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The macroscopic organization of reconstituted M13 coat protein-phospholipid systems. An EPR spectroscopy and polarizing microscope study

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The coat protein of the bacteriophage M13 in the α-helical state is reconstituted in macroscopically oriented systems of dioleoylphosphatidylcholine that are prepared by squeezing the reconstituted material between glass plates. The coat protein dramatically influences the macroscopic orientation of the multibilayers, as is investigated by polarizing microscopy and EPR spectroscopy of the cholestane spin label embedded in the bilayers. It is found that with increasing amounts of protein the spontaneous macroscopic orientation of the reconstituted system decreases. This effect is proposed to be due to an increase of the apparent viscosity of the lipid-protein systems with increasing amounts of protein. This is assumed to arise from a sticky effect of the C- and N-terminal protein parts that extend into the aqueous phase between the bilayers.

Introduction

Macroscopically oriented lipid multibilayer systems have been of great value in the study of lipid systems and biological membranes by spin probe EPR [1–19]. Usually these systems have been prepared by squeezing the lipid material between glass plates. By analyzing the angular-dependent properties of these systems by various spectroscopic techniques, detailed information about the molecular dynamics and structure of lipids could be obtained. The possibility of macroscopic orientation would be interesting as well in the study of lipid-protein interaction, but in the literature only a few papers have been presented on this subject. EPR spin-label experiments have been published on macroscopically oriented lipid-protein model membranes containing Folch-Lees proteolipids [20] and the peptide gramicidin A [21–22], and solid-state NMR spectroscopy has been applied for the peptide M2δ, a helical segment of the nicotinic acetylcholine acceptor [23]. Clearly macroscopic orientation of lipid-protein systems is difficult to carry out, and in this respect it has been noted by Tanaka and Freed [21] that the preparation of well-aligned multi-layers containing gramicidin A is a non-trivial, yet crucial task.

In this paper we have used the coat protein of the bacteriophage M13 reconstituted in macroscopically oriented phospholipid systems. M13 coat protein is a transmembrane model protein that has been well-studied recently using various biochemical and biophysical methods (for a review, see Ref. 24). In the course of our work, we found that M13 coat protein dramatically influenced the macroscopic orientation of the multibilayers, making it necessary to study this effect in more detail. Therefore, the macroscopic orientation was systematically studied of bilayers constituted of dioleoylphosphatidylcholine (DOPC) and containing different amounts of α-helical M13 coat protein between glass plates by measuring the EPR spectra of the cholestane spin label and by using polarizing microscopy. It is found that with increasing protein concentration the spontaneous macroscopic orientation decreases. In this paper we will analyse this effect and offer an explanation for the results obtained.

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; L/P, lipid to protein molar ratio; NMR, nuclear magnetic resonance.

Materials and Methods

Cholestane spin label (3β-doxyl-5α-cholestane) is obtained from Aldrich (Milwaukee, WI, USA). DOPC is obtained from Avanti (Alabaster, MI, USA) or Sigma (St. Louis, MO, USA) and used without further purification.

Bacteriophage M13 and its coat protein are obtained as described by Spruijt et al. [25]. A stock solution of the phospholipids and cholestane spin label in chloroform is prepared with a lipid/spin label molar ratio of 100:1. The chloroform is removed with nitrogen gas and lyophilized for at least 4 h to remove remaining chloroform. This lipid mixture is solubilized in buffer (50 mM cholate, 10 mM Tris-HCl, 0.1 mM Na_2EDTA, 150 mM NaCl (pH 8.0)) by sonicating for 3–6 min to obtain a clear solution. At this stage M13 coat protein solubilized in the same buffer is added up to the desired lipid to protein (L/P) ratio. This is followed by dialysis at room temperature against a 100 fold excess buffer (10 mM Tris-HCl, 0.1 mM Na_2EDTA, 150 mM NaCl (pH 8.0)) for a total of 48 h changing the buffer every 12 h.

After the dialysis procedure the reconstituted lipid-protein complexes are concentrated with a centrifuge at 40000 rpm at 4°C for 6 h. Various portions of the pellet are then spread over the central area (far from the edges) of glass plates (size 4×8 mm). These glass

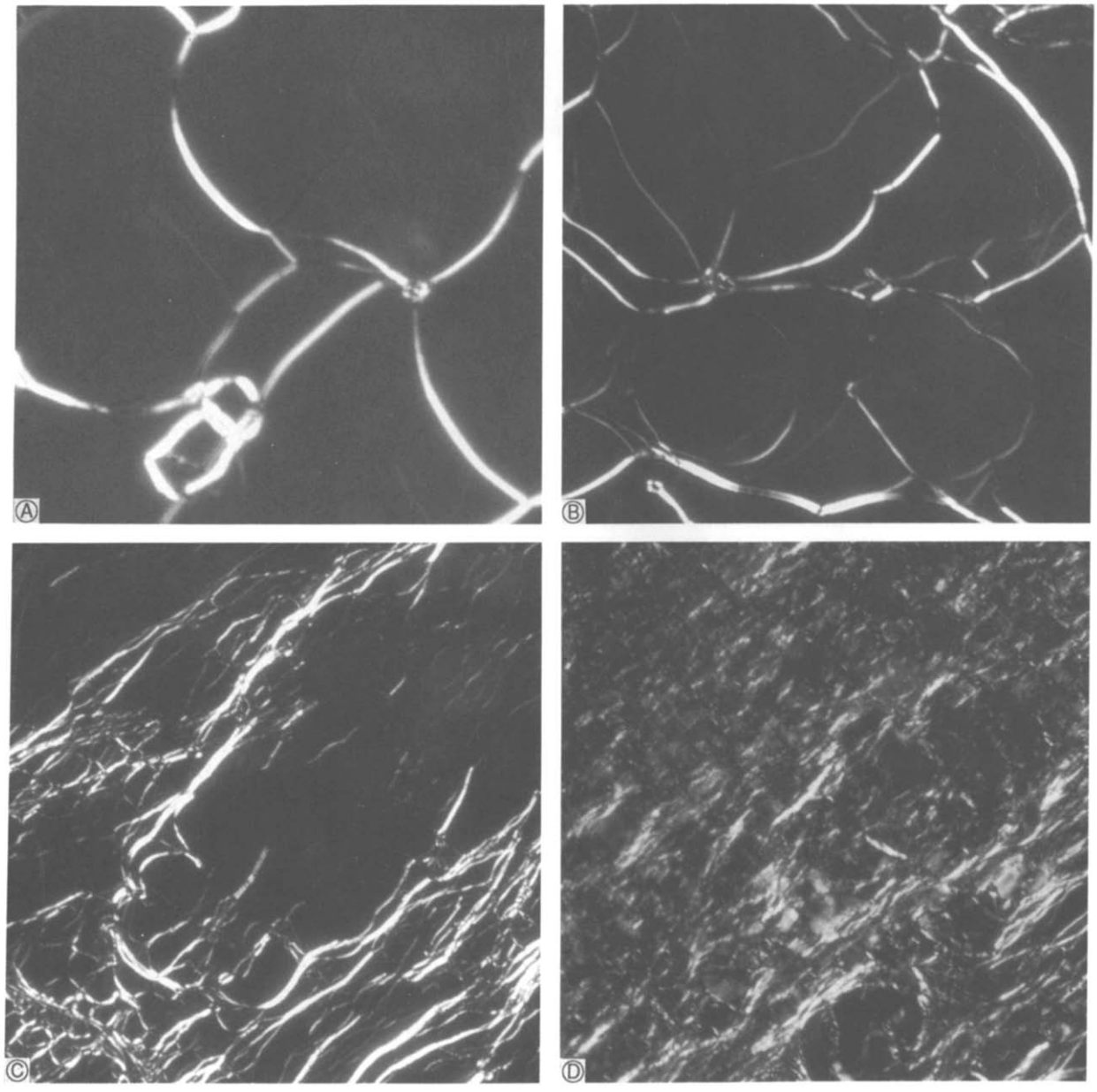

Fig. 1. Photographs through a polarizing microscope with crossed polarizers of macroscopically oriented DOPC with various amounts of M13 coat protein. (A), L/P ∞; (B), L/P 72; (C), L/P 27 and (D), L/P 12. The enlargement factor is 100.

plates are left at 40°C in a 80% hydrated atmosphere above a saturated solution of K_2SO_4 for at least 10 h and then covered with a second glass plate. Pressure is applied from the top of the glass plates to orient the lipid-protein bilayers parallel to the glass surface, as checked by polarizing microscopy. Polarizing microscopy is carried out using a standard polarizing microscope with crossed polarizers, equipped with a first order red plate and a camera. The procedure for preparation of all the oriented samples is the same both in the presence and in the absence of M13 coat protein to allow comparison of the results among different samples. The oriented glass plates are returned to the hydrated atmosphere for 1–4 days. No additional mechanical pressure is applied to the glass plates during equilibration. After the experiments, the aggregation state, L/P ratio and the incorporation of the coat protein are determined as described by Spruijt et al. [25]. The relative contribution of α-oligomers and β-polymers in the sample is obtained from an integration of the high-performance size-exclusion chromatography elution patterns. The water content of the samples is determined gravimetrically.

The EPR measurements are carried out at room temperature (23°C) with a Bruker ESP 300E EPR spectrometer operating in the X-band (around 9 GHz). About six oriented samples between glass plates are placed in a Teflon holder connected to a goniometer and inserted in a quartz tube in the microwave cavity. The oriented samples are rotated at different angles θ of the normal to the glass plates with respect to the direction of the magnetic field. The $\theta = 0°$ angle is determined on the basis of the lowest hyperfine splitting between the EPR lines. EPR settings are: 5 mW microwave power, 0.25 mT modulation amplitude, modulation frequency 100 kHz, 10 ms time constant, 20 s scan time, 10 mT scan width and 332 mT centre field. Up to 10–12 spectra are accumulated to improve the signal to noise ratio. Difference spectra are taken directly on the Bruker ESP 300E EPR spectrometer, using the Bruker software, by subtracting the EPR spectrum of the pellet of a sample prior to orientation (unoriented material) and the spectrum of the same system oriented between glass plates. The end point of the spectral titration was determined by eye, taking care of generating a flat baseline and minimal distortion of the resulting difference spectrum.

Results

Polarizing microscopy

Photographs were taken of the samples oriented between glass plates through a polarizing microscope to study their morphology (see Fig. 1). In Fig. 1, it can be seen that in pure DOPC multibilayers without M13 coat protein, large dark spots are present of material that has an uniaxial orientation (the optical axis of the material is perpendicular to the glass plates) and a small amount of light passes through a few light lines arising from unoriented material of which the optical axes are not normal to the glass plates. This indicates that most of the bilayers are oriented parallel to the glass plates. However, at decreasing L/P ratio, the amount of light passing through the polarizing microscope is increasing and the black areas become smaller in size, indicating that more material becomes unoriented. It was found that the observations through the polarizing microscope in the presence of the first-order red plate were less clear, probably due to the fact that in this case the presence of the red-violet colour dominates the microscope images (photographs not shown). The normal preparation time of all samples took four days. However, if this time was reduced, i.e., from four days to one day, the amount of oriented material for all samples was less, indicating that the macroscopic ordering of the samples improves with time (results not shown). However, no further improvement of macroscopic ordering was detected after four days. No attempts have been made to quantify the relative amounts of oriented and unoriented material, since the image gives only a small part of the sample, and a quantitation would require the analysis of several microscope images.

EPR spectroscopy

The study of the cholestane spin label in the sample provides a way to quantitate the macroscopic orientation of the material, since the complete sample is measured. The EPR spectra at $\theta = 0°$ are very suitable for this purpose [20], since the oriented and unoriented component are well-resolved. This is illustrated in Fig. 2 were the effect of increasing protein content on the $\theta = 0°$ EPR spectra of macroscopically oriented DOPC is given. Clearly in these spectra a strong effect of the protein can be observed. The $\theta = 0°$ EPR spectrum at $L/P = \infty$ is characteristic for an oriented bilayer system, with a small amount (about 20%) of unoriented material indicated by a second component. However, at decreasing L/P ratio the second spectral component increases in intensity. This component is invariable upon rotating the sample at different angles θ, supporting the interpretation that it arises from unoriented material in the sample. By subtracting the EPR spectrum of this unoriented material, the oriented spectra could be recovered. The relative intensity of the unoriented component in the $\theta = 0°$ EPR spectra as a function of the L/P ratio is quantitated by a digital double integration, as given in Table I. This result is in qualitative agreement with the polarizing microscope observations.

It is possible that the molecular orientation of the cholestane spin label within the bilayer is affected by

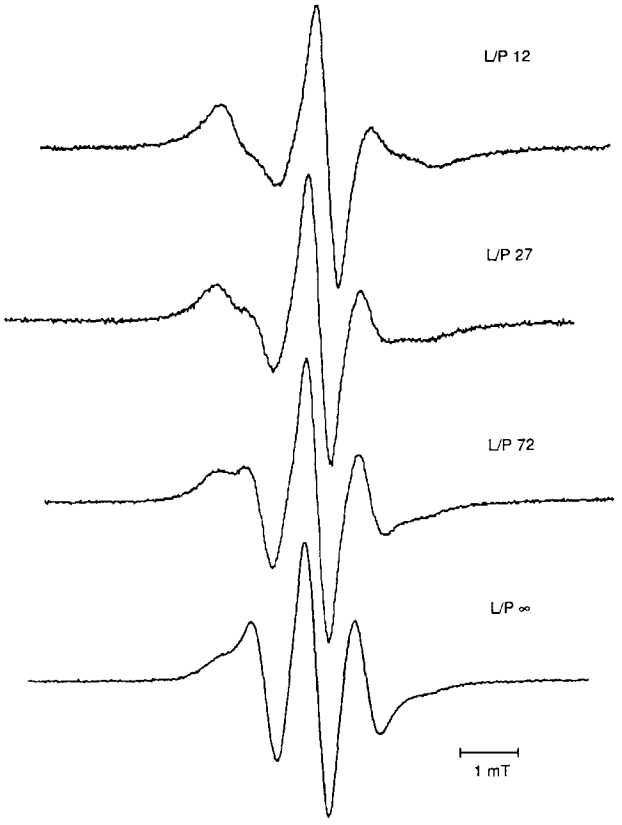

Fig. 2. $\theta = 0°$ EPR spectra of the cholestane spin label in macroscopically oriented DOPC with various amounts of M13 coat protein.

the presence of the protein. A change of the molecular order parameter would then tend to change in particular the spectral lineshape of the oriented component in the $\theta = 0°$ EPR spectra. The difference spectra (results not shown) do not indicate a strong effect of the protein on the lineshape and the effect on the double integration of the lineshape, and thus the results shown in Table I, is negligible.

After the EPR experiments, the relative contribution of α-helical and β-polymeric M13 coat protein in the samples was determined. This result is presented in Table I as well. The water content of all samples is $30 \pm 10\%$ by weight, independent of the L/P ratio. This is in agreement with results found by Korstanje et al. [11] for macroscopically oriented multibilayers of various phospholipid systems.

TABLE I

Various experimental parameters as a function of the L/P ratio of oriented M13 coat protein / DOPC multibilayers

L/P ratio	% unoriented [a]	% α-helix [b]
∞	20	–
72	40	90
27	85	78
12	> 90	67

[a] The error is $\pm 10\%$.
[b] The error is $\pm 5\%$.

Discussion

Preparation of well-oriented multibilayer systems is important for advanced angular-dependent spectroscopic studies. This would enable to obtain detailed information about the lipid-protein interaction and relative orientation of the molecules. Here we have used the coat protein of the bacteriophage M13 in the α-helical form, as a model protein for studying the effect of protein on the macroscopic orientation. In the experiments the spontaneous orientation is investigated of lipid-protein material squeezed between glass plates, i.e., no additional forces are applied on the glass plates during equilibration, and all samples are treated in a similar way to enable a mutual comparison of the effect of the M13 coat protein on the macroscopic orientation.

When reconstituted in phospholipid systems, M13 coat protein can adopt two different forms: an α-helical and a β-polymeric form. In this paper we have studied the α-helical form, which is the biologically active state of the protein. Lipid-protein samples with different L/P ratios were macroscopically oriented between glass plates and hydrated at a constant relative humidity. It turns out that all samples contain an equal amount of water, facilitating a mutual comparison of the results. However, due to the length of the preparation procedure (4 days), the α-helical protein has partly been converted into the β-polymeric form. This effect is stronger at higher protein contents in the sample (see Table I). Although the conditions of the lipid-protein reconstitutes were optimized to delay this conversion, it was not possible to completely suppress this effect.

From Table I, it is clear that although all samples underwent the same preparation procedure, the macroscopic ordering as sensed by the cholestane spin label decreases at decreasing L/P ratio. This observation is supported by the images obtained from the polarizing microscope (Fig. 1). Qualitatively similar results have been obtained of systems consisting of M13 coat protein embedded in oriented multibilayers of DMPC at 30°C (data not shown). This indicates that the temperature with respect to the gel to liquid crystalline phase transition temperature is not playing a major role in the results of Table I. The macroscopic ordering increases with time. A period of four days is taken as a practical limit for the preparation of all samples. A longer period would induce more unwanted β-polymers, whereas a shorter time would give less ordering. Therefore, the results indicate that the equilibration period is sufficient for the samples without protein to obtain a high degree of ordering (about

80%), but the protein containing samples have not achieved such a high ordering. This suggests that the apparent viscosity of the lipid-protein systems increases with increasing amounts of protein.

It has been found by Sanders et al. [26] that M13 coat protein in the β-polymeric state gives rise to phospholipids in a strongly stressed hexagonal phase close to the protein aggregates. These β-polymeric protein-induced lipids could be responsible for the reduction of macroscopic order, as observed in Figs. 1 and 2. There are two arguments that this effect is not the main reason for the reduction of macroscopic ordering with increasing protein contents. (1) It has been found that β-polymeric protein induces strongly immobilized boundary lipid, due to the large size of the aggregates. However, no bound component is observed in the EPR spectra of Fig. 2, indicating that the effect of β-polymeric protein is small; (2) The amount of β-polymers increases with time, whereas the amount of unoriented material decreases with time. This means that the presence of β-polymers does not directly affect the macroscopic order. For these reasons, we tend to explain the results in terms of a model that takes into account the effect of α-helical coat protein on the apparent viscosity of the lipid-protein systems.

In the preparation method that is employed here, macroscopic ordering arises from lining up of bilayers of the lipid-protein system along the glass wall of the supporting glass plates. This effect is caused by the adsorption of the head groups of the phospholipids to the glass wall. This first adsorbed bilayer will allow other bilayers to adsorb parallel to the glass wall, thereby generating a macroscopically oriented system. This process involves several changes in the macroscopic organization of the sample, that is initially in an unoriented vesicular or liposomal state. At increasing viscosity, these processes are retarded, and the ordering process slows down. Therefore, we should address the question why M13 coat protein is able to increase the apparent viscosity, and thus the interaction between the bilayers.

The major coat protein of M13 consists of one polypeptide chain of 50 amino acids. In the protein three specific domains can be distinguished: a hydrophobic core (amino acids 21–39) is flanked by an acidic N-terminus (residues 1–20) containing negatively charged glutamic and aspartic acids, and a basic C-terminus (residues 40–50), which contains positively charged lysines. It has been found by Sanders et al. [27] that the hydrophobic region of M13 coat protein, which is in an α-helical conformation [28], perfectly matches the lipid bilayer, resulting in a relatively small distortion of the bilayer structure of the lipid system. Also the C- and N-terminal regions are found to be in an almost 100% α-helical structure [24,28], indicating that they are sticking out in the aqueous phase, between the bilayers. The distribution of the C- and N-terminal regions throughout each bilayer leaflet is random. The membrane surface of a system containing a high amount of M13 coat protein, therefore, can be conceived as a sticky and hairy surface. These protein extremities will hinder the movement of two bilayers with respect to each other and thus increase the lateral macroscopic viscosity. Another effect that may play a role is an electrostatic interaction between the C- and N-terminal parts of the coat protein of two opposing bilayers, even though the protein molecules are randomly oriented in the bilayer after reconstitution. At a high concentration of terminal parts net attractive electrostatic interaction can increase the bilayer-bilayer interaction. This idea is supported by the observation that the aggregation of lipid-protein vesicles increases with an increasing amount of protein (data not shown). The ideas presented here are also in agreement with the observation that phospholipid systems containing the peptides gramicidin A [21] and M2δ [23] have been found to form well-oriented bilayers. In these cases the peptides do not have terminal amino-acid residues exposed to the water layer, and consequently the lateral viscosity is much less influenced by the protein.

The results found here indicate that the macroscopic orientation of lipid-protein samples is affected by the molecular properties of the protein. Under the conditions used in this paper, a full macroscopic orientation of M13 coat protein in DOPC and DMPC could not be achieved. The glass plate system is one of the best ways in obtaining a high macroscopic orientation of phospholipid systems, but it requires the vesicular and liposomal micro-structures to fuse and rearrange during orientation. Alternative methods that do not need such a rearrangement could be a better approach for orienting lipid-protein systems. This has been demonstrated recently for the peptide M2δ mixed with phospholipids in organic solvents that are applied to glass plates where the solvent is evaporated [23].

Recently, it has been proposed by Shon et al. [29] on the basis of solid-state ^{15}N-NMR spectroscopy in oriented phospholipid bilayers that the amphipathic N-terminal helix of the coat protein of the related bacteriophage Pf1 is parallel to the plane of the membrane. For M13 coat protein, the relative orientation of the N-terminal part is not known, but it is possible that the N-terminal helix, which has an amphipathic nature, is similarly associated with the membrane surface [30]. An N-terminal helix parallel to the membrane surface is expected to have a very marked effect on the surrounding phospholipid molecules, but spectroscopic experiments carried out on the phospholipids in reconstituted lipid-protein systems do not give evidence for such an effect [24,26,27,31]. Also, the interpretation offered in this paper supports an extended conformation of the N-terminal part. This may be related to the

membrane-bound virus assembly: an extended helix structure could be the conformation prior to assembly, since the coat protein in the intact virus has an extended, slightly bent, helical structure [32].

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