Volume 223, number 1, 148–154 FEB 05211 October 1987

Interaction of melittin with negatively charged phospholipids: consequences for lipid organization

A.M. Batenburg, J.H. van Esch, J. Leunissen-Bijvelt[+], A.J. Verkleij[+] and B. de Kruijff*

Department of Biochemistry, [+]Department of Molecular Cell Biology and **Institute of Molecular Biology and Medical Biotechnology, State University of Utrecht, Padualaan 8, Utrecht, The Netherlands*

Received 20 August 1987

A characterization of the structural alterations induced by melittin in model-membranes of dioleoylphosphatidic acid and egg phosphatidylglycerol is presented, based on the use of ^{31}P-NMR, freeze-fracture electron microscopy and small angle X-ray scattering. In accordance with earlier findings on the cardiolipin-melittin system, melittin is found to have an inverted phase inducing effect on these negatively charged lipids, in contrast to the influence on zwitterionic phospholipids. In phosphatidic acid this is expressed in the formation of an H_{II} phase; in phosphatidylglycerol a less ordered, non-lamellar structure with low water content is adopted.

Melittin; Hexagonal H_{II} phase; Protein-lipid interaction; ^{31}P-NMR; Nonbilayer lipid structure; Freeze-fracture electron microscopy

1. INTRODUCTION

Melittin, a 26 residue long, basic and amphiphilic polypeptide, is one of the best studied model peptides with respect to lipid-protein interactions [1,2]. Its structure has been shown to change from a complete random coil to 70% α-helix in a lipid-bound situation [3]. Phospholipid molecular properties, as reflected in acyl chain packing, also change as a consequence of the melittin-lipid interaction ([1,2] and references cited therein) and a distinctly stronger binding to negatively charged lipids was observed.

Correspondence address: A.M. Batenburg, Dept of Biochemistry, State University of Utrecht, Padualaan 8, Utrecht, The Netherlands

Abbreviations: (DO)PA, (dioleoyl-)phosphatidic acid; (DO)PS, (dioleoyl-)phosphatidylserine; (DO)PC, (dioleoyl-)phosphatidylcholine; PG, phosphatidylglycerol; R_i, phospholipid to peptide molar ratio; SUV, small unilamellar vesicles

Studies on the implications for supramolecular organization of the lipids are of later date and show for phosphatidylcholines vesiculation of the multilamellar structures above the gel to liquid-crystalline transition and formation of small discoïd structures at gel phase temperatures; at high peptide-lipid ratios very small particles are formed independent of the lipid physical state, which were interpreted as mixed melittin-phospholipid micelles [4,5]. Binding to the negatively charged cardiolipin however resulted in large structures composed of the hexagonal H_{II} phase [6]. Furthermore a correlation was demonstrated between the type of structures formed in PC versus cardiolipin systems and the depth of penetration of the peptide [7].

Since cardiolipin is a lipid which already has a tendency to adopt inverted lipid structures [8], we thought it of interest to investigate the macroscopic organization of complexes formed between melittin and other negatively charged membrane phospholipids. PA, PS and PG were

Published by Elsevier Publishers B.V. (Biomedical Division)
00145793/87/$3.50

chosen because they vary in that order in their ability to adopt inverted structures, for instance upon interaction with divalent cations, or upon protonation [8–10].

2. MATERIALS AND METHODS

The cardiolipin sodium salt was purified from bovine heart [11]; DOPC was synthesized as reported before [12]. The conversion of egg PC and DOPC to egg PG, DOPS and DOPA by phospholipase D catalysed transesterification was also described before [13]; lipid purity was confirmed by thin layer layer chromatography. Melittin from commercial sources (Serva, Sigma) appeared to be severely contaminated with phospholipase and the peptide was therefore isolated from whole bee venom (Serva) by Sephadex G-50 gel filtration [14], followed by covalent chromatography with thiopropyl-Sepharose (Pharmacia) as described elsewhere [7]. Incubation of the egg-yolk PC liposomes with melittin thus obtained (R_i = 4) in a 10 mM Ca^{2+} containing buffer for 20 h at 37°C, did not result in detectable amounts of degradation products; the absence of phospholipase activity was also checked after most of the experiments. [^{3}H]H_2O (5 mCi/ml) was from Amersham. All other chemicals were at least of analytical grade. For all experiments a buffer containing 25 mM piperazine-1,4-bis(2-ethanesulfonic acid), 100 mM NaCl, 1 mM EDTA, pH 7.3, was used. Lipid dispersions were formed by hydration of the dry lipid film in buffer; SUV were obtained by sonication of lipid dispersions as described before [15]. Phospholipid was determined as inorganic phosphate after perchloric acid destruction [16,17].

Binding experiments were performed by following the effect of titration with SUV on the fluorescence spectrum of a 10 μM melittin solution [7] with a Perkin Elmer MPF3 fluorimeter. The data on the shift of the fluorescence maximum were analysed with a nonlinear regression program using the equation $K_D = [P] \cdot [L_N] \cdot [PL_N]^{-1}$ in which [P] and [L_N] are the concentrations of free peptide and free peptide binding sites (each formed by N lipid molecules) respectively [18].

^{31}P-NMR experiments were carried out at 121.5 MHz with a Bruker MSL 300, using broadband decoupling as described before [19]. Lipid-melittin complexes were made by addition of a 1.0 mM melittin solution to a 2 mM unsonicated lipid dispersion and isolated with >95% lipid recovery by a 30 min, 27 000 × g centrifugation. The procedure for the X-ray diffraction measurements is described in [15]. Freeze-fracturing was performed on NMR-samples, after plunge-freezing with a Reichert Jung KF 80 [20] without the use of cryoprotectants. Replicas were analysed with a Philips 420 microscope.

To determine the water content and water-lipid ratios of the structures formed by PA and PG upon saturation with melittin, ~ 1 μmol lipid was suspended in 180 μl buffer containing 5 μCi ^3H_2O. After addition of the appropriate amount of melittin in 20 μl buffer, two cycles of freeze-thawing, a 30 min, 27 000 × g centrifugation, thorough removal of supernate and a quick (< 5 s) rinsing of the sticky, oil-like-pellet, the material was dissolved by sonication in 300 μl ethanol/chloroform 2:1 (v/v), whereafter the lipid was determined as P_i and the water content by scintillation counting and comparison with the supernate.

In the extraction experiments [15] 20 nmol melittin in 100 μl buffer was added to 430 μl of chloroform/methanol (5:8, v/v) containing 500 nmol lipid phosphorus, resulting in a one-phase system; a good phase-separation without interfacial emulsion was induced by addition of 370 μl chloroform and 100 μl of buffer, whereafter the melittin remaining in the aqueous phase was determined on the basis of its fluorescence.

Circular dichroism was measured with a Jobin-Yvon Auto-Dichrograph mark V, using 1 mm cuvettes, containing peptide at a concentration of 0.1 mg/ml; the peptide-lipid molar ratio was 1:50.

3. RESULTS AND DISCUSSION

Binding of melittin to negatively charged phospholipids can be accurately studied by following the fluorescence of tryptophan-19 [7,21]. The blue-shift, which is slightly more pronounced than the average 15 nm shift reported for PC systems [3,7,22], and the quantum yield increase (fig.1) indicate a transfer to a more hydrophobic surrounding. The stoichiometry of the melittin-lipid interaction, as determined from the blue-shift, could be calculated to be one melittin molecule

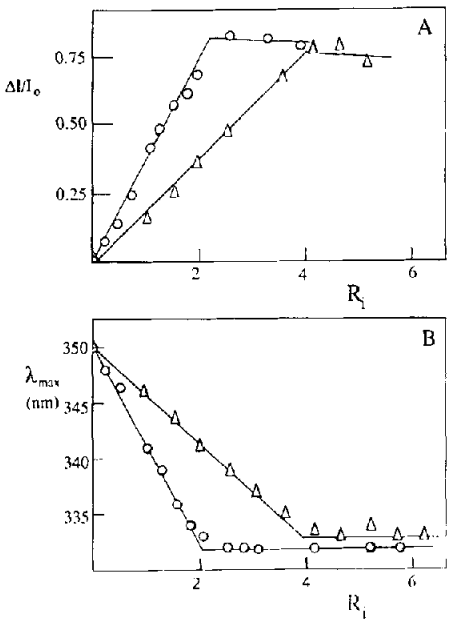

Fig.1. Effect of binding of melittin to DOPA (○) and egg (△) SUV on tryptophan fluorescence intensity (A) and wavelength of maximal fluorescence (B). Experiments were performed as described in section 2. I_0 is the emission intensity in the absence of lipids. ΔI is the increase of intensity upon lipid addition, measured at 335 nm.

bound to 3.97 ± 0.08 and 2.02 ± 0.06 lipids for egg PG and DOPA, respectively. This is in good agreement with data on saturated species dimyristoyl-PA and dipalmitoyl-PG [21], the phosphatidates apparently behaving as diacids at this pH [21]; similar to the case of cardiolipin, binding leads to compensation of four out of the six positive peptide charges. The K_D values were calculated to be $1.3 \pm 0.6 \times 10^{-8}$ M and $2.0 \pm 1.5 \times 10^{-8}$ M for PG and PA, respectively, similar to what was found for binding to bovine heart cardiolipin [6].

In contrast to observations on melittin-PC systems [4,5] the binding of melittin to DOPA dispersions caused a distinct increase of turbidity, at lipid-protein molar ratios $R_i = 10$ or less, leading to direct precipitation of the lipid. This is a first indication of a reorganization of the phospholipid. Phosphorus NMR (fig.2) of the pure lipid shows a spectrum with high field peak and low field shoulder and a residual chemical shift anisotropy $\Delta\sigma = 38$, which is typical for a partially averaged, axially symmetrical chemical shift anisotropy tensor encountered for phospholipids in extended bilayers [23] and henceforth will be called a bilayer spectrum. Addition of melittin causes a ratio dependent induction of an isotropic component with affecting the $\Delta\sigma$ of the bilayer spectrum of the bulk lipids; the bilayer spectrum is completely replaced at $R_i = 8$. In intermediate situations increasing the temperature leads to a larger fraction of the lipids experiencing isotropic motion (fig.2B′); this change shows considerable hysteresis upon cooling, as observed earlier for polymorphic transitions involving inverted lipid structures [24]. Since the samples were concentrated by centrifugation, the isotropic signal cannot be due to small structures (discs, micelles, small vesicles) but instead reflects high curvature within large structures. Small angle X-ray measurements at these ratios (fig.3A) reveal an increased X-ray scattering around 60 Å when compared with a pure lipid dispersion (not shown) but no sharp reflections and thus no ordered stacking within the resolution limits, whereas electron microscopy in accordance with this visualizes highly curved bilayer structures fused to a three-dimensional network with particles at the intersections (fig.4A) morphologically similar to the so called 'honeycomb' or 'sponge' structures [25], and regular patterns of stacked lipidic particles.

At $R_i = 4$, the NMR signal is no longer isotropic, but instead is characterized by a low field peak and high field shoulder (fig.2D) and a $\Delta\sigma$ of only 14 ppm, typical for lipids organized in cylinders such as found in the H_{II} phase [9]. This is well confirmed by the electron micrographs, exclusively showing the expected striated H_{II} phase patterns (fig.4C), and by the sharp X-ray reflections at 65 and 38 Å (fig.3B), corresponding to the 1 and $1/\sqrt{3}$ reflections of a hexagonal phase with a tube diameter of 76 Å. The melittin-phosphorus ratio at which this H_{II} phase occurs correlates well with that of the cardiolipin-melittin H_{II} phase [6]. A difference with the cardiolipin case is, that saturation is not yet attained at this R_i in the DOPA system (fig.1). Upon saturation the NMR spectrum is changed again (fig.2E) and an isotropic signal is seen, broader than the previously mentioned signal at high R_i. In this situation a diffuse X-ray scatter profile (fig.3C) is observed in agreement with freeze-fracture visualizing granular

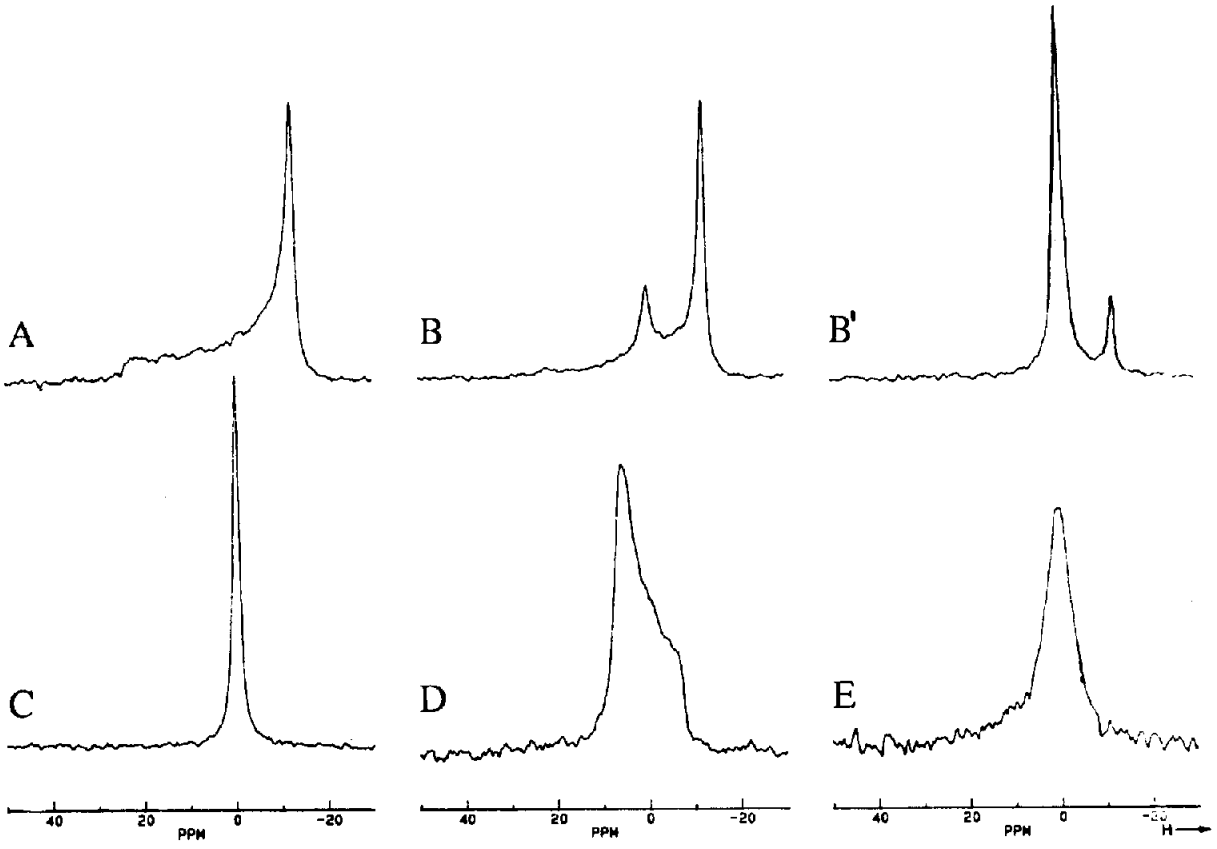

Fig.2. 121.5 MHz ^{31}P-NMR spectra of DOPA dispersions at 25°C (B': 45°C) before (A) and after (B–E) addition of various amounts of melittin. R_i = 20 (B and B'), R_i = 8 (C), R_i = 4 (D) and R_i = 2 (E). Linebroadening: 75 Hz.

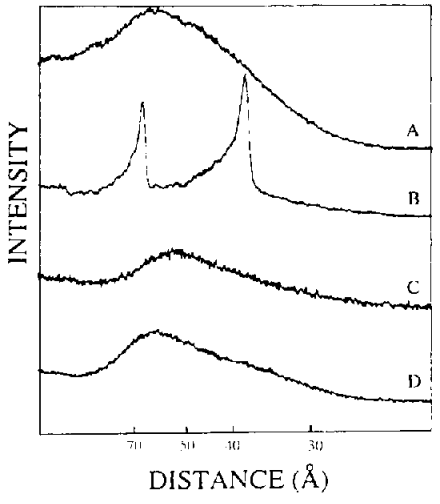

Fig.3. Small angle X-ray scatter patterns of melittin-DOPA (A–C) and melittin-egg PG (D) samples. R_i = 8 (A), R_i = 4 (B and D) and R_i = 2 (C).

structures without regular stacking (fig.4D). On the basis of the morphology and relation with the H_{II} phase, we suggest that this structure is composed of an inverted type of element, held together by hydrophobic forces. Addition of melittin to DOPS and egg PG to low R_i again resulted in immediate precipitation of the lipid, indicating that the induced isotropic ^{31}P-NMR signal (fig.5, shown for PG) as in the PA case, is attributable to lipid molecules experiencing fast diffusional motion along highly curved, large structures, rather than in tumbling small objects. The small isotropic peak in the pure lipid spectrum most likely reflects small vesicular structures. In contrast to the melittin-DOPA complex, at none of the studied R_i values was any evidence found for hexagonal H_{II} phase in egg PG, either with phosphorus NMR (fig.5), or with X-ray scattering (not shown). When egg PG was saturated with melittin (R_i = 4),

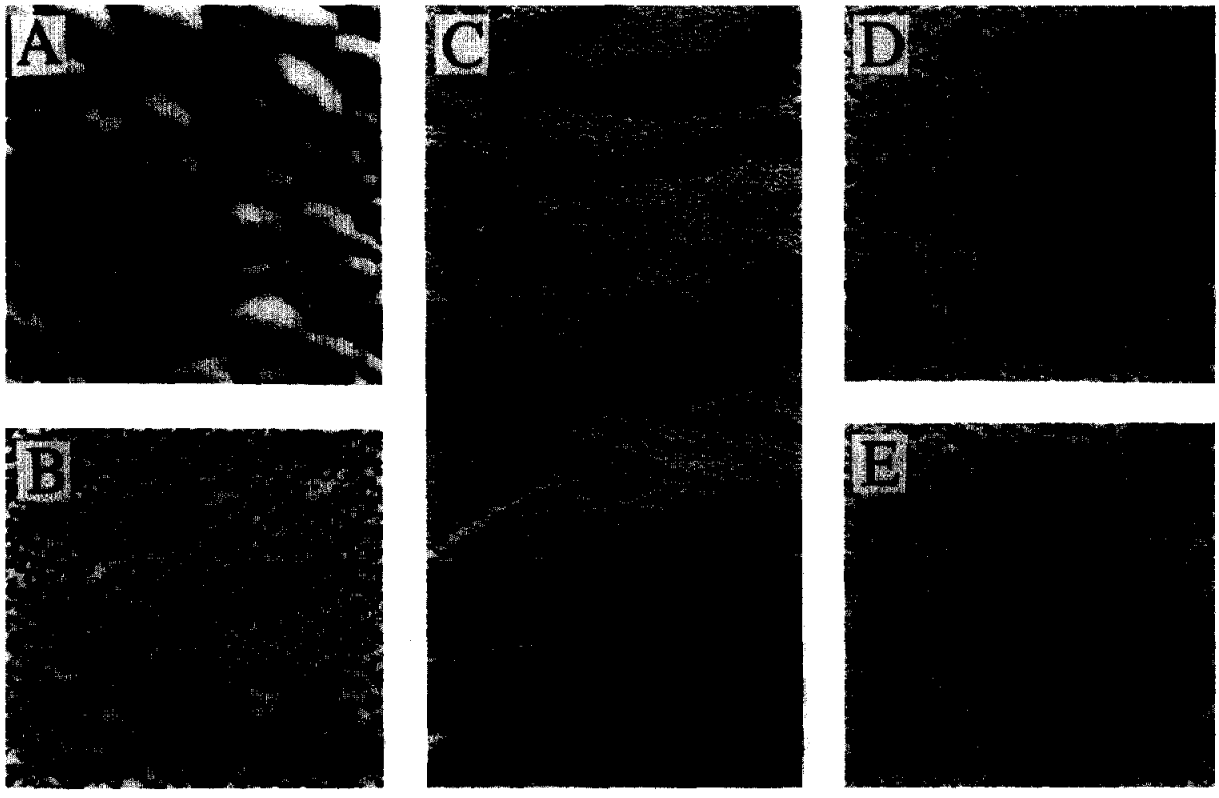

Fig.4. Freeze-fracture morphology of melittin complexes of DOPA (A–D) and egg PG (E). R_i = 8 (A and B), R_i = 4 (C and E) and R_i = 2 (D). Replicas of the pure lipid systems revealed a smooth bilayer structure only (not shown). Magnification: 73 000 × (panel A, 88 000 ×).

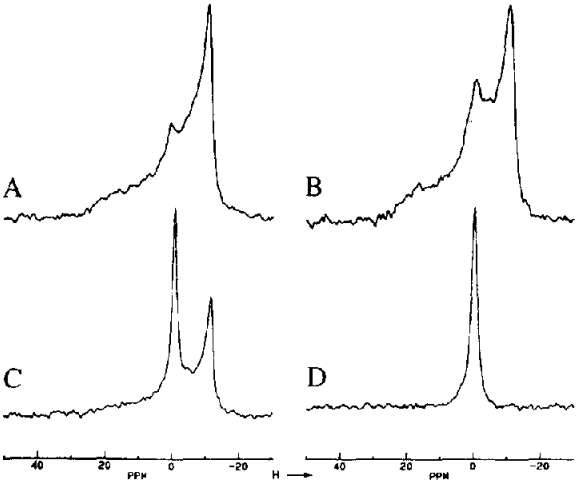

Fig.5. 121.5 MHz ^{31}P-NMR spectra of egg PG dispersions before (A) and after (B–D) addition of various amounts of melittin at 25°C. R_i = 25 (B), R_i = 10 (C) and R_i = 4 (D).

the situation is characterized by a diffuse X-ray scatter maximum around 60 Å (fig.3D, also observed with DOPS at R_i = 4, not shown), and by a purely isotropic ^{31}P-NMR signal; freeze-fracture replicas show irregularly stacked, granular structures, morphologically similar to what is seen in DOPA saturated with melittin. Using ^{3}H-labelled water, the water content of this very viscous, translucent phase was determined to be 20 ± 5% on a weight base is (P_i:H_2O = 1:18 ± 5 mol/mol), which is in agreement with the values that can be calculated for inverted lipid structures, for the melittin-DOPA H_{II} phase for instance being ~ 25%.

To support the structural data, the extractability of melittin into an organic phase in the presence of egg PG was studied, in comparison to other phospholipids (fig.6). The efficient extraction observed, similar to that in the presence of car-

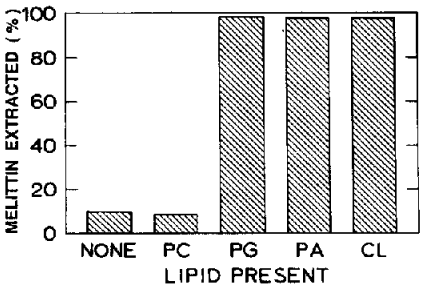

Fig.6. Extraction of melittin into an organic phase in the presence of various lipids. Experimental details are given in section 2. CL = bovine heart cardiolipin.

diolipin or DOPA, proves that melittin and egg PG together can form chloroform-soluble complexes, most easily conceived as inverted lipid structures in which the aqueous core contains the polar parts of the peptide, shielded from the organic solvent. It is noteworthy that egg PC, which is known to form micellar, 'oil in water' structures with melittin [4,5], does not stimulate extraction of melittin, although the amount of lipid present is enough to bind the major part of the melittin [26] and that the bilayer stabilizer [27] poly-L-lysine (polymerization degree n = 40) is not extracted by negatively charged lipids (Jordi, W., unpublished).

In conclusion the data indicate that melittin has a type II phase inducing effect on model membranes of negatively charged phospholipids in general. In contrast to the H_{II} phase inducing ability of the strictly hydrophobic peptide gramicidin however [28], the type of the structure actually formed is also influenced by the phase preference of the negatively charged lipid involved [8], leading in cardiolipin and PA, at a melittin-phosphorus ratio of 1:4, to the hexagonal H_{II} phase, whereas PG, PS and, at very high peptide content, PA adopt a less ordered, optically isotropic phase with a low water content which awaits further characterization. Since circular dichroism pointed to a similar α-helix content of melittin in cardiolipin when compared with egg PC (R_i = 50), as judged from the ellipticity at 221 nm (not shown), the opposite effects of melittin on zwitteronic and negatively charged membrane lipids with respect to supramolecular structure [4–6] are not correlated with the major differences in secondary structure. This justifies one of the assumptions included in the rationale for this dualism that was presented earlier [7], which was based on the different mode of insertion of the melittin in two the types of lipid, indicated by fluorescence quenching experiments.

Taking into account the preference for negatively charged phospholipids and the evidence presented for a phase segregation in systems of mixed negative and zwitterionic lipids [21], it can be imagined that melittin or melittin-like peptides in biological membranes will induce inverted or concave rather than convex lipid structures. This is of particular interest for the process of protein translocation across membranes, for which the N-terminal extensions of these proteins, closely resembling melittin with respect to size, amphiphilicity and secondary structure, have been proven to be indispensible, and for which on the other hand, inverted lipid structures conceivably could function as vehicles for transport of the polar parts of the protein.

ACKNOWLEDGEMENTS

We thank W.S.M. Geurts van Kessel for synthesis and purification of the lipids. These investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON), with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES

[1] Vogel, H. and Jähnig, F. (1986) Biophys. J. 50, 573–582.
[2] Dufourcq, J., Dasseux, J.-L. and Faucon, J.-F. (1984) Bact. Protein Toxins 24, 127–138.
[3] Vogel, H. (1981) FEBS Lett. 134, 37–42.
[4] Dufourc, E.J., Faucon J.-F., Fourche, G., Dufourcq, J., Gulik-Krywicki, T. and Le Maire, M. (1986) FEBS Lett. 201, 205–209.
[5] Dufourcq, J., Faucon, J.-F., Fourche, G., Dasseux, J.-L., Le Maire, M. and Gulik-Krywicki, T. (1986) Biochim. Biophys. Acta 859, 33–48.
[6] Batenburg, A.M., Hibbeln, J.C.L., Verkleij, A.J. and De Kruijff, B. (1987) Biophys. Acta, in press.
[7] Batenburg, A.M., Hibbeln, J.C.L. and De Kruijff, B. (1987) Biochim. Biophys. Acta, in press.
[8] De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., Van Echteld, C.J.A. and Taraschi, T.F. (1985) in: The enzymes of Biological Membranes (Martonosi, A.N. ed.) pp. 131–204, Plenum, New York.

[9] Tolcock, C.P.S., Cullis, P.R. and Gruner, S.M. (1986) Chem. Phys. Lipids 40, 47–59.
[10] Smaal, E.B., Nicolay, K., Mandersloot, J.G., De Gier, J. and De Kruijff, B. (1987) Biochim. Biophys. Acta 897, 453–466.
[11] Smaal, E.B., Romijn, D., Geurts van Kessel, W.S.M., De Kruijff, B. and De Gier, J. (1985) J. Lipid Res. 26, 633–637.
[12] Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid Res. 2, 168–229.
[13] Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
[14] King, T.P., Sobotka, A.K., Kochoumian, L. and Lichtenstein, L.M. (1976) Arch. Biochem. Biophys. 172, 661–671.
[15] Batenburg, A.M., Bougis, P.E., Rochat, H., Verkleij, A.J. and De Kruijff, B. (1985) Biochemistry 24, 7101–7110.
[16] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–389.
[17] Rouser, G., Fleischer, S. and Yamamoto, A. (1975) Lipids 5, 494–496.
[18] Hille, J.D.R., Donné-Op den Kelder, G.M., Sauve, P., De Haas, G.H. and Egmond, M.R. (1981) Biochemistry 20, 4068–4073.
[19] Chupin, V., Killian, J.A. and De Kruijff, B. (1987) Biophys. J. 51, 395–405.
[20] Sitte, H., Edelmann, L. and Neumann, K. (1987) in: Cryotechniques in Biological Electron Microscopy (Steinbrecht, R.A. and Zierold, K. eds.) Springer, Berlin, in press.
[21] Faucon, J.-F., Bernard, E., Dufourcq, J., Pérolet, M. and Bougis, P. (1981) Biochimie 63, 857–861.
[22] Georghiou, S., Thompson, M. and Mukhopadhyay, A.K. (1981) Biochim. Biophys. Acta 642, 429–432.
[23] Seelig, J. (1978) Biochim. Biophys. Acta 515, 105–140.
[24] Cullis, P.R. and De Kruijff B. (1979) Biochim. Biophys. Acta 559, 399–420.
[25] Verkleij, A.J. (1984) Biochim. Biophys. Acta 779, 43–63.
[26] Dufourcq, J. and Faucon, J.-F. (1977) Biochim. Biophys. Acta 467, 1–11.
[27] De Kruijff, B., Rietveld, A., Telders, N. and Vaandrager, B. (1985) Biochim. Biophys. Acta 820, 295–304.
[28] Killian, J.A., Van den Berg, C.W., Tournois, H., Keur, S., Slotboom, A.J., Van Scharrenburg, G.J.M. and De Kruijff, B. (1986) Biochim. Biophys. Acta 857, 13–27.