FEBS Letters 350 (1994) 104–108

FEBS 14347

The transit sequence of a chloroplast precursor protein reorients the lipids in monogalactosyl diglyceride containing bilayers

Vladimir Chupin, Ron van 't Hof, Ben de Kruijff*

Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received 11 May 1994; revised version received 21 June 1994

Abstract

The interaction of the chloroplast precursor protein of ferredoxin with mixed model membranes composed of ^{2}H chain labeled monogalactosyl diacylglycerol and phosphatidylcholine was studied by ^{2}H and ^{31}P NMR. The bilayers were found to have special chain packing properties which most likely are the result of a specific arrangement of head groups at the interface. The precursor and not the corresponding apoprotein induced a bilayer→isotropic transition in lipid organization as a result of the transit sequence–lipid interaction. The implications of these observations for proteins import into chloroplasts are indicated.

Key words: Chloroplast; Transit sequence; Monogalactosyl diglyceride; Membrane structure; ^{2}H NMR; ^{31}P NMR

1. Introduction

The majority of choroplast proteins are encoded on the nuclear genome and synthesized in the cytosol as precursors containing an N-terminal extension, the transit sequence [1,2]. This is necessary [3,4] and sufficient [5] for chloroplast-specific targeting and translocation across the chloroplast envelope membrane. Different envelope proteins appear to be involved in import [2] which is driven by ATP hydrolysis [6]. The precursor of the stromal protein ferredoxin (preFd) follows this general import pathway and is, in purified form, translocation-competent by itself. Recently, it was shown that a synthetic peptide corresponding to the transit sequence of preFd enters the functional import pathway without making use of protease-sensitive components on the outer surface of the envelope [7]. This suggests the occurrence of functional interactions between the transit sequence and membrane lipids early in the pathway. Studies involving phospholipase-treated choroplasts also suggested that lipids are involved in protein import [8].

Chloroplasts have a unique lipid composition and are rich in glycolipids not found elsewhere in the plant cell [9]. PreFd efficiently and specifically inserts into monolayers made of lipids extracted from the outer envelope membrane [10]. The insertion is mediated by the transit sequence and appears to be a general feature of transit sequences. The transit sequence has a high affinity for monogalactosyldiglyceride (MGDG). Therefore, this lipid is a possible candidate for the specific transit sequence-envelope lipid interaction. The affinity for phosphatidylcholine (PC), which is also an abundant compontent of the outer membrane, is much less, but introduction of low concentrations of MGDG already causes maximal insertin of the transit sequence, indicating a special arrangement of the lipid molecules in this system [10]. One possibility which emerges from these studies is that transit sequence–MGDG interactions contribute to organelle-specific targeting and allow insertion of the transit sequence into the outer membrane. Insertion is paralleled by a random coil→partial α-helix transition of the peptide [11], a possible prerequisite for further recognition in the translocator.

What the consequences of the transit sequence–MGDG interaction are for the organization of the membrane lipids and whether mixed MGDG/PC systems have special packing features, is not known, but of special interest, given the fact that MGDG is a typical type II non-bilayer membrane lipid. Type II lipids have been proposed to play several key roles in membrane functioning, including protein translocation [12].

To answer these questions, we studied the interaction of preFd with model membranes composed of ^{2}H chain-labeled MGDG and PC using broad line ^{31}P and ^{2}H NMR which are well-established non-perturbing lipid-structure probing techniques [13,14].

*Corresponding author.

Abbreviations: PreFd, preferredoxin; apoFd, apoferredoxin; MGDG, 1,2-dioleoyl-3-*O*-(β-*D*-galactopyranosyl)-*rac*-glycerol; $^{2}H_4$-MGDG, 1,2-di-[11-$^{2}H_2$]-oleoyl-3-*O*-(β-*D*-galactopyranosyl)-*rac*-glycerol; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; $^{2}H_4$-PC, 1,2-di-[11-$^{2}H_2$]-oleoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; NMR, nuclear magnetic resonance; FPLC, fast protein liquid chromatography.

2. Materials and methods

Deuterium depleted water was obtained from ISOTEC Inc., USA. Deuterium labeled and unlabeled lipids: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-di-[11-$^{2}H_2$]-oleoyl-*sn*-glycero-3-phosphocholine ($^{2}H_4$-PC), 1,2-dioleoyl-3-*O*-(β-*D*-galactopyranosyl)-*rac*-glycerol

SSDI 0014-5793(94)00734-9

(MGDG) and 1,2-di-[11-$^2H_2$]-oleoyl-3-*O*-(β-*D*-galactopyranosyl)-*rac*-glycerol ($^2H_4$-MGDG) were synthesized according to established methods [15–17]. PreFd from *S. pratensis* was purified from an *E. coli* overproducer as described by Pilon et al. [18] and stored in 25 mM Tris-HCl (pH 7.5), 8 M urea, 1 mM DTT, in Surfacil (Pierce Chemical Co., USA) coated Eppendorf cups at −20°C. Apoferredoxin (apoFd) was prepared from the holoprotein by removing the iron-sulfur cluster as described [19], and stored in 150 mM Tris/HCl (pH 7.5), at −20°C.

Polypeptides were, prior to the experiments, reduced by an incubation for 30 min at 25°C in 25 mM Tris-HCl (pH 7.5), 8 mM urea, 25 mM DTT, or in 150 mM Tris/HCl (pH 7.5), 25 mM DTT, for preFd and apoFd, respectively. The polypeptides were subsequently desalted by FPLC gel filtration over a fast desalting column (Pharmacia, Sweden), equilibrated in 20 mM Tris/HCl (pH 7.6), 100 mM NaCl, 2 mM DTT. Fractions were collected in Surfacil coated Eppendorf cups and placed on ice. After determination of the protein concentration according to Bradford [20] using bovine serum albumin as reference, the polypeptides were directly used for sample preparation. Polypeptide concentrations ranged between 0.5 and 0.9 mg/ml. Desired amounts of stock solutions of lipids in chloroform were mixed in a 10 mm NMR tube (total amount of lipid 15 μmol) and dried under a stream of nitrogen followed by storage under high vacuum for at least 20 h. Lipid films were hydrated by adding 1 ml of buffer (100 mM NaCl, 10 mM HEPES, 2 mM DTT, pH 7.4) or 0.8–1.8 ml of protein solution in the same buffer, followed by dispersion of the lipids by mechanical agitation. In order to reduce the amount of naturally occurring deuterium, the samples were lyophilized and rehydrated with deuterium-depleted water.

^2H and ^{31}P NMR spectra were recorded at 20°C at 46.1 MHz and 121 MHz, respectively, on a Bruker MSL-300 Spectrometer as described before [16].

The stability of preFd and apoFd during the NMR experiments was verified by SDS-PAGE electrophoresis (Laemmli) under non-reducing conditions [21]. Both pre- and apoFd migrated as a single band at their correct position after the NMR experiments. Freeze-fracturing was performed on NMR-samples, after plunge-freezing with a Reichert Jung KF 80 without the use of cryoprotectants [22]. Replicas were analyzed with a Philips 420 microscope.

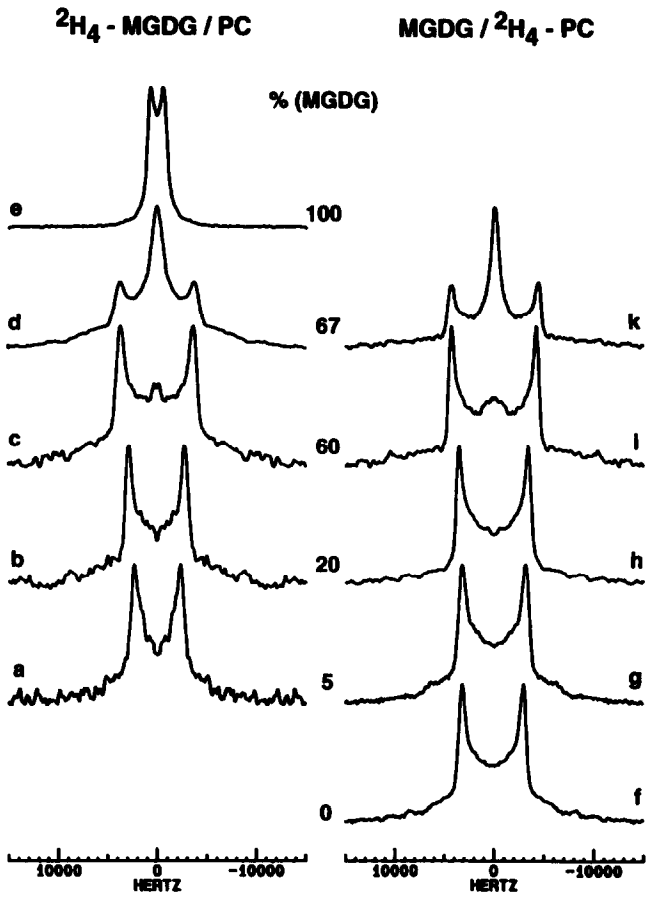

Fig. 1. ^2H NMR spectra of mixed $^2H_4$-MGDG/PC (a–e) and MGDG/$^2H_4$-PC (f–k) dispersions as a function of the MGDG content.

3. Results and discussion

3.1 Lipid organization in mixed MGDG/PC model membranes

MGDG in pure form is a lipid which, in hydrated form under physiological conditions, does not organize in bilayers but instead prefers to organize in the hexagonal H_{II} phase [17,23]. This is exemplified in the ^2H NMR spectrum e of pure $^2H_4$-MGDG by the small value of the quadrupolar splitting ($\Delta\nu_q$ = 1.4 (kHz)) which is due to the geometry of the H_{II} phase and the diffusion of the MGDG molecules around the tubes of which this phase consists.

Incorporation of increasing amounts of non-labeled PC results in the appearance of a new spectral component with an approximately four fold larger $\Delta\nu_q$ value, typical for a bilayer organization of this lipid [16,17] (Fig. 1, left lane). This is a reflection of the bilayer stabilizing activity of PC. Above 40% PC all MGDG is organized in bilayers. At intermediate DOPC concentrations, an isotropic ^2H NMR signal is present. This is often observed under conditions intermediate between the bilayer and the H_{II} phase and most likely results from lipid aggregate structure such as inverted cubic phases in which the molecules undergo rapid isotropic motion [24].

The right lane of Fig. 1 shows the ^2H NMR spectra of the corresponding lipid mixtures but now with the PC

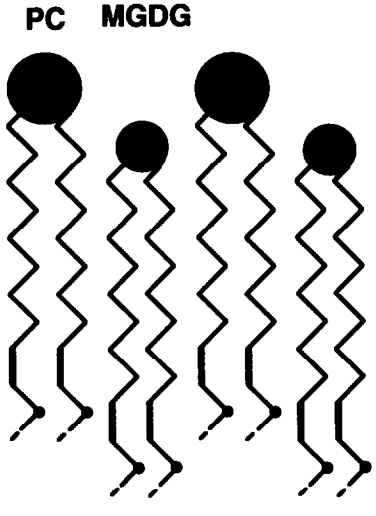

Fig. 2. The schematic representation of part of MGDG/PC model membrane. The model emphasizes the relative displacement of the head groups and consequences for the localization of the label at the 11-position in MGDG and PC molecules. The position of the deuterium label is indicated by a black dot.

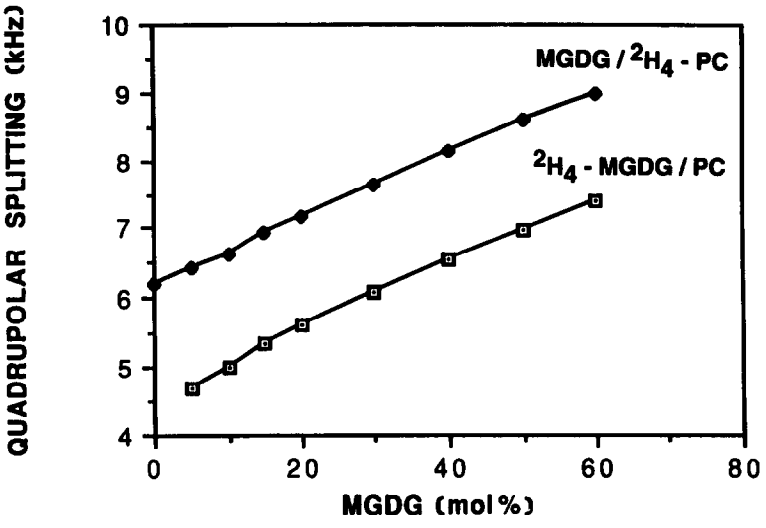

Fig. 3. Effect of MGDG concentration on the quadrupolar splitting of mixed $^2H_4$-MGDG/PC (□) and MGDG/$^2H_4$-PC (◆) dispersions.

molecule carrying the labeled oleoyl chains. In the absence of the unlabeled MGDG the PC is organized in bilayers with a $\Delta\nu_q$ of 6.3 kHz. Up to 60 mol% MGDG a bilayer organization is maintained but at and above 60 mol% MGDG, an isotropic signal is observed demonstrating that both the MGDG and PC are present in the intermediate isotropic phase. ^{31}P NMR measurements confirmed the macroscopic organization of the lipids (data not shown). Fig. 2 shows $\Delta\nu_q$ values of the individual lipids in mixtures which are organized in bilayers. Several effects are apparent. Firstly, the fact that $\Delta\nu_q$ of both PC and MGDG are affected upon increasing the MGDG concentration demonstrates that the two lipids sense each other's presence in the bilayer and therefore are not segregated into large domains. Secondly, $\Delta\nu_q$ increases with the MGDG fraction which is the behavior expected when a type II lipid is incorporated into a bilayer. Due to the cone-shape of type II molecules [25], the interface has the tendency to become concave, which is inhibited by the opposing monolayer resulting in a frustrated bilayer with a more close acyl chain packing (increased average acyl chain length) and thus a larger value of $\Delta\nu_q$. Thirdly, the $\Delta\nu_q$ value of MGDG is always *smaller* than that of PC. In fact, in mixtures where both lipids are ^2H-labeled, two well-separated doublets can be observed in the spectra, the smaller one corresponding to that of MGDG (data not shown). This is an unexpected result. For the reasons stated above, a pure type II lipid brought into a bilayer organization, for instance by lowering the temperature, always has a tighter acyl chain packing (and thus a larger $\Delta\nu_q$ value) than a typical bilayer forming lipid with a large head group, such as PC [16]*. This means that the mixed PC/MGDG bilayer has special properties. We propose that this is due to vertical displacement of the two lipid molecules with respect to each other in the bilayer. The PC head group would be more extended into the aqueous phase, thereby stretching the attached acyl chains, resulting in a larger quadrupolar splitting for the deuterons at the 11-position of the acyl chains. The 11-position of the acyl chain on the MGDG molecule is now located more closer to the methyl end of the oleoyl chain in DOPC, and therefore is more disordered, resulting in a decreased $\Delta\nu_q$ value. The galactose groups are more buried and are possibly located at the height of the ester carbonyl region of the PC molecules where they might be anchored via hydrogen bonding. Taking into account the size of phosphorylcholine, the galactose head group is expected to be exposed to its aqueous phase. A model illustrating this organization is shown in Fig. 3.

3.2. *Effect of preFd on lipid organization*

Low concentration of preFd when brought into contact with mixed $^2H_4$-MGDG/PC bilayers cause the appearance of an isotropic signal in both ^2H and ^{31}P NMR spectra of the samples (Fig. 4.). The $\Delta\nu_q$ value of the doublet in the ^2H NMR spectra did not significantly change. Besides for the appearance of the isotropic component, the ^{31}P NMR spectra did not change significantly. The broad line shape is diagnostic for the bilayer organization of the phospholipid [13,14]. The amount of isotropic signal increases with the MGDG content. From the spectra it can be estimated that in equimolar mixtures some 19% of the MGDG and 17% of the PC molecules undergo isotropic motion at the NMR timescale. For this condition, this implies that per preFd

*The value of $\Delta\nu_q$ of PC at 20°C is 6.3 kHz. It is not possible to obtain a ^2H NMR spectrum of the $^2H_4$-MGDG in a liquid crystalline bilayer by lowering the temperature, because the acyl chain became crystalline [17], but a value of $\Delta\nu_q$ of 9 kHz at 20°C for pure $^2H_4$-MGDG can be estimated from Fig. 2 by extrapolation.

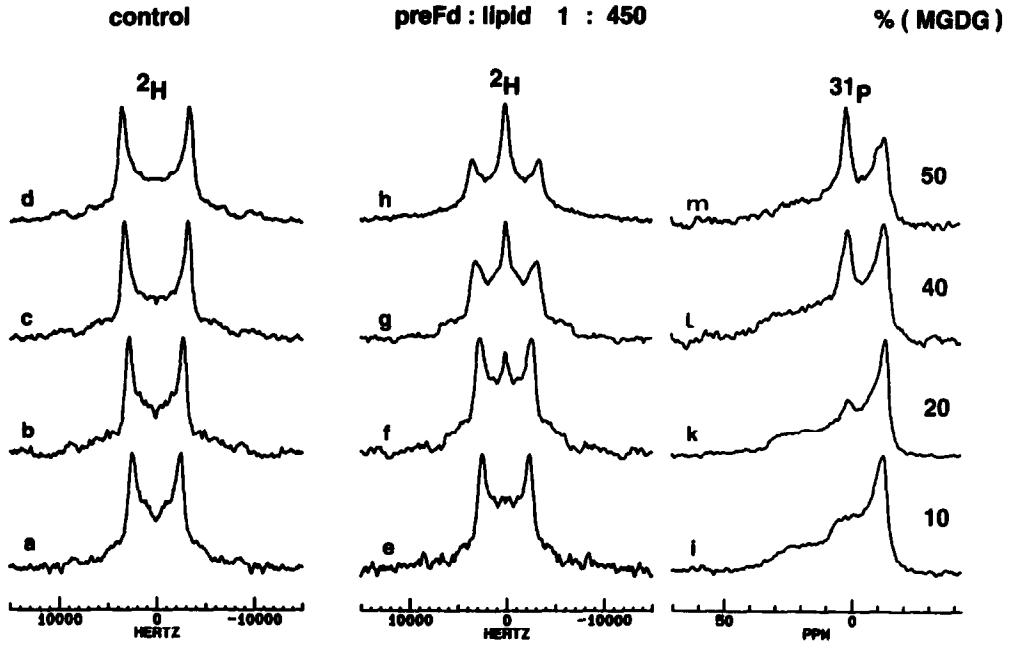

Fig. 4. ^{2}H NMR (a–h) and ^{31}P NMR (i–m) spectra of mixed $^{2}H_4$-MGDG/PC dispersions in the absence (a–d) and in the presence (e–m) of preFd (molar ratio of preFd:lipid = 1:450) as a function of the MGDG content: (a, e, i) 10; (b, f, k) 20; (c, g, l) 40; and (d, h, m) 50% (mol) of $^{2}H_4$-MGDG.

molecule present, some 100 lipid molecules underwent a bilayer→isotropic transition.

Fig. 5. shows that the induction of the isotropic signal depends on the amount of preFd present and is specific for the precursor. Addition of the apoprotein does not result in detectable spectral changes, demonstrating that the transit sequence is responsible for the observed effects. The exact nature of the isotropic lipid structure induced by the precursor proteins is not known. We propose it to originate from highly curved lipid structures in which the lipid molecules can undergo rapid diffusionial reorientation. Freeze-fracture electron microscopy failed to give an unambiguous answer on the precise nature of the isotropic structure, but on the

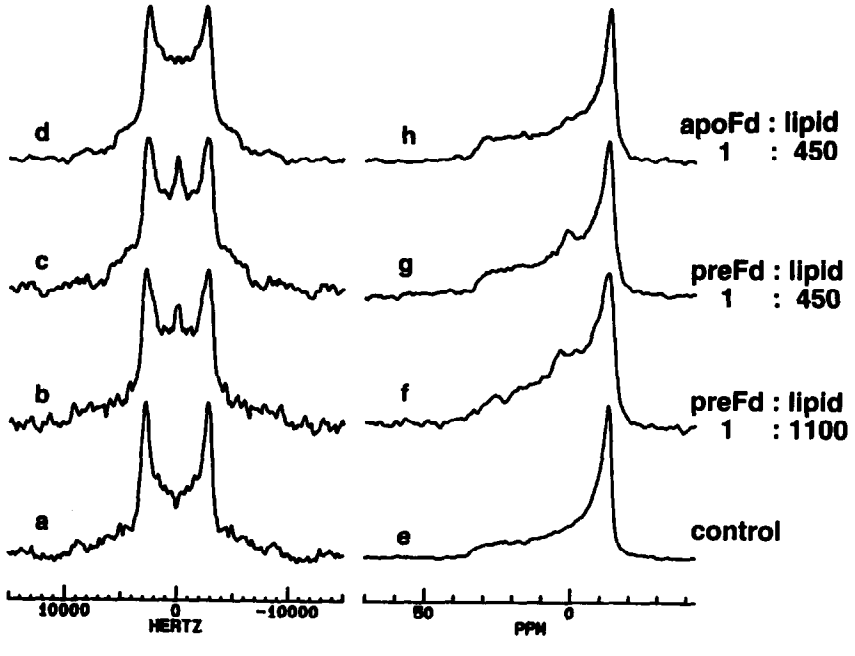

Fig. 5. ^{2}H NMR (a–d) and ^{31}P NMR (e–h) spectra of mixed $^{2}H_4$-MGDG/PC dispersions (molar ratio 1:4) membranes as a function of preFd and apoFd content: (a, e) protein free; (b, f) preFd:lipid (mol) = 1:1100; (c, g) preFd:lipid (mol) = 1:450; (d, h) apoFd:lipid (mol) = 1:450.

smooth bilayer fracture faces at several places locally higly curved domains were visible (data not shown).

The most simple explanation for the observed precursor induced bilayer destabilization is that the transit sequence, upon inserting into the lipid layer, shifts the bilayer↔type II structure equilibrium more towards the latter, resulting in the formation of the intermediate type II isotropic structure observed in the pure lipid system at higher MGDG concentrations.

3.3. Implications for protein import

Mixed bilayers of MGDG and DOPC are shown to exhibit special packing properties which likely are the result of an irregular arrangement of the galactose and phosphatidylcholine head groups on the surface (Fig. 3). Such a head group organization could give rise to insertion sites for the transit sequence and might explain why preFd already inserts with maximal efficiency in PC monolayers when only 5 mol% MGDG is present [10].

Chloroplast envelope membrane-specific insertion of the transit sequence could occur similarly, thereby determining or contributing to organelle-specific targeting. The other major finding of this study is the strong bilayer destabilizing activity of the transit sequence. The lipids in chloroplast membranes are in majority organized in a bilayer which is labile, due to the high content of the type II non-bilayer lipid MGDG. The transit sequence on the incoming precursor shifts the equilibrium more towards the non-bilayer situation, which could cause local defects in membrane lipid organization, which could facilitate further insertion or affect translocator function.

Conditions which promote type II structure formation in general facilitate interbilayer contact formation and fusion [26]. Chloroplast protein import appears to take place at sites of close contact between the outer and inner envelope membrane [27,28]. Transit sequence-lipid interactions could therefore be involved in the formation of such sites.

Acknowledgements: We thank A.J. Verkleij and J. Blijvelt for the freeze-fracturing and K. Brouwer for preparing the manuscript. This research was supported by a visitor grant from the Netherlands Organization for Scientific Research (NWO) to V. and by the Netherlands Foundation for Biological Research (BION) and the Neterlands Foundation for Biophysics with financial aid from the Netherlands Organization for Scientific Research (NWO).

References

[1] Chua, N.-H. and Schmidt, G.W. (1978) Proc. Natl. Acad. Sci. USA 75, 6110–6114.
[2] De Boer, A.D. and Weisbeek, P. (1991) Biochim. Biophys. Acta 1071, 221–253.
[3] Reiss, B., Wasmann, C.C. and Bohnert, H.J. (1987) Mol. Gen. Genet. 209, 116–121.
[4] Smeekens, J., Geerts, D., Bauerle, H.J. and Weisbeek, P. (1989) Mol. Gen. Genet. 261, 178–182.
[5] Van den Broek, G., Tinko, M.P., Kausch, A.P., Cashmore, A.R., Van Montagnu, M. and Herrera-Estrella, J. (1985) Nature 313, 358–363.
[6] Olsen, L.J. and Keegstra, K. (1992) J. Biol. Chem. 267, 433–439.
[7] Van 't Hof, R. and De Kruijff, B. (1994) manuscript in preparation.
[8] Kerber, B. and Soll, J. (1992) FEBS Lett. 306, 71–74.
[9] Douce, R., Block, M.A., Dorne, A.J. and Joyard, J. (1984) Subcell, Biochem. 10, 1–84.
[10] Van 't Hof, R., Van Klompenburg, W., Pilon, M., Kozubek, A., De Korte-Kool, G., Demel, R.A., Weisbeek, P.J. and De Kruijff, B. (1993) J. Biol. Chem. 268, 4037–4042.
[11] Horniak, L., Pilon, M., Van 't Hof, R. and De Kruijff, B. (1993) FEBS Lett. 334, 241–246.
[12] Killian, J.A., De Jong, A.M.Ph., Bijvelt, J., Verkleij, A.J. and De Kruijff, B. (1990) EMBO J. 9, 815–819.
[13] Seelig, J. (1977) Q. Rev. Biophys. 10, 353–418.
[14] Seelig, J. (1978) Biochim. Biophys. Acta 515, 105–140.
[15] Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid Res. 2, 168–229.
[16] Chupin, V., Killian, J.A. and De Kruijff, B. (1987) Biophys. J. 51, 395–405.
[17] Chupin, V., Smirnova, S. and De Kruijff, B. (1994) Recl. Trav. Chim. Pays-Bas 113, 237–240.
[18] Pilon, M., De Boer, A.D., Knols, S.L., Koppelman, M.H.G.M., Van der Graaf, R.M., De Kruijff, B. and Weisbeek, P.J. (1990) J. Biol. Chem. 265, 3358–3361.
[19] Pagani, S., Bonomi, F. and Cereletti, P. (1984) Eur. J. Biochem. 142, 361–366.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Laemmli, U,.K. (1970) Nature 227, 247–253.
[22] Sitte, H., Edelmann, L. and Neumann, K. (1987) in Cryotechniques in Biological Electron Microscopy (Steinbrecht, R.A. and Zierold, K. eds.) Springer, Berlin.
[23] Shipley, G.G., Green, J.P. and Nicols, B.W. (1973) Biochim. Biophys. Acta 311, 531–544.
[24] Lindblom, G. and Rilfors, L. (1989) Biochim. Biophys. Acta 988, 221–256.
[25] Israelachvili, J.M., Mitchell, D.J. and Ningham, B.W. (1977) Biochim. Biophys. Acta 470, 185–201.
[26] De Kruijff, B. (1987) Nature 329, 587–588.
[27] Pain, D., Kanwar, Y.S. and Blobel, G. (1988) Nature 331, 232–236.
[28] Schnell, D.J. and Blobel, G. (1993) J. Cell Biol. 120, 103–115.