Evidence for a homogeneous lateral distribution of lipids in a bacterial membrane

A photo cross-linking approach using anthracene as a photoactivable group

J. de Bony, G. Martin, M. Welby and J.F. Tocanne*

Centre de Recherche de Biochimie et de Génétique Cellulaires du CNRS, 118 route de Narbonne, 31062 Toulouse Cédex, France

Received 7 May 1984; revised version received 25 June 1984

A new photo cross-linking method has been developed for the study of the lateral distribution of lipids in natural membranes, which uses anthracene as a photoactivable group. This method, which rests on the potentiality of anthracene to form covalently bound dimers upon irradiation around 340–380 nm has been applied to the membrane lipids (dimannosyl diacylglycerol, phosphatidylglycerol, phosphatidylinositol) of the bacterium *Micrococcus luteus*. These glyco- and phospholipids were anthracene labelled by metabolically incorporating the synthetic 9-(2-anthryl)nonanoic acid. The following sequential procedure was used: (i) dimerization of the anthracene-labelled lipids in the membrane by irradiation of the intact cells at 360 nm; (ii) extraction of the lipids and thin-layer chromatography in the first dimension to separate the various lipid dimers from the monomers; (iii) partial dedimerization of the lipid dimers by illumination of the chromatogram at around 250–280 nm; (iv) chromatography in the second dimension to separate the native lipid monomers from the corresponding residual lipid dimers. On account of the occurrence of the 3 hetero dimers phosphatidylglycerol-dimannosyl diacylglycerol, phosphatidylinositol-dimannosyl diacylglycerol and phosphatidylglycerol-phosphatidylinositol after irradiating the cells, it is concluded that in this bacterial membrane, dimannosyl diacylglycerol, phosphatidylglycerol and phosphatidylinositol are homogeneously distributed.

Micrococcus luteus *Anthracene* *Photo cross-linking* *Phospholipid* *Glycolipid* *Lipid lateral distribution*

1. INTRODUCTION

In its very definition, the widely used fluid mosaic model of membranes [1] supports the concept that both lipids and proteins are free to laterally diffuse in the bilayer, implying a random lateral distribution of lipids and proteins. However, recent investigations suggest that biological membranes might be heterogeneous in the lateral direction, both with respect to their lipid and protein components [2–9]. Nevertheless, it should be noted that these studies do not account for the lateral distribution of lipids and proteins at a molecular level as they give only a 'macroscopic' outlook of membrane organization. To go further in the description of membranes and to be able to investigate the lateral distribution of lipids at a 'microscopic' level, in terms of neighbourhood relationships, we recently proposed a new photochemical cross-linking approach which uses anthracene as a photoactivable group [10]. This group, which is well suited for labelling the

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

hydrophobic core of the membranes, forms 9-9′, 10-10′ covalently bound dimers upon irradiation in the near ultraviolet (340–390 nm) [11]. Therefore, after incorporation of anthracene into the membrane lipids, the photodimerization reaction can be used for studying their lateral distribution.

We have described the synthesis and the physical and phase properties of 9-(2-anthryl)nonanoic acid [10] and various corresponding anthracene phospholipids [21]. A very simple and versatile photo cross-linking method has been proposed for investigating the lateral distribution of anthracene phospholipids in bilayer structures [21] and the anthracene fatty acid has been shown to be incorporated metabolically into the membrane lipids of the bacterium *Micrococcus luteus* [12].

Here, the method has been applied in situ to the membrane lipids of this bacterium. Data presented here indicate that in this bacterial membrane, the anthracene-labelled phospholipids and glycolipids are laterally homogeneously distributed.

2. MATERIALS AND METHODS

2.1. *Chemicals and apparatus*

Synthesis of 9-(2-anthryl)nonanoic acid has been described [10]. DEAE–cellulose was obtained from Bio-Rad (USA). All chemicals used were of analytical grade.

Photodimerization of anthracene phospholipids was performed on an optical bench of our fabrication [21]. Liposomes or cell suspensions were irradiated at 360 nm directly in a UV cuvette under magnetic stirring. UV absorption spectra were run on a Perkin-Elmer λ5 spectrophotometer.

2.2. *Cell growth*

M. luteus (ATCC 4698-4) was grown at 30°C under agitation in a peptone-containing medium supplemented with the anthracene fatty acid, under experimental conditions identical to those in [12]. Cells were harvested at the beginning of the stationary phase. They were washed by centrifugation twice with a 0.2% (w/v) aqueous solution of the non-ionic detergent WR 1339 (Ruger, USA), then twice with distilled water to remove most of the residual non-incorporated anthracene fatty acid. The last supernatant water phase was checked to contain no anthracene fatty acid. At this stage, bacteria were divided in two batches and submitted to the following treatment.

2.3. *Lipid photodimerization in intact cells*

The first batch of cells was irradiated at 360 nm for 3 min. This was enough time to reach a nearly complete collapse of the anthracene characteristic 340–390 nm UV absorption signal [10] which could be detected directly with the intact cells and despite the rather high absorbance (*A* 1.5) of the bacterial suspension. Lipids were extracted [13] and analyzed by thin-layer chromatography (TLC) as described below.

2.4. *Lipid photodimerization in liposomes*

Lipids were extracted from the second batch of cells and purified on a DEAE–cellulose column [14]. For dimerization experiments, the pure lipids or their mixtures were suspended in the form of liposomes and irradiated at 360 nm until a nearly complete collapse of the anthracene UV spectrum at 340–390 nm (2 mn). Then the lipids were recovered for TLC analysis as in [21].

2.5. *Thin-layer chromatography*

Each lipid sample was analyzed by TLC on PF 254 silica Allurolle (Merck, FRG). The eluting solvent was chloroform/methanol/acetic acid/water (65:25:10:4, v/v). The chromatograms were observed by fluorescence. Partial dedimerization of the lipid photo-dimers was achieved by illuminating the chromatograms at 254 nm with a conventional UV lamp.

3. RESULTS

Cardiolipin, dimannosyl diacylglycerol, phosphatidylglycerol and phosphatidylinositol constitute the major lipids in the membrane of *M. luteus* [16]. 9-(2-Anthryl)nonanoic acid is incorporated into each of these lipids, at a rate of about 5%, exclusively at the *sn*-1 position and without any degradation [12].

Hence, it was possible to apply, in vivo, the photo cross-linking method we have developed, in vitro, for the study of the lateral distribution of lipids in membranes [21]. As the dimer form of the anthracene lipids separates well from the monomer form by TLC and as illumination of the dimers in the UV (250–280 nm) leads to a partial restitution

of the native monomer molecules [15], the following procedure was used: (i) dimerization of the anthracene-labelled lipids in the membrane by irradiation at 360 nm; (ii) extraction of the lipids and TLC in the first dimension; (iii) partial dedimerization of the dimers by direct illumination of the chromatogram at 254 nm; (iv) chromatography in the second dimension.

3.1. *Photodimerization of phosphatidylglycerol, dimannosyl diacylglycerol and their mixture*

These two compounds constitute the major components of the membrane lipids of *M. luteus* [16]. For the sake of identification of the corresponding homo and hetero dimers, phosphatidylglycerol and dimannosyl diacylglycerol were suspended in the form of liposomes and assayed for dimerization alone or mixed together in a 2:1 molar ratio which approximates that found for the two lipids in the bacterium. Cardiolipin and phosphatidylinositol

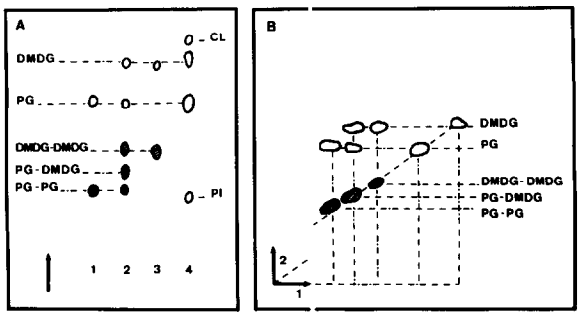

Fig.1. Photodimerization in vitro of purified anthracene-labelled lipids from *M. luteus*. (A) Thin layer chromatogram of: (i) pure phosphatidylglycerol (PG); (ii) phosphatidylglycerol/dimannosyl diacylglycerol 2:1 mixture; (iii) pure dimannosyl diacylglycerol (DMDG), after irradiation at 360 nm (dimerization) of the corresponding liposome suspensions; (iv) total lipid extract from non-labelled control cells (CL, cardiolipin; PI, phosphatidylinositol). (B) Thin-layer chromatogram obtained after chromatography in the first dimension of the 360 nm-irradiated PG/DMDG 2:1 mixture, followed by direct illumination of the chromatogram at 254 nm (partial dedimerization of the lipid dimers) and migration in the second dimension. The eluting solvent was chloroform/methanol/acetic acid/water (65:25:10:4, v/v). Note that the spots represent the contour of the spots observed by illuminating the chromatograms at 254 nm. Their size does not reflect the actual amount of the corresponding lipid monomers and dimers.

represent only a few percent of the lipids of *M. luteus* [16]. They were not tested for dimerization since the amount of purified lipids obtained was too low.

As can be seen in fig.1, phosphatidylglycerol and dimannosyl diacylglycerol homo dimers separated well from the corresponding monomer. It should be stressed that, in fact, each lipid dimer is expected to exist in the form of two head-to-head isomers which are not separated by this chromatography technique [21]. Note also that the dimer form of phosphatidylglycerol migrated practically like phosphatidylinositol.

Irradiation of the lipid mixture yielded the 3 photo dimers which are expected for a homogeneous distribution of the lipids in the bilayer, i.e., the phosphatidylglycerol (PG-PG) and dimannosyl diacylglycerol (DMDG-DMDG) homo dimers and the phosphatidylglycerol-dimannosyl diacylglycerol (PG-DMDG) hetero dimer. The occurrence of such a hetero dimer is clearly demonstrated by fig.1B, which was obtained by applying the above sequential procedure to the lipid mixture. As the illumination step at 254 nm only partly restitutes the lipid monomers from the corresponding dimer, a homo dimer will be characterized in the second dimension by two spots, i.e., the residual dimer along with the monomer, while a hetero dimer will be characterized by 3 spots, i.e., the residual dimer along with the two constitutive monomers. Indeed, on the diagonal line of fig.1B, one can observe successively, from top to bottom, first the non dimerized dimannosyl diacylglycerol (DMDG) and phosphatidylglycerol (PG), then the residual dimannosyl diacylglycerol homo dimer (DMDG-DMDG), the residual phosphatidylglycerol-dimannosyl diacylglycerol hetero dimer (PG-DMDG) and the residual phosphatidylglycerol homo dimer (PG-PG). Above the diagonal line and on the corresponding vertical line, appear the characteristic lipid monomers obtained after partial dedimerization of each lipid dimer and migration in the second dimension, namely, dimannosyl diacylglycerol (DMDG), dimannosyl diacylglycerol (DMDG) and phosphatidylglycerol (PG) and finally phosphatidylglycerol (PG).

3.2. *Photodimerization of lipids in intact cells*

Intact bacteria were irradiated at 360 nm. Lipids

were extracted and the total lipid extract was submitted to the sequential procedure, as above. On the corresponding chromatogram shown in fig.2, one can clearly identify, in the left part, the dimannosyl diacylglycerol (DMDG-DMDG) and the phosphatidylglycerol (PG-PG) homo dimers and the 3 heterodimers: phosphatidylglycerol-dimannosyl diacylglycerol (PG-DMDG), phosphatidylinositol-dimannosyl diacylglycerol (PI-DMDG), phosphatidylglycerol-phosphatidylinositol (PG-PI). No phosphatidylinositol homo dimer was detected.

To the right, one can observe two spots which might correspond to the cardiolipin monomer and homo dimer. Note that this attribution remains ambiguous as due to its chemical structure cardiolipin is susceptible to forming an internal homo dimer. Such a point deserves further investigation. Above these two spots, there is one spot which corresponds to the non-dimerized phosphatidylglycerol (PG), one which is the superimposition of non-dimerized cardiolipin (CL) (traces), dimannosyl diacylglycerol (DMDG) and anthracene fatty acid (AFA) (traces) and finally a large spot which corresponds to the bacterial neutral lipids (NL).

In any case, no hetero dimers containing either cardiolipin or the anthracene fatty acid or neutral lipids were detected on the chromatograms.

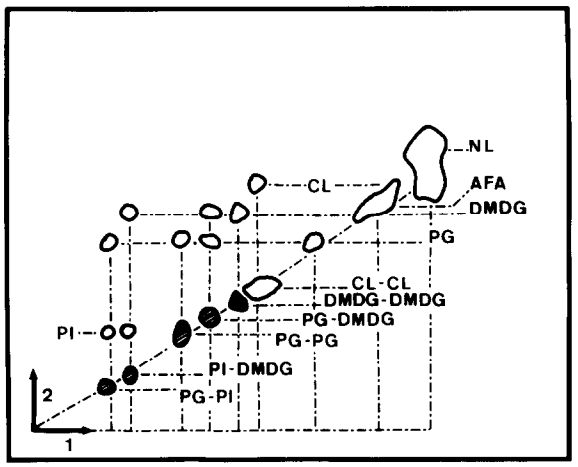

Fig.2. Photodimerization in vivo of the anthracene-labelled lipids in *M. luteus*. Obtained after chromatography in the first dimension of the total lipid extract from 360 nm irradiated (dimerization) intact cells, followed by illumination of the chromatogram at 254 nm (partial dedimerization of the lipid dimers) and migration in the second dimension. The eluting solvent was chloroform/methanol/acetic acid/water (65:25:10:4, v/v). CL, cardiolipin; AFA, anthracene fatty acid; DMDG, dimannosyl diacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; NL, neutral lipids. Note that the spots represent the contour of the spots observed by illuminating the chromatograms at 254 nm. Their size does not reflect the actual amount of the corresponding lipid monomers and dimers.

4. DISCUSSION

Taking advantage of the possibility to incorporate metabolically 9-(2-anthryl)nonanoic acid into the membrane lipids of the bacterium *M. luteus*, the method we have developed for the study, in vitro, of the lateral distribution of lipids in artificial membranes [21] has been applied, in vivo, to the membrane lipids of this bacterium. It proves to be operational. After irradiation at 360 nm of intact anthracene-labelled cells, extraction and TLC of the lipids (fig.2), 5 lipid dimers are clearly identified: the dimannosyl diacylglycerol (DMDG-DMDG) and phosphatidylglycerol (PG-PG) homo dimers and the phosphatidylglycerol-dimannosyl diacylglycerol (PG-DMDG), phosphatidylinositol-dimannosyl diacylglycerol (PI-DMDG) and phosphatidylglycerol-phosphatidylinositol (PG-PI) hetero dimers. Such a result suggests that in the bacterial membrane, dimannosyl diacylglycerol, phosphatidylglycerol and phosphatidylinositol are homogeneously distributed.

One could argue that given a time scale of 1 min, the products of the photodimerization reaction are not representative of the exact lipid distribution within the membrane. This is only partly true. On the grounds of a lipid lateral diffusion coefficient of 10^{-8} $cm^2 \cdot s^{-1}$ [17], an instantaneous picture of the membrane would require an illumination time shorter than 1 μs. This is the time it takes for a lipid molecule to jump from one position to the next. It is clear that if transient lipid domains exist in the membrane, they will not be detected with an illumination time as long as 1 min. In fact, it should be observed that the photo dimerization reaction is a very fast process which proceeds within a few nanoseconds (anthracene fluorescence lifetime: 4 ns [18]), a time scale 3 orders of

magnitude lower than that of lipid lateral diffusion. Therefore, providing that the photo products do not perturb the initial lipid distribution in the membrane, illuminating the cells for 1 min corresponds to an integration in time of a series of instantaneous pictures of the lipid distribution. An illumination time of 1 min is short compared to the lifetime of the bacterial cells (division time of *M. luteus*: 3 h). Therefore, if preferential lipid–lipid or lipid–protein interactions exist in the membrane, with functional significance, they would present a certain character of permanency and should be detected by our method. At this stage and from another point of view, one could still argue that a detailed analysis of the lateral distribution of the lipids in the membrane is linked to the possibility of quantitating the various lipid homo to hetero dimer ratios. No attempt was made here to determine these ratios. Nevertheless, any extensive lipid segregation in the membrane should be detected by our method, without measuring these ratios. This method allowed us to demonstrate a calcium-induced phase separation in anthracene-phosphatidylcholine/anthracene-phosphatidic acid mixture [21]. In contrast, to detect microheterogeneities in the lipid distribution would require a very accurate determination of the lipid homo to hetero dimer ratios, an aspect of the problem which deserves further investigation, together with the above question of the illumination time. In any case, at this level of investigation and on account of the occurrence of the above 3 hetero dimers in the membrane of *M. luteus*, we believe it likely that in this membrane, the anthracene-labelled dimannosyl diacylglycerol, phosphatidylglycerol and phosphatidylinositol are homogeneously distributed in the lateral direction, a conclusion which does not exclude the existence of microheterogeneities near or around the membrane proteins.

It has been shown that in the configuration used, anthracene only slightly perturbs the lipid molecular packing [10] and that the anthracene phospholipids do not tend to segregate in domains when mixed at low or moderate concentration ($< 10\%$) with other lipids [10]. Therefore, the conclusion of a homogeneous lateral distribution found for the above 3 anthracene lipids more than likely applies to the corresponding non-labelled lipids present in the bacterial membrane. Such a conclusion is not in contradiction with the remarkable miscibility properties phosphatidylglycerol and dimannosyl diacylglycerol exhibit in monolayer and in bilayer systems [19]. A recent differential thermal analytical investigation of the thermotropic lipid phase transitions in fatty acid-homogeneous membranes of *Acholeplasma laidlawii* B also suggest the existence of a homogeneous lipid pool in these cells [20].

The above results demonstrate the potentiality of anthracene as a reversible photo cross-linker reagent for the study of the lateral distribution of lipids in a natural membrane and they provide, for the first time, information at a molecular level. Structure and organization of biomembranes are certainly very subtle and complex and new tools are required for describing the membrane assembly at a microscopic scale, both in terms of organization and dynamics of the various components. Remembering that anthracene is fluorescent and that its photo-dimers are not [10], it is worth noting that this group can be used not only for topological studies of lipids in membranes but also for investigating their lateral diffusion rate [22,23].

REFERENCES

[1] Singer, S.J. and Nicholson, G.L. (1972) Science 175, 720–731.
[2] Wunderlich, F., Ronai, A., Speth, V., Seelig, J. and Blume, A. (1975) Biochemistry 14, 3730–3735.
[3] De Laat, S.W., Van der Saag, P.T., Elson, E.L. and Schlessinger, J. (1979) Biochim. Biophys. Acta 558, 247–250.
[4] Stier, A. and Sackmann, E. (1973) Biochim. Biophys. Acta 311, 400–408.
[5] Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295.
[6] Gounaris, K., Sundby, C., Andersson, B. and Barber, J. (1983) FEBS Lett. 156, 170–174.
[7] Tucker, A.N. and White, D.C. (1971) J. Bacteriol. 108, 1058–1064.
[8] Bevers, E.M., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1978) Eur. J. Biochem. 84, 35–42.
[9] Severs, N.J. and Robenek, H. (1983) Biochim. Biophys. Acta 737, 373–408.
[10] De Bony, J. and Tocanne, J.F. (1983) Chem. Phys. Lipids 32, 105–121.

[11] Bowen, E.J. and Tanner, D.W. (1955) Trans. Faraday Soc. 51, 475–481.
[12] Welby, M. and Tocanne, J.F. (1982) Biochim. Biophys. Acta 689, 173–176.
[13] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[14] Rauser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C. and Bauman, A.J. (1969) Methods Enzymol. 14, 272–317.
[15] Chandross, E.A. (1965) J. Chem. Phys. 43, 4175–4176.
[16] Johnson, J.H. and Grula, E.A. (1980) Can. J. Microbiol. 26, 658–665.
[17] Vaz, W.L.C., Derzko, Z.I. and Jacobson, K.A. (1982) Cell Surface Rev. 8, 83–136.
[18] Shinitzky, M., Dianoux, A.C. and Weber, G. (1971) Biochemistry 10, 2106–2113.
[19] Lakhdar-Ghazal, F. and Tocanne, J.F. (1981) Biochim. Biophys. Acta 644, 284–294.
[20] Silvius, J.R., Mak, N. and McElhaney, R.N. (1980) Biochim. Biophys. Acta 597, 199–215.
[21] De Bony, J. and Tocanne, J.F. (1984) Eur. J. Biochem., in press.
[22] Teissié, J., Tocanne, J.F. and Baudras, A. (1978) Eur. J. Biochem. 83, 77–85.
[23] Theretz, A., Teissié, J. and Tocanne, J.F. (1984) Eur. J. Biochem., in press.