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Protein–Lipid Interactions: Recombinants of the Proteolipid Apoprotein of Myelin with Dimyristoyllecithin†

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ABSTRACT: Recombinants of the proteolipid apoprotein (PLA) of bovine myelin with dimyristoyllecithin (DML) have been prepared under conditions which maximize the opportunity for hydrophobic interactions. X-ray diffraction studies show that the overall lamellar structure of the lecithin is preserved when PLA is incorporated into the DML liposomes. Both above and below the temperature of the DML order–disorder transition, maximally hydrated DML/PLA recombinants have a larger bilayer repeat distance than maximally hydrated DML, attributable to increased intercalation of water between the bilayers. In a DML/PLA recombinant containing 6.3 wt % protein at 37 °C, a single phase is present in which the protein is homogeneously distributed throughout the bilayers. At 10 °C, two phases are present: a DML/PLA complex phase, and a small amount of free DML. Differential scanning calorimetry of DML/PLA recombinants shows the presence of a shoulder on the high temperature side of the DML order–disorder transition peak which increases in size with increasing protein content. This shoulder represents the order–disorder transition of a population of DML molecules whose acyl chain melting is influenced by its association with the protein. The amount of this "boundary" lipid was estimated from calorimetry measurements to be 4 mg of DML per mg of proteolipid apoprotein. Model calculations indicate that this quantity of "boundary" DML corresponds to three to four concentric layers of phospholipid around the PLA molecule. This long-range ordering of phospholipid acyl chains by an integral membrane protein suggests that the fluidity of the interior of natural membranes may be influenced not only by the length and degree of unsaturation of the phospholipid acyl chains and by the presence of cholesterol, but also by the characteristics of the integral proteins present.

Proteolipids, operationally defined as protein–lipid complexes soluble in chloroform–methanol mixtures, were first isolated from bovine white matter tissue by Folch and Lees (1951). The proteolipid apoprotein (PLA),[1] which is the delipidated form of the white matter proteolipid, was later shown to be the most abundant protein of the myelin sheath (Autilio et al., 1964; Gonzalez-Sastre, 1970). The amino acid composition of PLA (composed of greater than 50% apolar residues), its solubility in chloroform–methanol, and the presence of 2–3% covalently bound fatty acids in its structure (Stoffyn and Folch-Pi, 1971) suggest a role as an integral membrane protein in myelin (for a review, see Folch-Pi and Stoffyn, 1972). Recent freeze-fracture electron microscopy studies (Pinto da Silva and Miller, 1975) also suggest that this protein is deeply embedded in the myelin lipid apolar region. The myelin proteolipid apoprotein has been shown to be highly α-helical in 2:1 chloroform–methanol (Sherman and Folch-Pi, 1970), a solvent in which the protein is monomeric (Nguyen Le et al., 1976). PLA can be converted to a water-soluble form (Folch-Pi and Stoffyn, 1972; Lees et al., 1976) in which the protein is present as a large aggregate (Zand, 1968) of low helical content (Sherman and Folch-Pi, 1970). In addition, proteolipids have been found in a variety of nonneural tissues (Folch-Pi and Sakura, 1976) and subcellular preparations such as mitochondria (Cattel et al., 1971; Tzagoloff and Meagher, 1972) and sarcoplasmic reticulum (MacLennan et al., 1972). The widespread distribution of proteolipids suggests that this class of proteins may be a ubiquitous component of biological membranes, although their functions remain unclear.

Dimyristoyllecithin (DML) and other homogeneous chain-length saturated lecithins have been shown by a variety of techniques to undergo two reversible thermal transitions (Chapman et al., 1967; Hinz and Sturtevant, 1972). The DML thermal pretransition (ΔH = 0.67 cal/g), a broad transition with the peak maximum at around 12 °C, has been shown recently by Janiak et al. (1976) to correspond to a structural

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[1] Abbreviations used: PLA, proteolipid apoprotein; DML, dimyristoyllecithin; DSC, differential scanning calorimetry; DPL, dipalmitoyllecithin; Ans, 8-anilino-1-naphthalenesulfonate.

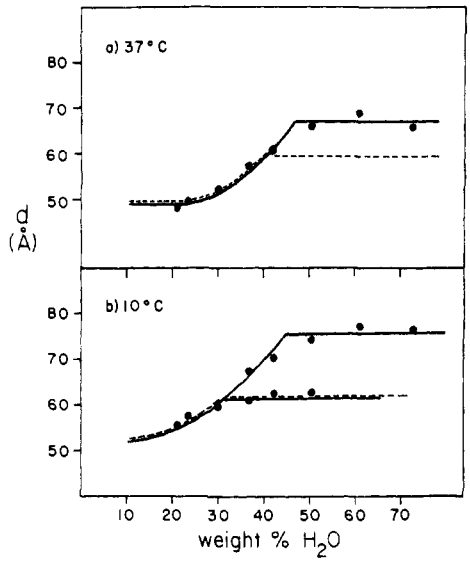

FIGURE 1: Bilayer repeat distance *d* (lipid layer plus water layer) for a DML/PLA recombinant at 6.3 wt % PLA, as a function of water content. Experimental points are derived from three to five orders of diffraction. Solid line: DML/PLA recombinant. Dashed line: data of Janiak, M. J., Shipley, G. G., and Small, D. M. (in preparation), for DML in water. (a) At 37 °C; (b) 10 °C.

change from a lamellar rigid-chain structure, commonly called the gel phase ($L_{\beta'}$ in the nomenclature of Luzzati and Tardieu, 1974), to another rigid-chain lamellar structure which possesses a ripple in the plane of the lamellae of period 120–160 Å ($P_{\beta'}$, as first described by Tardieu et al., 1973). The order-disorder transition (ΔH = 9.7 cal/g), a sharp transition with the peak maximum at 24 °C, is a change from the rippled lamellar rigid-chain structure to a lamellar liquid crystalline structure (L_α) in which the DML hydrocarbon chains are melted.

The solubility of PLA in lipophilic solvents such as chloroform–methanol allows solutions of PLA and phospholipids to be prepared in a manner which enhances apolar interaction between the two. The solvent can be removed and the mixtures hydrated to form liposomes (Bangham and Horne, 1964) so that the effects of PLA on phospholipid bilayers may be examined. In this paper we report x-ray diffraction and calorimetric observations on the effect of PLA on the structure and thermal transitions of dimyristoyllecithin. Our data indicate the presence of a boundary layer of lipid around the protein and allow a quantitative assessment of the size of this boundary layer.

Materials and Methods

Myelin Proteolipid Apoprotein. Proteolipids were extracted from freshly dissected bovine white matter and delipidated by extensive dialysis against chloroform–methanol mixtures according to the procedure of Folch-pi and Stoffyn (1972). Chemical analysis of PLA showed it to contain 0.08% phosphorus and 3.1% fatty acid.

Dimyristoyllecithin. Glycerylphosphorylcholine was prepared from egg yolk lecithin according to Chadha (1970) and was reacted with myristic anhydride to form dimyristoyllecithin by the method of Cubero Robles and Van Den Berg (1969). DML was purified by chromatography on silicic acid and shown to be greater than 99% pure by thin-layer chromatography. Myristic acid was purchased from Nu-Chek Prep (Elysian, Minn.).

DML/PLA Recombinants. For differential scanning calorimetry (DSC), stock solutions of PLA and DML in chloroform–methanol (2:1, v/v) were mixed in appropriate proportions, added to Perkin-Elmer DSC pans (50-μL capacity), dried down under N_2, and desiccated under vacuum overnight. An equal weight of doubly distilled H_2O was added to each pan, and the pans were sealed The sealed samples were equilibrated in an oven at 45 °C for 6 h.

Similarly, samples for x-ray diffraction were prepared in acid-washed glass tubes. X-ray samples were equilibrated by repeated centrifugation through the constricted tubes for 6 h at 45 °C. The tubes were opened at room temperature and aliquots taken for x-ray diffraction and determination of water content by gravimetric analysis.

Differential Scanning Calorimetry (DSC). Calorimetric data were obtained on a Perkin-Elmer DSC-2 differential scanning calorimeter, at a scanning rate of 1.25 °C/min, at sensitivities in the range 0.5–2.0 mcal/s. Baselines were drawn using the protocol described in recent studies of protein denaturation (Privalov and Khechinashvili, 1974; Jackson and Brandts, 1970). Peak areas were determined by planimetry and enthalpies calculated on the basis of the known amount of DML in each pan. Samples were also run at 5 °C/min for quantitation of the pretransition enthalpy and for attempted observation of protein denaturation.

X-Ray Diffraction. Nickel-filtered Cu Kα radiation from an Elliot GX6 rotating anode generator was focussed by cameras using either toroidal mirror (Elliot, 1965) or double mirror (Franks, 1958) optics. Samples were held in sealed thin-walled glass tubes in a sample holder kept at constant temperature by a circulating solvent heating/cooling system.

Results

X-Ray Diffraction. X-ray diffraction patterns of recombinants of DML with the proteolipid apoprotein were recorded at 10 and 37 °C. At 37 °C, DML/PLA recombinants containing 6.3 wt % PLA (DML:PLA = 15:1, w/w) exhibit three to seven low-angle diffraction lines (depending on water content) in the ratio 1.0:0.5:0.33 . . . , indexing on a one-dimensional lamellar lattice. A plot of the bilayer repeat distance *d* (lipid layer plus water layer) vs. water content for this recombinant is shown in Figure 1a. Below 41 wt % H_2O, the values for *d* are identical with those observed for DML alone at the same water contents (Janiak, M. J., Shipley, G. G., and Small, D. M., in preparation). At higher water contents, the recombinant continues to hydrate until it reaches a maximum bilayer repeat of 67 Å, which is 7.5 Å greater than that for maximally hydrated DML. At all water contents studied, a single diffuse wide-angle line centered at 4.5 Å is observed, characteristic of the melted chain conformation of the phospholipid.

When the DML/PLA recombinants are cooled to 10 °C, a temperature below both the DML order-disorder transition and pretransition, the swelling behavior illustrated in Figure 1b is observed. At water contents greater than 30 wt %, diffraction from two phases is observed: (1) lamellar diffraction from a DML/PLA complex phase which reaches a bilayer repeat distance of 75.5 Å at 45 wt % H_2O, and (2) a single diffraction line (very weak) which corresponds to the first order of diffraction from a "free" DML phase which has the same *d* as maximally hydrated DML at the same temperature. (At lower protein contents, higher orders of lamellar diffraction are seen for this excess "free" DML phase.) The maximum bilayer repeat distance of 75.5 Å for the DML/PLA complex

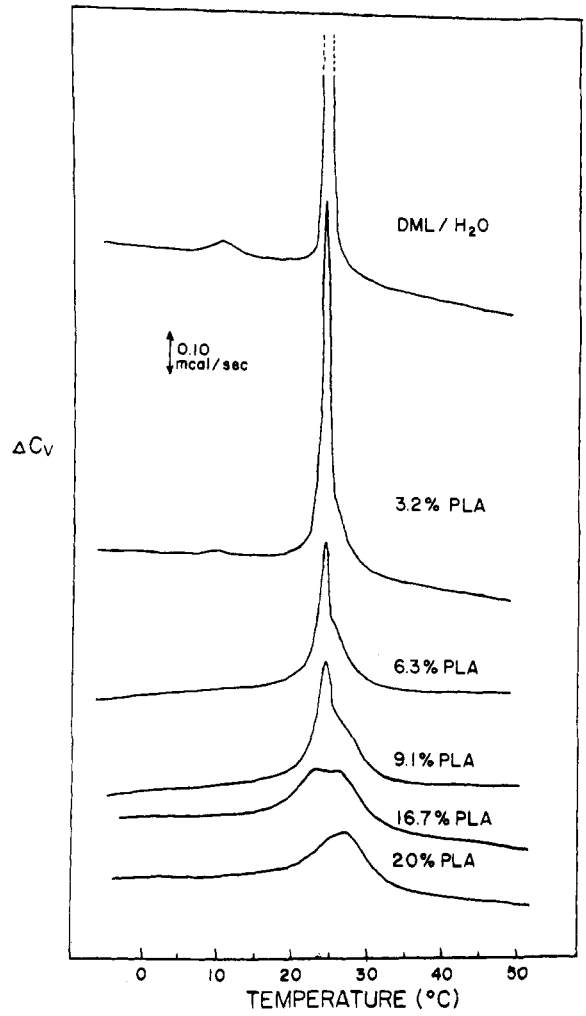

FIGURE 2: DSC thermograms of DML and DML/PLA recombinants. All traces shown are heating runs obtained at 1.25 °C/min. Each sample contained 7–9 mg of DML and varying amounts of protein.

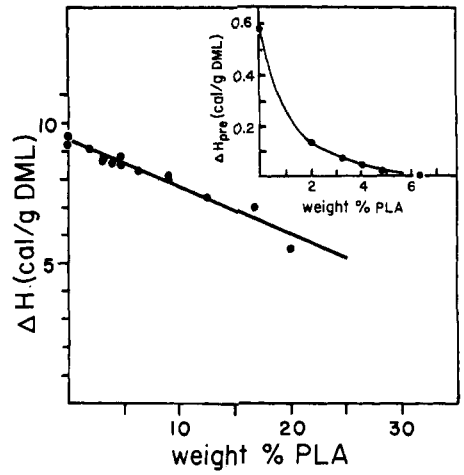

FIGURE 3: Enthalpy of the order-disorder transition of DML/PLA recombinants as a function of protein content. Insert: Enthalpy of the pretransition of DML/PLA recombinants as a function of protein content.

phase is 14 Å greater than that for maximally hydrated DML alone. At water contents less than 30 wt %, only one set of lamellar reflections is observed (4–5 orders of diffraction), giving *d* spacings identical with those for DML alone at the same water contents. Since two phases are observed above 30 wt % H_2O, it is probable that, below this water content, two phases are present whose bilayer repeat spacings are sufficiently similar to preclude resolution due to the limitations of the geometry of our x-ray camera (estimated resolvable difference ~0.5–1.0 Å). In all cases, at 10 °C, a single sharp wide angle reflection centered at 4.1 Å is observed, indicating that the DML acyl chains are ordered in the DML/PLA complex phase, as they are for hydrated DML at this temperature. No wide angle reflection at 4.5 Å, characteristic of melted hydrocarbon chains, was observed in these recombinants at 10 °C.

The intensity of the diffraction from the free DML phase at 10 °C is very weak, indicating that, at 6.3 wt % PLA, the protein is interacting with the large majority of the phospholipid in forming the DML/PLA complex phase, leaving only a small amount of free DML. In recombinant mixtures containing less PLA, the ratio of the intensities of the diffraction lines from the free DML and the DML/PLA complex phase (I free/I complex) becomes larger with decreasing protein content. At a higher protein content (9 wt % PLA), no diffraction characteristic of free DML is observed. Recombinants at 6.3 wt % PLA prepared in 20 mM or 1 M KCl exhibited the same bilayer repeat spacings as were observed in the absence of salt.

Differential Scanning Calorimetry. DSC thermograms (Figure 2) of DML and DML/PLA recombinants (in excess water) were obtained at a heating rate of 1.25 °C/min. There are a number of obvious changes in the calorimetric behavior of DML in the presence of the proteolipid apoprotein. The thermal pretransition decreases in enthalpy upon addition of small amounts of PLA and disappears by 6.3 wt % PLA. The order-disorder, or chain-melting, transition (peak maximum 24 °C) broadens and decreases in height with increasing amounts of protein, with the concurrent appearance of a shoulder on the high-temperature side of the peak. The peak temperature of the major order-disorder peak of the recombinants is identical with that observed for pure DML and remains unchanged with increasing protein content. At 20 wt % PLA, the major order-disorder peak is not observed, and only the broad peak corresponding to the shoulder remains, with the peak maximum at 26 °C.

The total enthalpy of the order-disorder transition (including the shoulder) decreases linearly with increasing protein content (Figure 3). The enthalpy of the thermal pretransition (ΔH_{pre}) of DML decreases nonlinearly with increasing protein content (Figure 3, insert). By 2 wt % PLA, ΔH_{pre} has fallen to 20% of the value for pure DML and is zero at 6.3 wt % PLA.

After studying the behavior in the range of the DML thermal transitions, each sample was heated to 120 °C at 5 °C/min. In no case was an enthalpy or discontinuous heat capacity change observed which might correspond to protein denaturation. After cooling, a subsequent run through the DML thermal transition range showed a decrease in the size of the shoulder, an increase in the size of the main peak, and an increase in the size of the pretransition if a pretransition was originally present (% PLA < 6.3 wt %). The 6.3 wt % PLA recombinant exhibited a pretransition after heating to 120 °C, even though no pretransition was previously observed. Recombinants containing 9.1–20 wt % PLA did not exhibit a pretransition before or after heating to 120 °C.

Discussion

Our x-ray diffraction results indicate that, at the DML/PLA ratios studied, incorporation of the myelin proteolipid apoprotein into DML liposomes does not change the overall

lamellar structure. At water contents below the maximum hydration of DML (41 wt % H_2O at 37 °C, 30 wt % H_2O at 10 °C), the bilayer repeat distance d for a recombinant containing 6.3 wt % PLA does not differ significantly from that observed for pure DML. In addition, the intensity distribution of the diffraction from the recombinants does not differ significantly from that observed for pure DML at the same water contents, suggesting that the bilayer electron density profile would not be significantly different at these protein contents. While these observations do not directly locate PLA in any portion of the DML bilayer, they are consistent with a position deeply embedded in the bilayer, with a minimum amount of protein located in the polar interbilayer region. Such a deeply embedded location for PLA in myelin has been suggested by the observation of negligible binding of Ans to the protein in myelin (Feinstein and Felsenfeld, 1975a), by the observation of particles (presumed to arise at least partly from PLA) in freeze fracture electron micrographs of myelin (Pinto da Silva and Miller, 1975), and by the apolar nature of the protein itself and its avidity for lipids (Folch-Pi and Stoffyn, 1972). In recombinants of the protein with phospholipids, a deeply embedded position was indicated by, again, reduced Ans binding (Feinstein and Felsenfeld, 1975b) and the presence of particles in freeze-fracture electron micrographs[2] (Papahadjopoulos et al., 1975).

At 37 °C, as the hydration of the 6.3 wt % PLA recombinant is increased past the point of maximum hydration for pure DML, the recombinant continues to hydrate until a maximum bilayer repeat of 67 Å is reached at 47 wt % H_2O. The increased bilayer repeat distance at maximum hydration (7.5 Å greater than that observed for maximally hydrated DML) is primarily due to the increased intercalation of water. This increased hydration suggests that the proteolipid apoprotein, while deeply embedded, plays a role at the polar surface of the bilayers and may extend into the interbilayer space. The increased intercalation of water is not simply due to protein-contributed charge on the bilayer surface since recombinants hydrated in the presence of salt behaved similarly to those prepared in the absence of salt. The forces which result in limited swelling of pure lecithins in water are poorly understood, and thus a quantitative analysis of the effect of PLA on the bilayer repeat distance is difficult.

When the recombinants are cooled to temperatures below the order-disorder transition (10–21 °C), the protein and DML associate in a lamellar DML/PLA complex phase (showing no evidence of a rippled $P_{\beta'}$ structure), separate from another phase which has the same bilayer repeat as hydrated DML. A similar observation has recently been reported by Kleemann and McConnell (1976) who, using freeze-fracture electron microscopy, studied recombinants of ATPase from sarcoplasmic reticulum with DML. When ATPase/DML recombinants were quenched from a temperature above the DML order-disorder transition, the protein was seen to be evenly distributed in the fracture plane. When quenched from below the transition, the protein was aggregated into patches, leaving other smooth patches presumably composed only of DML.

The calorimetric curves obtained as a function of PLA content are interpreted as follows. The pretransition enthalpy decreases nonlinearly with increasing protein content (Figure 3, insert), suggesting that this decrease is not due to direct stoichiometric binding of lipid to protein. The presence of a thermal pretransition at low protein contents (<6.3 wt % PLA) indicates that a population of DML is present whose thermal behavior is completely unperturbed by the presence of the protein. This population, which we refer to as "free" DML, decreases rapidly in size with increasing protein content, and comprises about 20% of the DML present at 2 wt % PLA (using the pretransition enthalpy behavior shown in Figure 3 (insert) to estimate the amount of "free" DML present). By this criterion, there is no "free" DML present at 6.3 wt % PLA, in contradiction to the x-ray diffraction results which show that, at 10 °C and 6.3 wt % PLA, there is, in fact, a very small amount of "free" DML present (Figure 1b). This difference is due to the greater sensitivity of the x-ray diffraction method, which shows no "free" DML at 10 °C in a recombinant mixture containing 9 wt % PLA.

The appearance of a shoulder on the high-temperature side of the order-disorder transition indicates the presence of a population of DML whose acyl chain melting is influenced by its association with the protein.[3] We refer to this population as "boundary" DML. To assess whether this effect of the proteolipid apoprotein on the DML order-disorder transition is due to hydrophobic interactions between the protein and the DML acyl chains, or to ionic interactions with the DML head groups, the effect of PLA on the crystal-isotropic melting transition of n-decane (at −30 °C) was studied (Curatolo, W., unpublished observations). Although the protein was insoluble in n-decane, it swelled macroscopically in the hydrocarbon, becoming gelatinous in form. Differential scanning calorimetry showed the appearance of a shoulder on the high temperature side of the n-decane melting transition in the presence of PLA, indicating that the protein is capable of forming hydrophobic associations with a simple hydrocarbon. We conclude that the effect of PLA on the DML order-disorder transition is probably largely due to hydrophobic interactions between the protein and the DML acyl chains.

The linear decrease in the total enthalpy of the order-disorder transition (including the shoulder) with increasing protein content may be the result of either or both of two possible phenomena. If PLA interacts with a first boundary layer of lipid so strongly that the lipid undergoes no thermal transitions, then increasing the protein content would decrease the ΔH in a linear fashion. In this case, the amount of lipid in the boundary layer can be obtained from the x intercept of the straight line in the ΔH vs. wt % PLA plot (Figure 3). This extrapolated value is 55 wt % PLA, or 0.8 mg of DML per mg of protein. However, if this were the case, we would expect the peak temperature of the transition to remain constant, with the sharp transition peak decreasing in area with increasing protein content, and broadening as the cooperative unit of the free (melting) DML becomes disrupted at high protein contents. The situation is more complicated since, starting at low protein contents, a broad transition (shoulder) appears whose peak temperature is approximately 2 °C higher than that of the free DML present. If we assume that the shoulder represents the transition of "boundary" DML, the decrease in total

[2] This study was performed with the N-2 protein of human myelin (Gagnon et al., 1971), which is chemically similar to the proteolipid apoprotein of bovine myelin.

[3] Papahadjopoulos et al. (1975), studying recombinants of the N-2 protein of human myelin with dipalmitoyllecithin (DPL), observed a broadening and decrease in enthalpy of the DPl order-disorder transition peak with increasing protein content, but no distinct shoulder was observed. The absence of an observed shoulder in this system may be due to heating rate effects. The calorimetric curves for the DPL/N-2 recombinants were obtained at a heating rate of 5 °C/min. Our DML/PLA system exhibits no shoulder when heated at 5 °C/min, but shows a distinct shoulder when heated at the slower rate of 1.25 °C/min.

TABLE I: Calculated Number of DML Molecules in Concentric Layers Around PLA.[a]

Cylinder height, h (Å)	Cylinder surface area (Å^2)	Axial ratio	Surface perimeter (Å)	No. of DML molecules			
				1st layer	2nd layer	3rd layer	4th layer
35	857	1	104	30	42	55	68
35	857	2	113	32	44	57	69
35	857	3	127	35	47	60	72
67	448	1	75	23	36	48	61
67	448	2	82	25	37	50	62
67	448	3	91	27	39	52	64

[a] PLA is modeled as an elliptical cylinder of height h and axial ratio a/b. The height h is perpendicular to the plane of the bilayer. Surface area is area of protein on bilayer surface and is equal to the molecular volume divided by h. Surface perimeter is the circumference of the protein elliptical cylinder.

enthalpy with increasing protein content must indicate a smaller ΔH for the order-disorder transition of the boundary population than for the free DML. As more protein is added, the population of DML giving rise to the shoulder increases at the expense of free DML, resulting in decreased total enthalpy. Assuming equilibrium as the sample is heated through the transition at 1.25 °C/min, the Gibbs equation, $\Delta G = \Delta H - T\Delta S$, reduces to $\Delta S = \Delta H/T$. Since the transition temperature for the boundary lipid is higher than that for free DML, and the enthalpy is lower, then the entropy difference between the initial and final states must also be smaller:

$$(S_{\text{liq cryst}} - S_{\text{gel}})_{\text{DML}} > (S_{\text{liq cryst}} - S_{\text{gel}})_{\text{"boundary" DML}}$$

This indicates that, for the boundary lipid in the recombinant, the acyl chains must be more disordered in the gel state or more ordered in the liquid crystalline state, or both. Raman spectroscopic data (W. Curatolo, unpublished observations) indicate that, in the presence of PLA, the acyl chains of DML are stabilized (have a larger trans/gauche ratio than pure DML at the same temperature) above the order-disorder transition and are disordered, to a small degree, below the transition.

The amount of phospholipid in the boundary layer can be estimated by observing the protein content at which the calorimetric peak for the order-disorder transition has no sharp component centered at 24 °C and consists only of the shoulder component. Figure 2 shows that this occurs at approximately 20 wt % PLA, which corresponds to a ratio of 4 mg of DML bound per mg of proteolipid apoprotein or a molar ratio of 142:1 DML/PLA, given a monomer molecular weight of 24 000 for PLA (Folch-Pi and Sakura, 1974). From an assumed partial specific volume of $\bar{v} = 0.74$ cm^3/g for PLA and the molecular weight, we have calculated an approximate molecular volume of 30 000 Å^3. If we model the protein as an elliptical cylinder which is completely embedded in the DML bilayer (as suggested by the freeze-fracture results of Papahadjopoulos et al., 1975), the height h of the protein cylinder (perpendicular to the bilayer plane) can be varied and the area of the protein at the bilayer surface (the area of the top of the protein cylinder) calculated. Assuming various axial ratios ($a/b = 1, 2, 3$) for the protein elliptical cylinder, the protein perimeter in the bilayer plane can be calculated for a range of protein shapes. Utilizing this calculated perimeter and the known dimensions of the DML molecule,[4] an approximation of the number of DML molecules which can be fit into layers around the protein molecule can be calculated.

Table I contains the results of such calculations for two general cases (at 37 °C): (1) the protein spans the hydrocarbon portion of the bilayer without extending into the water space ($h = 35$ Å),[4] and (2) the protein spans the hydrocarbon region and also extends halfway into the interbilayer water space on each side of the bilayer ($h = 67$ Å). The number of DML molecules in concentric layers around the protein is given for both cases at three PLA axial ratios. The number of DML molecules which can be fit in the 1st, 2nd, 3rd, and 4th layers is markedly insensitive to the variations in protein shape. The calorimetrically observed boundary population of DML in the DML/PLA recombinants was 142 molecules of DML per molecule of PLA. Table I shows that this number of DML molecules can be fit in three to four layers around the protein. Similar calculations using the structural parameters of DML at 10 °C[4] give the same result.

Jost et al. (1973), using spin label-electron spin resonance spectroscopy, observed the presence of 0.2 mg of strongly immobilized lipid per mg of protein in the system cytochrome oxidase/mitochondrial lipids. This corresponded to the amount of lipid which could be fit in one layer around the cytochrome oxidase complex (47 phospholipids per protein complex), given an approximation of the dimensions of the protein derived from electron micrographs. In the case of our DML/PLA recombinants, it seems that the protein is able to influence larger domains of DML, extending out several layers from the protein.

At protein contents greater than 6.3 wt % PLA, no "free" DML is present as judged by the absence of a pretransition, but a large fairly sharp component of the order-disorder peak is still present, in addition to the shoulder. This fairly sharp chain-melting peak derives from DML which is "perturbed", that is, which undergoes no pretransition but remains unbound. The transition peak for this "perturbed" population becomes broader as more protein is added because the cooperative nature of the transition is disturbed by the presence of the protein with its boundary lipid.

As a summary, a graphic representation of the populations of DML present in the DML/PLA recombinants is given in Figure 4. In recombinants containing less than 6.3 wt % PLA at 10 °C (Figure 4a), two phases are present (as observed by x-ray diffraction): a DML/PLA complex phase, and a "free" DML phase. The DML/PLA complex phase consists of protein with its "boundary" lipid layers, dispersed in "perturbed" DML which exhibits no pretransition and undergoes a broadened order-disorder transition. At protein contents greater than 6.3 wt % at 10 °C (Figure 4b), no "free" DML

[4] The surface area per molecule of DML (on the bilayer surface) is 60.8 Å^2/molecule at 37 °C, giving a surface radius of 4.4 Å. The lipid thickness at 37 °C is 35 Å. At 10 °C, the surface area per molecule is 50.2 Å^2/molecule, corresponding to a surface radius of 4.0 Å (Janiak, M. J., Shipley, G. G., and Small, D. M., in preparation).

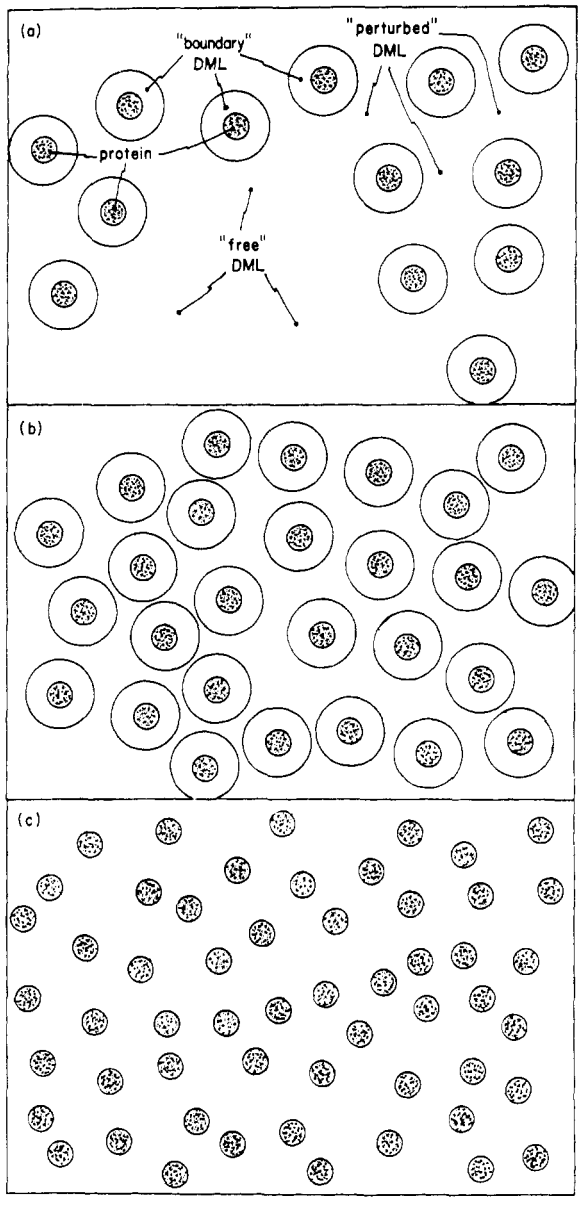

FIGURE 4: Graphical representation (looking down on the surface of the bilayer) of the various populations of DML present at 10 °C in DML/PLA recombinants. (a) Protein content less than 6.3 wt. %; (b) protein content between 6.3 and 20 wt % (no "free" DML is present); (c) protein content greater than 20 wt % (all DML is "boundary").

is present, and at greater than 20 wt % (Figure 4c) no "perturbed" DML is present. At 37 °C (not shown), only one phase is present, consisting of PLA with its "boundary" lipid distributed homogeneously throughout the liquid-crystalline (melted-chain) DML. There is no distinction between "free" and "perturbed" DML at this temperature since both these populations are in the melted-chain state.

The DML/PLA recombinants show no direct evidence of protein denaturation upon heating to 120 °C in the calorimeter. Circular dichroic spectra of the proteolipid apoprotein in sonicated recombinants with egg yolk lecithin show the presence of a significant amount of α-helix at room temperature, which cannot be denatured to a random structure by heating at 90 °C for 0.5 h (W. Curatolo, unpublished observation). The protein conformation, then, appears to be stabilized by the presence of phospholipid. Indirect evidence, however, suggests that, at high temperatures, "boundary" DML is released which subsequently exhibits thermal behavior similar to "free" or "perturbed" DML, depending on the original DML/PLA ratio. This effect is partially reversible since reequilibration at 45 °C for 6 h results in thermal behavior which is intermediate between that observed before and after taking the sample to 120 °C. We attribute these observations to partially reversible protein aggregation at high temperatures, keeping in mind that noncooperative unfolding of the protein may also be involved.

Whether our observations on DML/PLA recombinants can be extrapolated to interactions in the myelin sheath is unclear. The phospholipid composition of myelin is complex, containing only 20–25% phosphatidylcholine (Evans and Finean, 1965), and so interactions between PLA and other lipids such as phosphatidylethanolamine, phosphatidylserine, sphingomyelin, the glycosphingolipids, and particularly cholesterol (which also has a perturbing influence on phospholipid organization) are undoubtedly of importance. What is clear, however, is that the fluidity of the interior of natural membranes, thought to be a controlling factor in membrane protein-mediated phenomena, may in fact be strongly influenced by the membrane proteins themselves.

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Dynamic Structure of Lipid Bilayers Studied by Nanosecond Fluorescence Techniques†

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ABSTRACT: Molecular motions in liposomes of dipalmitoylphosphatidylcholine (DPPC) were studied by nanosecond fluorescence techniques. As a fluorescent probe for the hydrocarbon region, 1,6-diphenyl-1,3,5-hexatriene (DPH) was used. Time courses of fluorescence intensity $I_T(t)$ and emission anisotropy $r(t)$ of DPH embedded in DPPC liposomes were measured at various temperatures. The value of the fluorescence lifetime τ obtained from a single exponential decay of $I_T(t)$ was somewhat higher than that in liquid paraffin below the transition temperature T_t and decreased above T_t. Higher values of τ below T_t indicate the almost complete hydrophobic environment. The decay curves of $r(t)$ were separated into two phases: an initial fast decreasing phase of the order of one nanosecond and a second almost constant phase. This indicates that the orientational motion of DPH in the hydrocarbon region is described by a wobbling diffusion restricted by a certain anisotropic potential. The results were analyzed on the model that the wobbling diffusion is confined in a cone with a uniform diffusion constant. Though temperature dependence of the cone angle was sigmoidal, that of the wobbling diffusion constant was like the exponential function. The change in the cone angle at T_t was sharper than that in the wobbling diffusion constant at T_t. Estimated values of the viscosity in the cone were an order of magnitude smaller than the values of "microviscosity" which were estimated from the steady-state emission anisotropy without considering the restrictions on the rotational motion.

Structure and dynamics of lipids in biological membranes have been recognized as the essential factors in their functions and organization (Inesi et al., 1973; Racker and Hinkle, 1974). Phospholipids in model membranes such as aqueous dispersions or liposomes are known to be in a bilayer structure and the cooperative melting of their hydrocarbon chains, the crystalline-liquid-crystalline phase transition, takes place at a certain temperature (Träuble and Eibl, 1974; Sackmann et al., 1973; Chapman, 1975; Jacobson and Papahadjopoulos, 1975). Studies of dynamic properties or molecular motion of lipids in model bilayers, however, have not resolved several important questions.

Although the use of spin-label techniques for the dynamic studies of lipid bilayers is widespread, the information regarding molecular motion is restricted in most investigations. In describing the characteristics of molecular motion of membranes, Hubbell and McConnell (1971) have used the "order parameter", the orientational degree of hydrocarbon chain, which can be obtained from the electron spin resonance spectra assuming rapid anisotropic rotation of the probe. Recently, Israelachvili et al. (1975) proposed a method to analyze the line shapes of ESR[1] spectra assuming both tumbling within the confines of a cone and restricted rotations about the long axis of a probe. Rotational correlation times of tumbling and of restricted rotations of spin-labeled molecules embedded in lecithin bilayers were estimated by the method.

Fluorescence depolarization techniques are also useful to investigate dynamics of lipid bilayers (Badley et al., 1973). From the emission anisotropy measured under constant illumination, Shinitzky et al. (1971) estimated the "microviscosity" of membrane. The treatment to estimate "microviscosity", however, is not appropriate when the rotational diffusion of a fluorescent probe is restricted within a certain region such as a cone.

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, DL-α-dipalmitoylphosphatidylcholine; ESR, electron spin resonance.