THE MODULATION OF LIPID BILAYER FLUIDITY BY INTRINSIC POLYPEPTIDES AND PROTEINS

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1. Introduction

The importance of lipid-bilayer fluidity for biomembrane structure and function is now well appreciated [1–3]. The presence of molecules such as cholesterol within the lipid bilayer structure is known to modulate the bilayer fluidity [4]. Early results using monolayer systems and described as 'condensation effects' were explained and rationalised in terms of this lipid-modulation effect [5]. A whole range of physical techniques have supported this interpretation including the use of NMR spectroscopic methods [6–8].

The effects of intrinsic or integral proteins on lipid bilayer fluidity have been as yet less widely studied. The main technique so far has used ESR spin probes. Some workers have interpreted their results in terms of immobilised boundary lipids surrounding the proteins [9]. The precise nature of this immobilisation is however not well defined. Other workers have used reconstituted systems for such studies and described the ESR and enzymatic data in terms of annulus effects [10]. There appears however to be some controversy concerning these data [11] as well as confusion concerning the special effects of lipids upon intrinsic proteins [12].

In the present paper we extend our recent studies [13] of the modulating effect of an 'intrinsic' polypeptide (gramicidin A) upon lipid fluidity using physical techniques including monolayer methods. We use the results to try to provide insight into the situation of intrinsic proteins in membrane structures. ('Captive-lipids' [14] should not be present with this simple intrinsic polypeptide.) The modulating effect of intrinsic proteins on lipid bilayers is of considerable importance for understanding the dynamics and function of biomembrane structures.

2. Experimental

L-1,2-dipalmitoyl-α-lecithin and egg yolk lecithin were obtained from Fluka Chemicals and Lipid Products respectively. Gramicidin A was purchased from Koch-Light Laboratories. The purity of the lecithins was confirmed by thin-layer chromatography and in the case of L-1,2-dipalmitoyl-α-lecithin was also evident from the calorimetric data of the pure lecithin in water. Both the lecithins and the gramicidin A were used without further purification. The methyl ester of stearic acid spin-labelled in the 12 position was obtained from Synvar Ltd. Spin-labelled phospholipid was synthesised from egg yolk lysolecithin and palmitic acid, spin-labelled in position 10, as in [15].

The preparation of samples and determination of the Laser Raman and ESR spectra have been described in [13]. The monolayer experiments were carried out on a teflon trough fitted with fixed and moving teflon barriers. Appropriate amounts of the components, (the gramicidin A dissolved in 1:1 chloroform: ethanol and the phospholipid dissolved in chloroform) were mixed in an Agla syringe and an aliquot of the solution spread to form the monolayer. The surface-pressure-area curves, measured by compression were determined at least three times. All the usual experimental precautions in such determinations were taken.

3. Results and discussion

Various studies of membrane-protein structures support the idea that they contain hydrophobic polypeptide segments, as suggested for the MN glycoprotein [16] of the erythrocyte membrane, and sometimes consisting of groups of helical polypeptides, as with bacteriorhodopsin [17]. In both cases the polypeptide segments span the lipid bilayer. The polypeptide gramicidin A (a pentadeca peptide) is a relatively simple polypeptide considered to form transmembrane channels by means of the association of 2 molecules. The length of the double molecule is in the range 30–37 Å dependent upon the particular helical conformation chosen. A structure based upon a $_{4(L,D)}$helix with 4.4 residues/turn has been proposed [18], but a $_{6(L,D)}$structure has also been considered [19]. The molecule is surface active and can form monolayers at the air–water interface [20,21]. For our purposes it is a rigid polypeptide structure which can orientate at an interface into a lipid monolayer or bilayer system. We examined this intrinsic polypeptide so as to ask the question how does the presence of such a polypeptide affect lipid fluidity and what does this tell us about the modulation of biomembrane structure fluidity particularly due to intrinsic proteins?

The surface pressure–area curves at 22°C for egg yolk lecithin containing increasing amounts of gramicidin A are shown in fig.1a. It can be seen that the effect of the polypeptide is to reduce the lateral compressibility of the mixed monolayer and also that the area/molecule of gramicidin A at a pressure of 30 dyne/cm is $\sim$ 130 Å^2.

The mean area/molecule as a function of the composition of the mixed monolayer is shown in fig.1b at 3 surface pressures. Comparison of the expected theoretical straight line which occurs in the case of ideal mixing with the experimental line, indicates that gramicidin A is exerting a 'condensing' effect similar to that observed with cholesterol. The 'condensing' effect decreases with decreasing surface pressure and

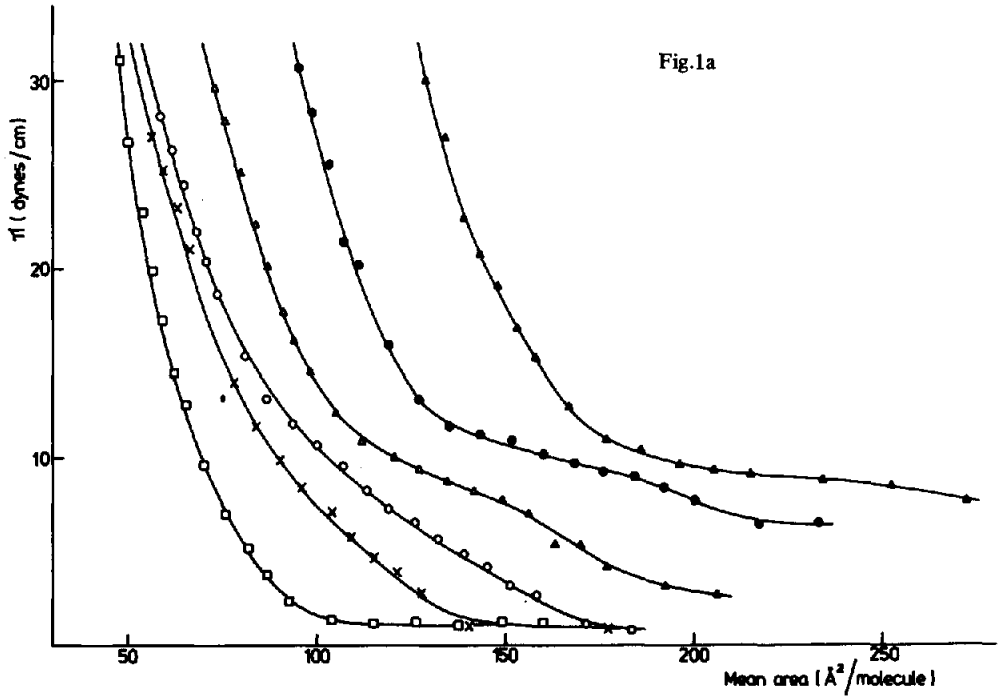

Fig.1a. Surface pressure–mean molecular area curves for mixed monolayers of egg-yolk lecithin and gramicidin A. (□– –□) pure egg-yolk lecithin; (X– –X) 15.1% gramicidin A; (○– –○) 32.3% gramicidin A; (△– –△) 49.7% gramicidin A; (●– – ●) 69.2% gramicidin A; (▲– –▲) pure gramicidin A.

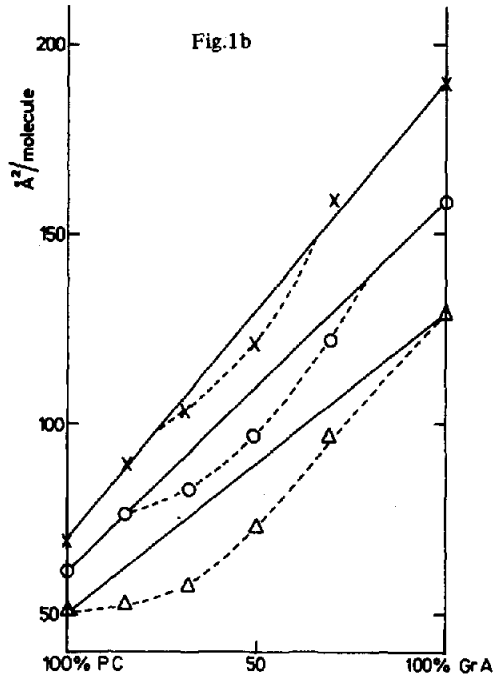

Fig.1b. Mean molecular area–mole fraction plots for egg-yolk lecithin and gramicidin A mixtures at 30 dyne/cm (△– –△); 15 dyne/cm (○– –○); 10 dyne/cm (×– –×).

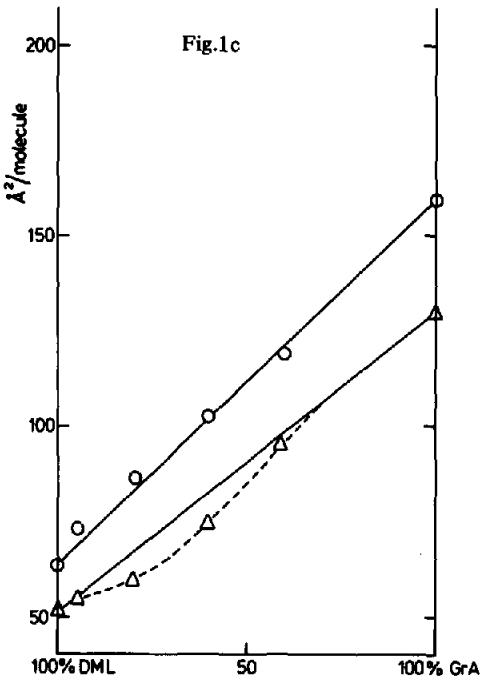

Fig.1c. Mean molecular area–mole fraction plots for dimyristoyl lecithin and gramicidin A mixtures at 30 dyne/cm (△– –△); 15 dyne/cm (○– –○).

very little deviation can be observed from ideality at a surface pressure of 10 dyne/cm. Similar behaviour has also been observed for mixed monolayers of dimyristoyl lecithin and gramicidin A at 23°C.

Previous studies of monolayers of gramicidin A have indicated limiting values for the area/molecule of 145 Å² [20,21]. It was suggested that due to difficulties in measuring the point of collapse area values measured at pressures greater than 10 dyne/cm were meaningless [21]. A determination of the molecular area of gramicidin A in a mixed egg-yolk lecithin/gramicidin A monolayer gave a value of ∿ 200 Å². However no allowance was made for the possible condensing effect of gramicidin A on neighbouring lecithin molecules. We assume that gramicidin A has the same molecular area in the mixed monolayers as it does in the pure monolayer. This enables us to calculate its condensing effect on phospholipids, e.g., egg-yolk lecithin. This is shown in fig.1b. A similar condensing effect but less pronounced is also seen with dimyristoyl lecithin (fig.1c). Maximum deviation from ideality in both cases occurs between 20 mol% and 40 mol% gramicidin A and in this region the area/molecule of the lecithin has been reduced to a value around 44 Å² which is typical of the cross-sectional area of a lecithin molecule with vertical all-*trans* chains [22].

The behaviour observed with the egg-yolk lecithin and the saturated lipid is understandable in view of the well known difference of their transition temperatures. The unsaturated lecithin is (at the temperature of the experiment) in its fluid state whilst the dimyristoyl lecithin is close to its T_c transition temperature. This 'condensation' effect can be compared with the effect observed with cholesterol [23].

It can be seen that with cholesterol maximum condensation occurs around 50 mol%, a higher value than that for gramicidin A, i.e., the 20–40 mol% for gramicidin A. This is a reflection of the larger size and more rigid nature of the polypeptide, gramicidin A, compared to the cholesterol molecule. Thus each molecule of gramicidin A 'condenses' a larger number

of lipid molecules. Thus the monolayer studies show that a rigid intrinsic polypeptide can modulate the lipid fluidity and lateral compressibility giving rise to a rigid structure dependent upon the relative concentration of polypeptide to lipid present in the system.

Raman data [13] are also useful for showing that a rigidifying effect upon the fluid lipid chain conformations can be produced by the presence of an intrinsic polypeptide. In particular the data indicate a decrease in the *gauche* rotamer content of the lipid alkyl chains at relatively low content of the polypeptide. The quantitative interpretation of data of this type is however not unequivocal. Recent publications differ in their approach to this problem [24,25]. We have followed [13] the treatment as in [24] which assumes that the band at 1130 cm^{-1} is the skeletal optical mode *K=O* vibration of a hydrocarbon chain in which every C–C bond is in a *trans* conformation. However, the *trans* segments of chains containing *gauche* rotamers may also contribute to the intensity of this band. However, a lower limit of 3 has been set [25] to the number of bonds in a *trans* segment such that it will still contribute to the 1130 cm^{-1} band. That the intensity contribution per *trans* bond in a segment is a constant is also assumed [25]. They then define an order parameter:

$$S_{trans} = \frac{(I_{1130}/I_{ref})\ \text{Observed}}{(I_{1130}/I_{ref})\ \text{Solid DPL}}$$

where (I_{1130}/I_{ref})Solid DPL represents fully ordered (all-*trans*) chains. There is, however, no simple relationship between S_{trans} and the number of *trans* bonds in a lipid chain if a certain minimum number of *trans* bonds is necessary for contribution to the Raman intensity. S_{trans} becomes a function of both the number and the arrangement of *gauche* rotamers. If we assume that the increased *trans* content is concentrated in the first lipid layer adjacent to the polypeptide then the 'equivalent' of $\sim$ 10 lipid molecules are affected. (About 6 lipid molecules form a tightly packed lipid layer around the gramicidin molecule in the $_{4(L,D)}$configuration.) We shall examine various aspects of the quantitative analysis of the Raman data in future publications.

When ESR spectra are studied (see fig.2) using spin probes, the presence of a new 'immobile component' is observed at very high concentrations of polypeptide to lipid. A typical ESR spectrum of the two components in the fluid phase is shown in fig.2a. The 3 line spectrum from strongly immobilised spin label is indicated by the arrows. This spectrum is superimposed on another 3 line spectrum typical of a highly-mobile spin label. This highly mobile component disappears on cooling the mixture to room temperature below the main phase transition of the pure lipid (fig.2a). Similar behaviour to this is observed when a spin-labelled phospholipid is used as a probe. Typical spectra from a wide range of lipid–polypeptide mixtures above the main phase transition are shown in fig.2b. The presence of such an 'immobilised component' requires that the rotational correlation time of the nitroxide group should be in the long correlation time regime ($> 3 \times 10^{-8}$ s) and by itself does not preclude exchange with excess fluid lipid at frequencies approaching 10^7 Hz [11]. It is significant that this new immobilised component is observed only at high concentrations of polypeptide. There will clearly be a decrease of exchange possibilities for the spin probes at these high polypeptide ratios.

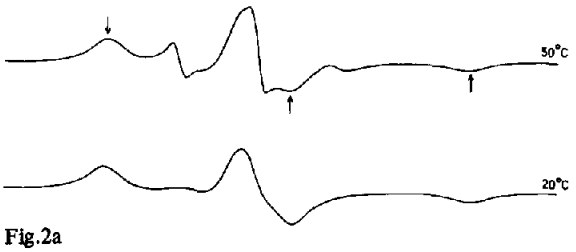

Fig.2a

Fig.2a. The ESR spectra of 2% (w/w) C_{12} spin-labelled methyl stearate in a dipalmitoyl lecithin/gramicidin A (2:1) dispersion at the temperatures shown.

We have attempted to simulate lipid–polypeptide arrangements in random arrays using a simple Monte-Carlo technique where the lipid chains and the polypeptide helix (gramicidin A) diameters are scaled to give convenient and practical arrays.

Photographs of 2 such random arrays simulating different ratios of lipid to polypeptide are shown in fig.3a,b. The arrays show a number of interesting features. By randomly mixing the 2 components local fluctuations in concentration occur which vary over a wide range of values. Polypeptide aggregates or clusters occur, i.e., polypeptide–polypeptide contacts

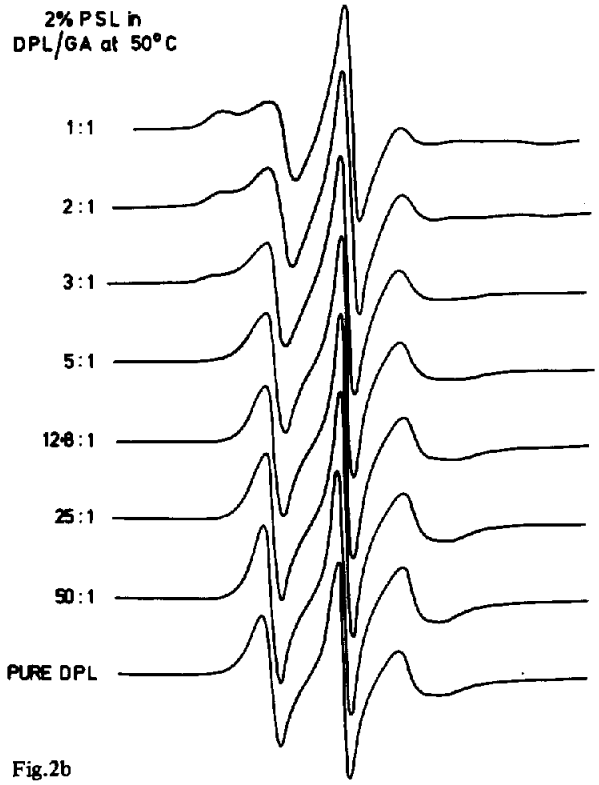

Fig.2b

Fig.2b. The ESR spectra at 50°C of 2% (w/w) phospholipid spin label in dipalmitoyl lecithin–gramicidin A dispersions with the indicated molar ratios.

occur, and their number increases as the polypeptide : phospholipid ratio increases. Moreover among the clusters can be seen lipid chains sandwiched between two or more protein molecules.

Random arrays of the type shown in fig.3 were used to obtain the contact data plotted in fig.4. The composition of the random arrays is plotted in terms of percentage polypeptide per pair of lipid chains. Figure 4 shows that the fraction of lipid chains in contact with one polypeptide rises to a relatively sharp maximum at a lipid : polypeptide ratio of ca. 2:1 and then rapidly decreases. The fraction of lipid chains making contact with 2 or 3 polypeptides begins to increase for ratios of ca. 8:1 and is still increasing at a 1:1 ratio which is the limit of our data.

As the proportion of polypeptide in the arrays increases, the fraction of lipid chains in contact with other lipid chains decreases smoothly. The number of lipid chains which are not contacted by a polypeptide is as small as 5% when an array contains 50% polypeptide.

Such a random arrangement of lipid and polypeptide and consideration of the multiple contacts of lipid with polypeptide may be useful in rationalising the growth of the ESR immobilised component as the content increases of polypeptide [13]. The multiple contacts of lipid with polypeptide are quite significant

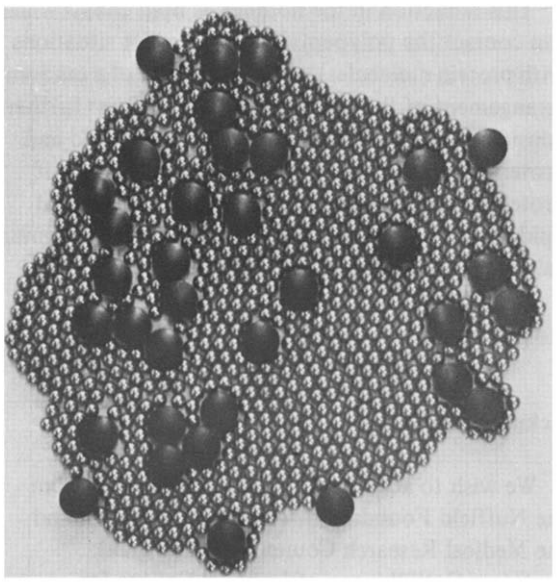

Fig.3a

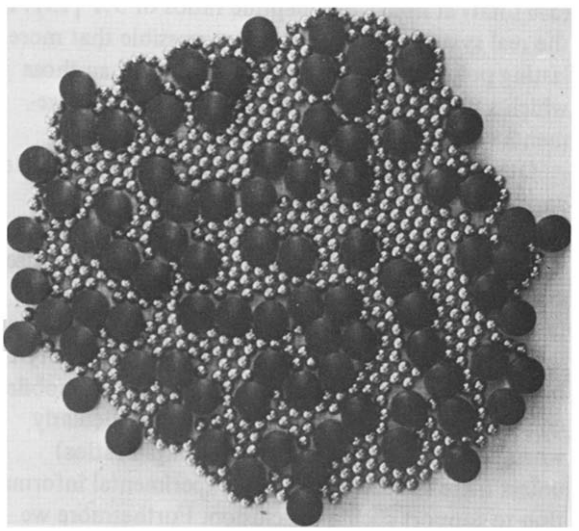

Fig.3b

Fig.3. Simulated lipid:polypeptide arrays with lipid:polypeptide ratios of (a) 10:1 and (b) 3:1.

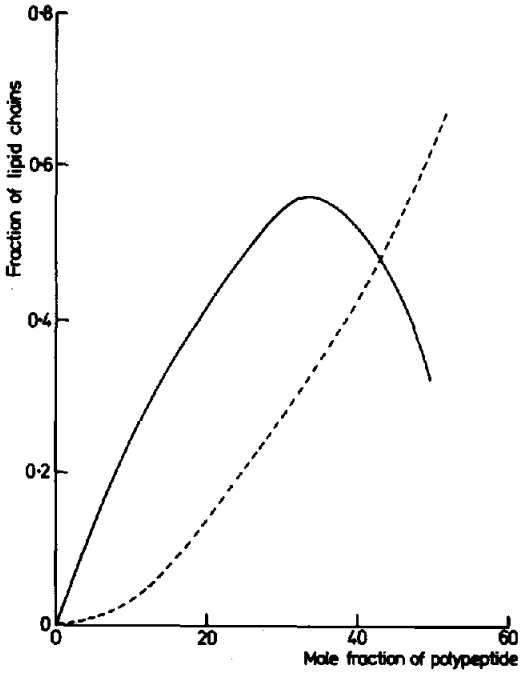

Fig.4. The fraction of lipid chains making contact with 1 (——) and 2 or 3 (– – –) polypeptides as a function of mol fraction of polypeptide. The data were determined from arrays of the type shown in fig.3.

(5% total) at lipid : polypeptide ratios of 5:1 [13]. In the real system of course it is also possible that more lasting polypeptide aggregates may form than those which spontaneously occur in the random arrangements which we have tried to simulate.

Other workers have observed similar ESR immobile components in protein–lipid complexes [9,10]. Thus with the reconstituted sarcoplasmic reticulum Ca^{2+}-ATPase systems the presence of such 'immobilised' components observed only at high protein : lipid ratios has been attributed to the presence of a special lipid annulus [10]. We consider that it is necessary to be cautious in the association of such ESR immobile components with special lipid effects (particularly when observed only at high protein:lipid ratios) unless there is other additional experimental information to support such a conclusion. Furthermore we suggest that contact data, for such protein–lipid systems, need examination similar to those we have shown in fig.4 (chosen to take into account the appropriate size of protein under examination). We can envisage that single lipid contacts with a protein can give rise to the modulated lipid detected by Raman spectroscopy and multiple contacts of a lipid with 2 or more proteins could give rise to further immobilisation of lipid. Such contact datum at least requires further examination and consideration. (We will discuss this elsewhere.)

An additional question relevant to these protein–lipid systems is: 'What is the number of contacts which are made between the lipid chains and an intrinsic polypeptide or protein?' A number of workers have attempted to rationalise their ESR and enzyme activity data by calculating the maximum number of lipid chains which are tightly packed around the protein circumference, i.e., the maximum coordination number and associating this number with some critical effect on enzyme activity and ESR data [9,10].

An interesting and important feature of the random arrays shown in fig.3 is that the number of lipid chains which on average contact the polypeptide is approx. 8. This can be contrasted with the *maximum* number of 11 lipid chains which could contact the circumference of the polypeptide. This decrease in coordination number is consistent with [26] in which packing two sizes of discs with relative diameters of 2:1 was shown to produce a reduction in the average coordination number from 6–4.7.

This reduction in the number of lipid chains which can contact the polypeptide (and in other situations with protein molecules) as a consequence of a random arrangement of the components also requires further consideration. If random arrangements of lipid and protein are considered to occur and particularly if protein–protein contacts take place, then the real lipid–protein contacts may be less than the maximum values presently chosen [9,10].

Acknowledgements

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