Biochimica et Biophysica Acta 903 (1987) 166–176
Elsevier

BBA 73664

Interactions of cytochromes b_5 and c with phospholipid monolayers

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(Received 20 January 1987)
(Revised manuscript received 27 April 1987)

Key words: Phospholipid monolayer; Cytochrome b_5; Cytochrome c

Monolayers of charged and neutral phospholipids at the air/water interface containing the cytochromes b_5 and c are studied by film balance techniques and by fluorescence microscopy. A new technique is introduced to obtain a defined and homogeneous protein distribution within the membrane. It is shown that both proteins preferentially partition into the fluid membrane phases coexisting with solid lipid domains, thus allowing formation of periodic protein distributions. Protein reconstitution in protein/lipid ratios up to 1:50 does not change the pressure, π_c, corresponding to the main lipid transition but changes the slope in the pressure/area isotherms. It also affects the pressure-induced lipid crystallization, in that the monolayer can be viewed as segregated into a protein-free and a protein-enriched phase. Whereas penetration of cytochrome c into the monolayer is highly dependent on lipid head group charge, this does not hold for cytochrome b. In both cases, monolayer penetration is monotonously reduced with increasing surface pressure, pointing to the dependence of hydrophobic protein–lipid interactions on hydrocarbon chain density.

Introduction

Many important biological processes depend on the conduction of a signal [1], electron, protein or energy [2], from a bilayer membrane to the adjacent water phase. To understand these processes, it is therefore of utmost importance to obtain a very detailed picture of the membrane/water interface. This includes the structure of hydrophobic and hydrophilic parts of the membrane and the arrangement of proteins in the membrane adjacent to it.

Physically, the most appealing way to study the interfacial structure resides in the use of model membranes composed of proteins and synthetic phospholipids [3]. With these molecules well-defined and pure systems can be prepared, allowing studies of even subtle structural changes via measurement of cooperative processes like phase transitions. Among these model systems the use of extended planar membranes appears to be a promising route [4]. These systems exhibit a well-defined geometry and curvature, allow independent variation of many parameters, such as molecular density, other molecular features and ionic

Abbreviations: DMPA, L-α-dimyristoylphosphatidic acid; DPPC, L-α-dipalmitoylphosphatidylcholine; DP- NBD-PE, L-α-dipalmitoylnitrobenzoxadiazolephosphatidylethanolamine; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide (lauryldimethylamine-*N*-oxide); EDTA, ethylenediaminetetraacetic acid, tetra sodium salt; cytochrome b, cytochrome b_5.

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conditions and the study of long-range in-plane transport processes.

Considerable progress has been made in recent years to develop preparative and analytical techniques to study stable planar membranes. It has become possible to prepare supported bilayer membranes in fluid as well as in solid lipid phases and to investigate protein influence on molecular diffusion [5]. To study processes predominantly located at the membrane/water interface it often suffices to build up lipid monolayers at the air/water interface. This is done in this work, where special use is made of the above-mentioned advantages of independently variable parameters. A new technique to reconstitute proteins into monolayers is introduced and applied to the partly membrane-penetrating proteins, cytochrome *c* and b_5. More detailed information on the microstructure of the film is presented by using recently developed microfluorescence techniques in combination with film balance measurements.

Materials and Methods

The phospholipids DPPC (Fluka, Buchs, Switzerland, DMPA (Sigma, Munich) and DP-NBP-PE (Avanti Polar Lipids, Inc., Birmingham, AL, U.S.A.) were specified to be 99% pure and used without further purification. The detergent LDAO was purchased from Fluka). The water was Millipore-filtered and divalent ion contamination was prevented by using $5 \cdot 10^{-5}$ M EDTA.

Cytochrome *b* was prepared from beef liver by the method described by Strittmatter et al. [6] and was fluorescently labelled using fluorescein isothiocyanate [7]. Horse heart cytochrome *c* (purity 95%, molecular weight 12385, type III) was obtained from Sigma, Munich.

Monolayers were prepared by spreading a 3:1 chloroform/methanol solution of the lipid or lipid/dye mixture on the water surface. In the experiments reported below, no buffer was used, in order to prepare a very simple subphase. This was obviously tolerated by the proteins, as reference experiments with cytochrome *c* in buffer solution have shown and as was observed previously for cytochrome *b* [8].

Monolayers were studied with a Wilhelmy-type film balance with an integrated fluorescence microscope [9] enabling a spatial resolution of 2 μm. Dyes or fluorescently labeled proteins at the air/water interface are excited through an objective lens in the bottom of the teflon trough, and the distribution of dye emission is detected via an SIT TV camera. Images stored via a video recorder are displayed after photographing from a TV monitor. They could also be analysed using the home-built image analysis system, BAMBI [10].

Experimental Results and Interpretation

A key problem to be solved when studying proteins in monolayers is the well-defined and functional arrangement of the proteins in the model membrane. For a fluorescent membrane protein of the photosynthetic apparatus we could show that a homogeneous or, in a defined way, heterogeneous molecular distribution could be achieved by a simple procedure [11]. Small droplets (approx. 1 μl) of a detergent solution (0.01 vol% LDAO) containing the protein could be spread on the hydrophobic surface of a fluid monolayer. Protein distribution and function could then be assayed by fluorescence microscopy and spectroscopy, respectively.

The proteins studied in this work are either water-soluble (cytochrome *c*) or have been made water-soluble by transforming them into a corresponding aggregation state after detergent dialysis (cytochrome *b*). Yet, reconstitution to obtain quantitative analysis was still a problem. Preparing the monolayer on a subphase with the protein dissolved has the following drawbacks:

(i) Protein diffusion into the monolayer is very slow (hours) [8,12], which leads to long standing times, and thus to possible denaturation before the experiment is started.

(ii) Within realistic times, only the protein-saturated monolayer can be studied quantitatively. In that case, however, the membrane distortion by the protein is so large that subtle changes, e.g., changes of phase transitions, cannot be quantified [8]. In addition, the protein concentration within the monolayer is difficult to determine.

(iii) Before forming the lipid monolayer, there may exist, on the water surface, a layer of fragments of denatured protein that, later on, may mix with the lipid layer.

An alternative reconstitution technique, injection of the protein into the subphase after lipid monolayer formation, presents the problem that the protein penetrates the monolayer close to the injection point, and hence is not distributed homogeneously, as shown below (Fig. 4d).

We therefore tried the reconstitution technique described above for membrane proteins, i.e., spreading of protein from detergent solution. Using fluorescently labeled cytochromes, we observed a microscopically homogeneous protein distribution in the fluid lipid phase and will demonstrate below to which extent the reconstitution is quantitative.

Fig. 1(a) compares pressure/area isotherms of pure DPPC monolayers with those measured after spreading the detergent solution containing no cytochrome *b* or cytochrome *b* in a protein/lipid ratio of 1 : 200, 1 : 100 and 1 : 20, respectively. The change in the slope of the isotherm is clearly pronounced at a pressure π_c of about 10 mN/m, corresponding to the onset of the fluid/gel phase transition. This transition is broadened but not shifted by protein reconstitution. Comparing the isotherms in the protein-free case, one realizes that the detergent increases the slope of the nearly horizontal part of the isotherm without increasing the molecular areas in the fluid and solid phases. This shows that within a measurement accuracy of 5% no LDAO is incorporated into the monolayer. The change in the coexistence phase, basically a hindrance of crystallization, which has also been observed after purposefully incorporating surface active impurities [13], may still be due to residual detergent or to detergent micelles adjacent to the monolayer in the subphase. On increasing the protein content, one observes an increase in molecular area, obviously due to the monolayer area taken by cytochrome *b*. This area is reduced with incresing surface pressure, because then the isotherms asymptotically approach the same molecular area.

These results are presented more clearly in Fig. 1(b), where the ordinate ΔF is derived by subtracting the molecular area per lipid in the pure DPPC monolayer from that measured after spreading the detergent solution for the same surface pressure. In the case of protein incorporation, ΔF can easily by converted into a partial

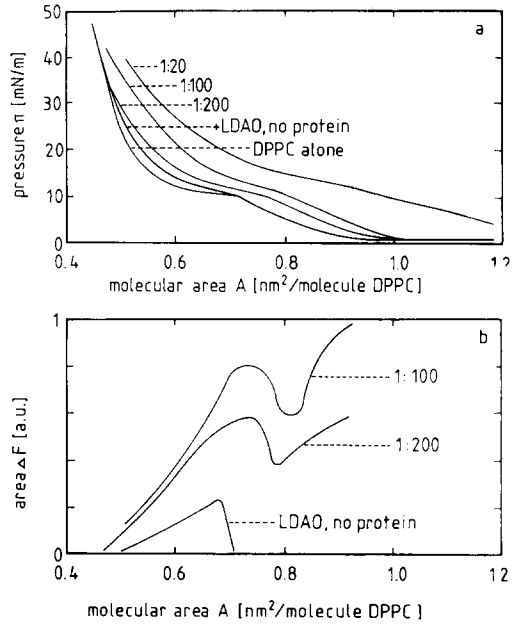

Fig. 1. (a) Surface pressure as a function of molecular area per lipid for DPPC monolayers before and after spreading of a 10^{-2} vol% LDAO solution containing no cytochrome *b* and cytochrome *b* to yield protein/lipid ratios as indicated. $T = 26\,^{\circ}$C, pH 5.6, $5 \cdot 10^{-5}$ M EDTA. (b) Area ΔF, determined by subtracting the molecular area of pure DPPC monolayer from the values measured as given in (a) after spreading for the same pressure, using the abscissa of (a).

protein area by dividing the value by the number of proteins. The quantitative interpretation is complicated by the fact that the protein influence on lipid crystallization also changes the area per lipid. Qualitatively, the curves in Fig. 1(b) can be understood as follows: If there is a protein-free detergent solution spread, ΔF rises steeply at the onset of the phase transition, since LDAO in the fluid phase causes a melting pressure increase. For fixed pressure this increases the mean area per lipid. The latter is finally reduced on further pressure increase or reduction of the area. This mechanism is also expected for protein incorporated, and likewise gives rise to the left maxima of the corresponding plots in Fig. 1b. The fact that ΔF approaches zero on further increase of the molecular area demonstrates that all cytochrome *b* would be squeezed out of the surface layer at a high enough surface pressure. However, in contrast to findings presented later for cytochrome *c*, there is no specific pressure above which this process takes

place. Concerning the shapes of the curves, the high onset starting at low pressure is due to pressure increase on reconstitution at fixed barrier position with the monolayer in the fluid phase. Then, compressing the barrier leads to a ΔF reduction, probably due to an area change of the protein. This may be due to the lateral compressibility of the LDAO/protein complex (see below). These features are qualitatively independent of protein concentration, but are roughly scaling with it in magnitude ΔF.

A similar behaviour of the isotherms is also observed for cytochrome *b* in DMPA monolayers (Fig. 2a, b)), the only pronounced difference from the DPPC monolayer being a change in the isotherm slope at a pressure of about 25 mN/m. This may be ascribed to a squeezing out of cytochrome *b* at this pressure.

In this case also the LDAO influence is negligible: As shown in Fig. 1(a) for DPPC monolayers, one observes a slight increase of the isotherm in the phase coexistence range but no effect in π_c.

The latter is much more pronounced considering the interaction of cytochrome *c* with DMPA monolayers (Fig. 3). This protein obviously also penetrates the monolayer, but apparently only at pressures below 25 mN/m. In this case the isoterms are time-dependent, which may be ascribed to the imperfect reconstitution and which is observed more clearly in Fig. 3(b). In fact, for cytochrome *c*, in contrast to cytochrome *b* addition, we did not observe a spontaneous but a gradual pressure rise. This may be due to cytochrome *c* not being taken up immediately and gradually disappearing from the subphase.

That cytochrome *c*/lipid interaction is largely of an electrostatic nature can be deduced from a comparison of its interactions with DMPA and DPPC monolayers. In the latter case, spreading of cytochrome *c* containing LDAO solution influences the isotherm in the same way as shown in Fig. 1 for the protein free case. Obviously, cytochrome *c* does not interact at all with DPPC membranes, in accordance with previous findings with lipid vesicles [12,14] and monolayers [15].

Information on protein distribution is obtained from fluorescence micrographs using fluorescently labeled protein. Figs. 4(a–c) present a series of

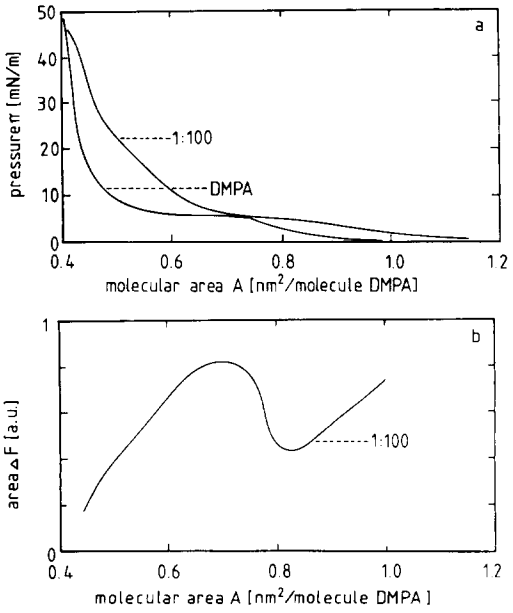

Fig. 2. (a) Surface pressure as a function of molecular area per lipid for DMPA monolayers before and after spreading of a LDAO solution containing cytochrome *b* to yield a protein/lipid ratio of 1:100. $T = 26\,°C$, pH 5.6, $5 \cdot 10^{-5}$ M EDTA. (b) Area ΔF derived from (a) as described under Fig. 1.

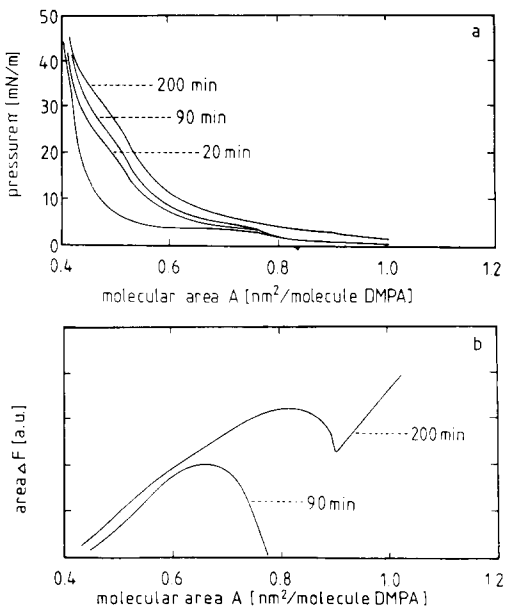

Fig. 3. (a) Surface pressure as a function of molecular area per lipid for DMPA monolayers before and various times (indicated in min) after spreading of a LDAO solution containg cytochrome *c* to yield a protein/lipid ratio of 1:100. (b) Area ΔF derived from (a) as described under Fig. 1a.

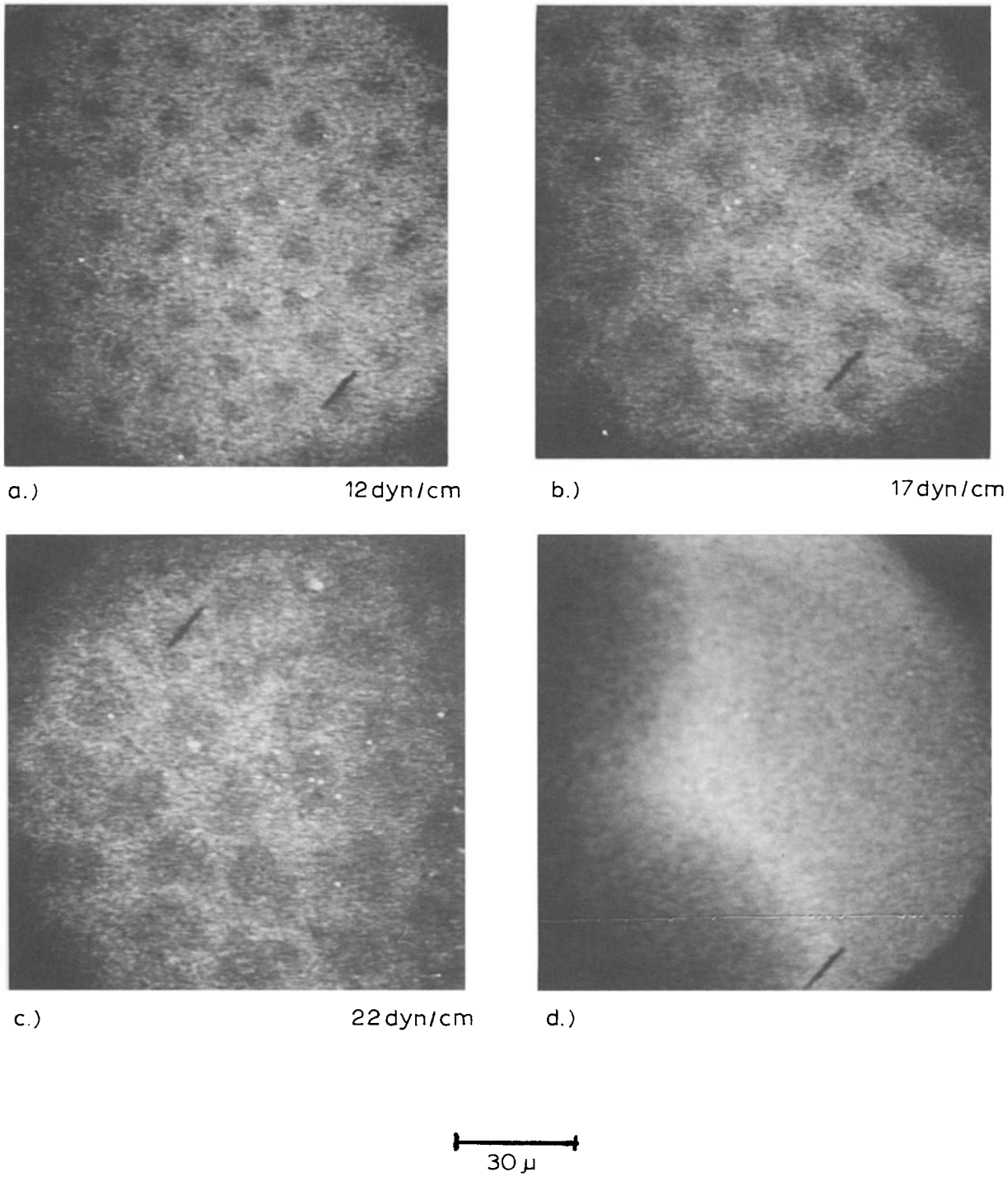

Fig. 4. Fluorescence micrographs of DPPC monolayers containing fluorescently labeled cytochrome *b* in a protein/lipid ratio of 1 : 50. (a–c): Protein incorporated by the described spreading technique and at pressures (indicated in mN/m) to keep the film in the solid/fluid phase coexistence region. (d): Protein added by subphase injection. $T = 25\,^{\circ}$C, pH 5.6, $5 \cdot 10^{-5}$ M EDTA.

micrographs on increasing the surface pressure within the phase coexistence region. Whereas for a pressure below $\pi_c = 10$ mN/m a homogeneous fluorescence distribution is observed, dark domains of rather uniform size appear at higher pressures. These domains are ascribed to gel-phase lipid coexisting with the fluid phase and have previously been visualized using surface-active dyes that predominantly partition into the fluid phase [16–18]. Due to the very low light level, the contrast is not as good as with other dyes. Yet one clearly observes a preferential partitioning of the protein into the fluid phase.

More quantitative information on protein distribution can be obtained from a densitometric analysis of the fluorescence micrographs, as one may assume a linear relation between protein concentration and fluorescence intensity. Thus, Fig. 5 shows a series of densitograms through vertical lines in the pictures on increase of the pressure (Fig. 5(a–d)), starting from the beginning of the phase coexistence range near π_c. The linear line also given corresponds to the baseline, consisting of camera noise, scattered light and reflected light passing the optical filters. The intensity maxima, clearly observable in the densitograms of Fig. 5, corresponding to the lower pressures, result from fluid areas, whereas the minima can be attributed to solid domains. The fact that the minimum intensity is somewhat above background indicates that a protein fraction is dissolved in the condensed phase. This fraction, however, is rather small, and from the contrast the partition coefficient for protein distribution between fluid and solid monolayer phases may be estimated to be near 5. On increasing the pressure one also observes a reduction of total intensity. This demonstrates that cytochrome *b* is squeezed out of the surface layer and does not reside near the interface.

The above finding, that the protein preferentially partitions into the fluid phase, seems to be very general, as similar patterns were also observed for cytochromes *b* and *c* in DMPA.

Fig. 4(d) also presents a typical observation of surface textures if the protein is injected into the subphase under the lipid monolayer. Extremely inhomogeneous protein distribution is established with a protein enrichment near lines separating areas of vastly different composition. Hence, it appears that the protein segregated within the surface layer forms a phase with distinguished lipid content, and the surface inhomogeneities cannot be removed within times of more than 10 h. Nevertheless, one can occasionally observe textures as shown in Figs. 4(a–c) if the monolayer is

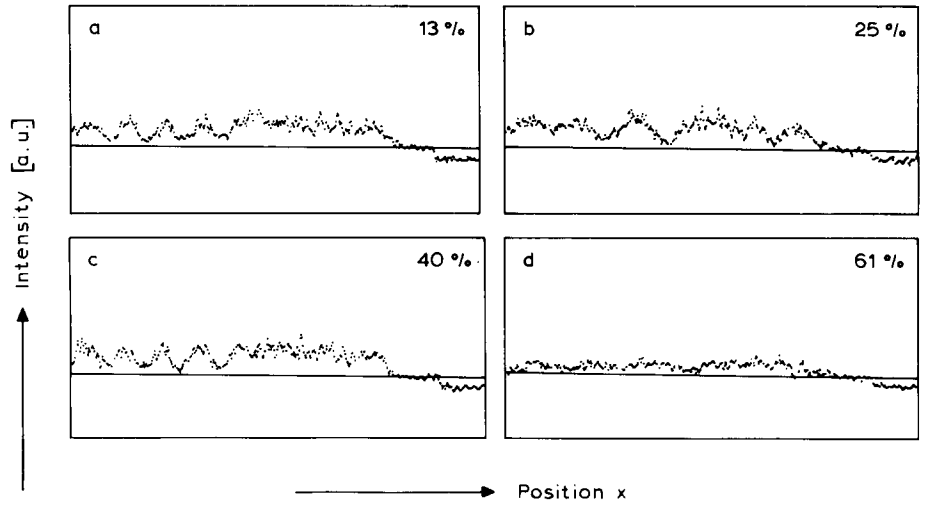

Fig. 5. Fluorescence intensity along a vertical line in the fluorescence micrographs taken under conditions of Figs. 4(a–c) for various degrees of crystallization, ϕ (indicated). The straight lines correspond to the background intensity detected in the absence of protein fluorescence.

maintained in the phase coexistence range, but this preparation procedure obviously yields no defined protein distribution.

Images as those presented in Figs. 4(a–c) can also be analysed to quantify the protein influence on lipid crystallization. (For simplicity we use the word crystallization, although we know from synchroton X-ray experiments that the condensed phase exhibits long-range orientational but short-range positional order [20].) From the measured dark area ratio S one may determine the degree of crystallization, ϕ, according to [19]:

$$\phi = 1 - (1 - S) \cdot \frac{A}{A_{fl}} \tag{1}$$

In Eqn. 1 A is the mean molecular area. A_{fl} is assumed as the molecular area in the fluid phase at the pressure π_c. The assumption of a constant value of A_{fl} does not introduce errors above 10% in determining ϕ, because for $S \approx 0$, A_{fl} is nearly constant due to the near horizontal isotherm slope and for $0.8 > S \gg 0$, where due to increased pressure A_{fl} may be reduced up to 20%, the second term in Eqn. 1 becomes less important.

Fig. 6 compares ϕ as a function of molecular area with the corresponding isotherms in the presence and absence of protein. In the latter case fluorescence micrographs were obtained using the dye DP-NBD-PE. In the absence of protein (Fig. 6(a)) ϕ depends linearly on molecular area A on increasing the pressure above π_c. The linear line can be understood as due to coexistence of two phases with molecular areas $A_{fl} = 74$ Å^2/molecule and $A_{solid} = 48$ Å^2/molecule. Obviously, the molecular area A_{solid} is larger than expected for a solid lipid phase, as deduced from pressure/area isotherms (A_{solid}(DPPC) = 44 Å^2/molecule). This indicates a density difference of the condensed phases at different pressures. The deviation of the $\phi(A)$ relationship at higher pressures (35 mN/m) may thus be explained by a structural change of the condensed phase.

These deviations from linearity are much more pronounced and qualitatively different if the monolayer contains protein (Fig. 6(b)). The $\phi(A)$ relation exhibits a plateau in the region of 70 to 60 Å^2/molecule, basically meaning that there is a range of pressures within which an overall density change influences the fluid and the condensed

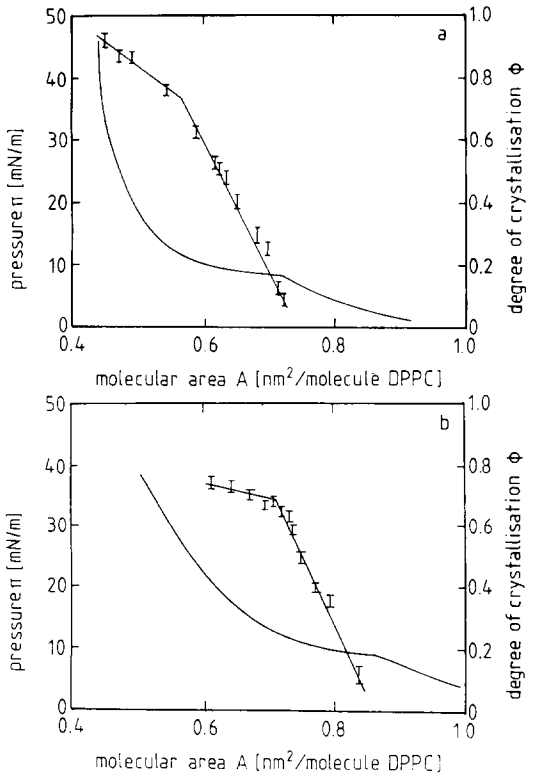

Fig. 6. Degree of crystallization ϕ, derived as described in the text, as a function of molecular area in comparison with the corresponding isotherm for a DPPC monolayer without (a) and with (b) cytochrome *b* in a protein/lipid ratio of 1 : 50. Conditions as in Fig. 4(a–c).

phases in a similar way and where the area ratios are not affected. This finding will be discussed more elaborately below.

Interesting aspects, that may reflect lipid specificity, result from kinetic studies. These have previously been performed in experiments after protein injection [8] but are difficult to analyze, as time constants are largely determined by transport through the subphase. The latter may depend on apparatus used due to different water depth, convection and stirring conditions, and thus do not allow for a molecular picture. The result of an alternative experiment is presented in Fig. 7: The monolayer is compressed to a high pressure where the protein is partly squeezed out of the surface layer. On quickly expanding the film to a new fixed barrier position, the pressure, $\pi(t)$, quickly drops and then rises again to a new equilibrium value $\pi(\infty)$ (Fig. 7(a)). The pressure relaxation

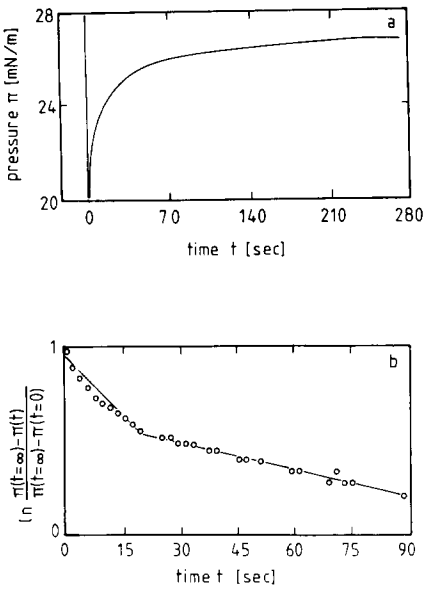

Fig. 7. (a) Pressure as a function of time following a fast pressure decrease of a DMPA monolayer containing cytochrome *b* in a protein/lipid ratio of 1:100. $T = 25\,^{\circ}$C, pH 5.6, $5 \cdot 10^{-5}$ M EDTA. Pressure before starting the experiment: 35 mN/m (b) $\ln[(\pi(t=\infty)-\pi(t)/(\pi(t=\infty)-\pi(t=0))]$ as a function of time for the measurement of (a).

cannot be described by a single algebraic function but as a sum of two exponential functions (Fig. 7b).

$$\pi(\infty) - \pi(t) = a \cdot e^{-t/\tau_1} + b \cdot e^{-t/\tau_2} \qquad (2)$$

The time constants τ_1 and τ_2 are 10 ± 5 and 70 ± 20 s, respectively, and independent of pressure $\pi(\infty)$ or starting pressure, but the amplitude ratio a/b depends on pressure. The fast component with relaxation time τ_1 is absent if the pressure is kept below π_c during the experiment ($a = 0$) and its amplitude increases with increasing surface pressure. Obviously it can be ascribed to the melting of crystalline domains. The slow component (relaxation time τ_2) then corresponds to diffusion, adsorption and incorporation of the protein.

The melting process, although in its details not the subject of this work, is visualized in Fig. 8. Expanding a compressed film with large solid domains with occasional dye inclusions (Fig. 8(a)) one then observes extended circular inclusions within these condensed areas (Fig. 8(b)). This indicates that melting does not occur by molecular detachment from the outer faces of the condensed

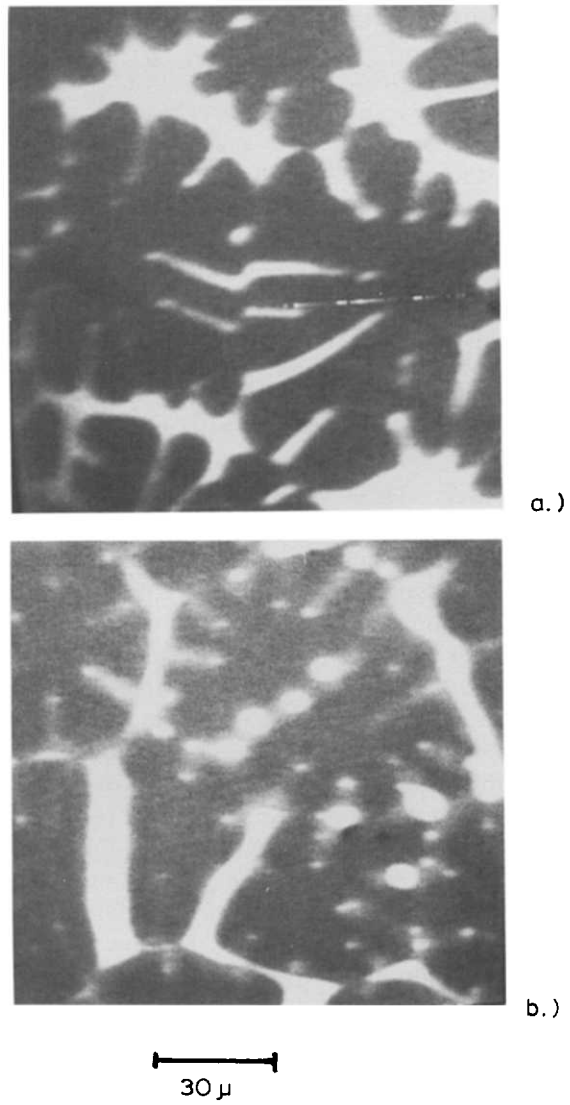

Fig. 8. Fluorescence micrograph of a DMPA monolayer containing cytochrome *b* in a lipid/protein ratio of 100:1 and additionally 1 mol% of DP-NBD-PE before (a) and 10 s after a pressure jump (b). Surface pressures, 20 mN/m (a) and 11 mN/ (b), $T = 25\,^{\circ}$C, pH 5.6, $5 \cdot 10^{-5}$ M EDTA.

domain, but that fluid phase inclusions are formed. It may be due to an elastic expansion of the crystalline domain on pressure reduction, causing a melting of areas inside the domain probably because of a locally high impurity content. The difference between a protein-containing layer and a protein-free one may be that proteins stabilize defects in the condensed phase, and thus counteract melting.

Discussion

Protein reconstitution

Before reconstitution the two proteins used in this work were present in water-soluble form, but they exhibit a different hydrophilicity. Whereas cytochrome *c* is strongly hydrophilic, cytochrome *b* is a membrane protein exhibiting large hydrophobic parts penetrating into the membrane. Reconstitution into the monolayer, therefore, occurs spontaneously and quantitatively. Assuming that all propteins spread are incorporated one calculates from the area increase of the monolayers with nominal protein/lipid ratios of 1 : 200 and 1 : 100 areas per cytochrome *b* of 760 and 700 Å^2, respectively. These values are in reasonable aggreement with published data [8,21,22] and demonstrate that almost all added protein is incorporated. The latter does not hold on increasing the protein/lipid ratio towards 1 : 20. In that case the saturation limit, reported as 1 : 32 for cytochrome *b* in DPPC [8], is exceeded and protein must be lost during reconstitution. Yet we should state that values given on protein cross-section represent upper limits, since protein incorporation also has a disordering influence on the membrane causing an increase in molecular area [23].

It is also conceivable that a minimum LDAO concentration (determined as 10^{-2} vol% is needed for quantitative reconstitution. For lower LDAO content the droplet does not spread on the hydrophobic lipid surface, obviously due to too high a surface tension. In addition, the monolayer has to be in a homogeneous fluid phase: Reconstitution at virtually zero pressure is imperfect, presumably due to areas of low-density ('gaseous') lipid through which material spread disappears into the subphase.

On the other hand, the hydrophilic cytochrome *c* is not spontnaeously incorporated. After spreading the LDAO/cytochrome *c* solution one does not observe a spontaneous but a gradual pressure rise and the pressure/area isotherms are still time dependent. Yet, also in this case reconstitution is nearly complete, because the protein area calculated from the surface area increase at π_c under the assumption of 100% incorporation (500 Å^2/molecule) yields a realistic value for this protein. The advantage of our protein-spreading technique probably results from the fact that even if the protein is not immediately incorporated, it is laterally homogeneously distributed and concentrated in the subphase near the interface.

Protein arrangement in the membrane

Besides comparing two proteins of different hydrophilicity, this study also compares two phospholipids with different head groups. Hence, from the drastically different findings on cytochrome *c* interaction with the negatively charged DMPA, in contrast to that with then eutral DPPC surface, one may conclude that the interaction is of electrostatic nature. This is in accordance with previous findings on monolayers [12,15] and on vesicles [14]. For cytochrome *b*, on the other hand, there is no direct involvement of lipid head group in the interaction. This can be deduced from the fact that the influence of the protein on the isotherms (Figs. 1 and 2) is very similar, the only pronounced difference being the well-defined pressure where the squeezing out of the protein is directly observable. In accordance with previous conclusions [8], it seems to be the hydrocarbon chains attracting the protein, and therefore the longer chain of DPPC stabilizes the protein position within the membrane. Nevertheless, also in this case we observe a reduction of protein content with increasing surface pressure. One reason for this may be that on partly crystallizing the membrane, protein is enriched in the fluid phase, thereby exceeding its saturation limit. This possibility can be rejected in view of the finding that the area changes for protein/lipid ratios of 1 : 200 and 1 : 100 in Fig. (b) are very similar. One would, however, expect that for the lower protein content the saturation limit in the fluid phase would be approached only for $\phi > 80\%$, at a much higher pressure than for the higher cytochrome *b* content. Therefore, we assume that it is the hydrocarbon chain density that has to be small enough to provide optimum protein solubilization, although we have no quantitative handle on this.

This work documents and stresses the importance of the lipid phase considering protein/lipid interactions. We have shown that cytochromes *b* and *c* as well as other membrane proteins preferentially partition into the fluid lipid phase. This is understandable, as the high packing densities in

the condensed phases do not allow for distortion due to protein penetration. Therefore, it is meaningless to compare lipids with different molecular features but also in different phases: The latter may govern protein incorporation into the membrane indpeendent of all other details. This is also why we have chosen the above two lipids exhibiting a well-pronounced phase change at a comparable pressure.

Crystallization and melting may also determine the kinetics of processes measured via changes in surface pressure and molecular area. This was demonstrated above, where we showed that pressure relaxations with time scales on the order of 10 s are due to melting of crystalline domains. These may or may not be followed by protein penetration, which is observed to occur on slower (diffusion limited) time scales. In view of this, one can also understand the break in the slope of the pressure versus time plot following injection of cytochrome *b* into the subphase under a DPPC monolayer [8]: It occurs at the transition pressure π_c, and lipid crystallization on further pressure increase reduces the fluid area accessible for protein penetration, and hence the penetration rate.

At the end of this subsection we should comment on the monotonous decrease in area per protein on increasing the surface pressure from 1 mN/m to π_c. This is also observed for pure cytochrome monolayers, but cannot be understod if the protein resembles a hard sphere. In that case, one would have to assume an area per protein of more than 1400 Å^2 at zero surface pressure and the area reduction would then be due to squeezing out of the protein on increasing the surface pressure. This large area, however, is inconsistent with data on cytochrome *b* size. Hence, we assume that at low pressures there are changes in the area per protein. These are not connected with the possible detergent environment, as they are also observed in the absence of detergent. They probably arise from either a pressure-induced cytochrome *b* structural or an orientational change.

Protein influence on membrane structure

In contrast to findings with proteins in bilayer vesicles [24], cytochromes *b* and *c* do not change the pressure π_c corresponding to the main transition of the lipid. This indicates that the monolayer surface can be viewed as a phase-separated system: An undistorted lipid phase with main phase transition at π_c and a protein-enriched phase undergoing no phase transition observable in the isotherm. This second phase may be the one responsible for the plateau in the $\phi(A)$ relation in Fig. 6(b). This means that there is a large protein fraction, that may be viewed as boundary lipid, which undergoes solidification at a pressure much larger than π_c.

It is interesting to speculate about the molecular origin of the plateau in the $\phi(A)$ relation. Its presence requires that the microscopically observed dark (solid) domains are about as compressible as the bright (fluid) areas and may have one or several of the following reasons:

(i) At the pressures considered the remaining fluid phase enriched in protein may be less compressible than expected for a typical fluid lipid. Thus, the protein 'strengthens' the fluid membrane. This is conceivable, but judged from the slope in the isotherms in the region of the $\phi(A)$ plateau, does not lead to a compressibility as would be typical for a solid lipid phase.

(ii) Due to protein incorporation the solid becomes more compressible. The reason for this may be that the solid phase is not a typical solid but exists of small grains with nonsolid space in boundary areas where protein may still be located. This protein may be squeezed most easily into the subphase or into the fluid lipid phase, thus causing an anomalously large area compressibility. This speculation is supported by Synchroton X-ray data on monolayers in the absence of protein, revealing low positional coherence length for the 'solid' phase [20], and by Monte-Carlo calculation pointing to the possibility of a small grain type structure [25]. We are at present preparing experiments to conclude on it by a more quantitative analysis of fluorescence microscopic data obtained with a more sensitive detection system. Indications that protein may exist in the solid phase also result from Fig. 5, where the fluorescence intensity level corresponding to this phase is shown to be above background.

Final remark

This study introduced a new technique to pre-

pare protein-containing monolayers and reported microscopic and quantitative data on monolayer penetration by proteins, stressing in particular the influence of different lipid phases. It can also be viewed as a necessary prerequisite for more sophisticated studies on the structure of proteins in membranes. Experiments presently being planned by our group are devoted to microscopically probe the membrane head group region by surface force [26] and and X-ray reflection measurements [27].

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft, through SFB 143.

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