of (a) DPPC and (b) DOMA monolayers on buffer solution.

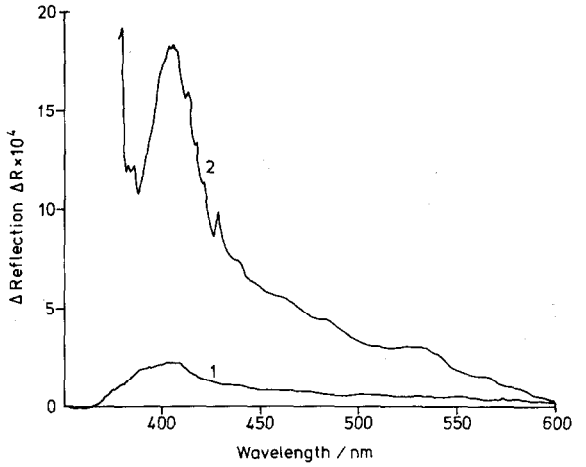

Fig.7. Reflection spectra obtained from cyt.*c* solution covered with a DPPA monolayer measured under $\alpha = 45.1°$ incidence (with respect to the normal) with linearly polarized light: ΔR_p electric vector oscillating (1) in the plane of incidence and (2) perpendicular to the plane of incidence. Surface pressure 30 mN/m. Subphase: 5 mg/l cyt.*c* in 1 mM Tris buffer, pH 7.5.

average orientation of chromophores at an air/water interface can be derived.

According to previously described theoretical calculations [19] the orientation of chromophores may be specified via an orientation parameter P. This parameter has the values $P = 0$ for an in-plane statistical distribution, $P = 1/3$ for an isotropic distribution and $P = 1$ when all dipoles are oriented normal to the surface. The calculated values of $\Delta R_s/\Delta R_p$ at an angle of incidence $\alpha = 45°$ for these situations are 5.128, −24.260 and −0.258, respectively [19]. From our experiments the $\Delta R_s/\Delta R_p$ value for an angle of incidence $\alpha = 45.1°$ is 5.55. The value of this ratio was also determined for $\alpha = 30.9°$ and found to be 1.96. The corresponding theoretical value for $\alpha = 30°$ is 1.79 for $P = 0$. From these results we conclude that the transition moments of cyt.*c* chromophores are oriented in the layer plane, signifying that the porphyrin plane is oriented parallel to the water surface. Earlier data for mixed monolayers of an amphiphilic porphyrin dye with different surface lipids [6,19] showed a statistical orientation of the transition moments in the monolayer plane. Our results indicate a similar orientation of the porphyrin that is located in the hydrophobic core of monolayer-bound cyt.*c*.

4. CONCLUSION

We have reported here the advantage of reflection spectroscopy as compared to other techniques for studying the adsorption of cyt.*c* to densely packed lipid monolayers. By using this direct method it was found that cyt.*c* is preferentially bound to monolayers of negatively charged phospholipids in the solid phase.

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