Hypothesis

The importance of non-planar bilayer regions in photosynthetic membranes and their stabilisation by galactolipids

Denis J. Murphy*

Botanisches Institut der Universität, Schloßgarten 3, 4400 Münster, FRG

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Photosynthetic membranes contain considerable regions of high surface curvature, notably at their margins, where the average radius of curvature is about 10 nm. The proportion of total membrane lipid in the outer and inner thylakoid margin monolayers is estimated at 21% and 13%, respectively. The major thylakoid lipid, monogalactosyldiacylglycerol, is roughly cone-shaped and will not form complete lamellar bilayer phases, even in combination with other thylakoid lipids. It is proposed that this galactolipid plays a role in: (a) stabilising regions of concave curvature in thylakoids; and (b) packaging hydrophobic proteins in planar bilayer regions by means of inverted micelles. This model predicts substantial asymmetries in the distribution of lipids both across and along the thylakoid bilayer plane.

Thylakoid *Marginal membrane* *Galactolipid* *Planar bilayer* *Heterogeneous lipid distribution*

1. INTRODUCTION

The fluid-mosaic model of membrane organisation was originally proposed by Singer [1,2] and later extended to chloroplast thylakoid membranes by Anderson [3]. Current models of photosynthetic membrane structure at the molecular level show it as principally consisting of a planar lipid bilayer formed of more or less cylindrically shaped lipid molecules: into this bilayer are embedded to varying extents the different membrane-associated proteins [4–8]. Such a representation takes no account of the deformation of the lipid bilayer [9] caused by the presence of the large amount of protein molecules and super-molecular complexes which together make up 50% (w/w) of the photosynthetic membranes of both plants [10] and photosynthetic bacteria [11,12]. The model also fails to represent the distribution and nature of the different lipid classes. Finally, there is no attempt to explain the nature of the tightly curved margins of the thylakoid sacs. Planar bilayer membranes seem to be found in most photosynthetic organisms. However, the huge amounts of non-bilayer-forming monoglycolipids also associated with these membranes [13–15] indicate that non-planar and non-bilayer regions probably play an important role in both the structure and function of such membranes. Here, the problem of how much of the photosynthetic membrane is deformed from a planar bilayer configuration is examined. A role is proposed for non-bilayer forming lipids in maintaining the stability of photosynthetic membranes and in maximising the amount of planar bilayer regions.

2. CONTRIBUTION OF NON-PLANAR MARGINAL MEMBRANE REGIONS

The photosynthetic membranes of higher plant chloroplasts [7], and algae [16], and even some

* Permanent address: ARC Research Group on Photosynthesis, University of Sheffield, Sheffield S 10 2TN, England

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bacteria [17] are made up of flattened sacs termed thylakoids. Distinctions have commonly been drawn between so-called granal and stromal thylakoids. Indeed, two such distinct populations of photosynthetic membranes may be separated following mechanical disruption of chloroplasts; e.g., by Yeda press treatment [6]. A laterally asymmetric distribution of many membrane proteins between the granal and stromal thylakoids has recently been demonstrated in various laboratories; e.g., the PSI LHCP complex [19,20], water-splitting protein [21], and ferredoxin–$NADP^+$-reductase [20]. Other membrane components such as plastoquinone [7] and the cytochrome *b–f* complex [22] are equally distributed between both types of membrane, and are regarded as electron carriers between the granal and stromal thylakoids [22].

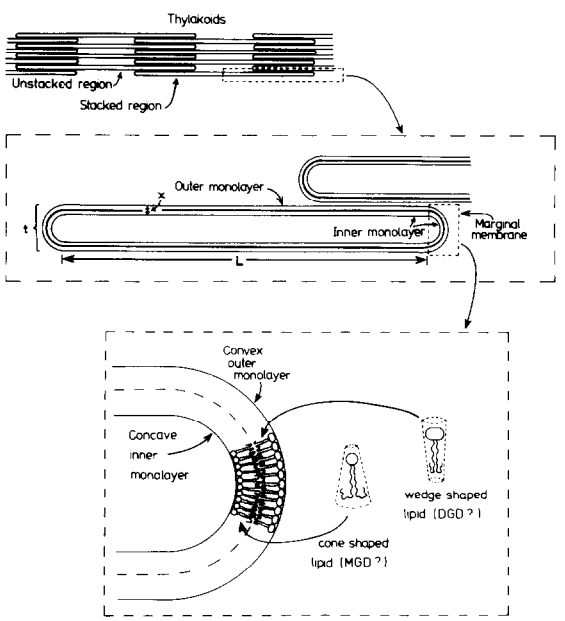

Fig. 1.(A) Schematic representation of thylakoid structure in cross-section. Thylakoid membranes are visualised as elongated flattened vesicles that overlap to different extents both in response to short-term environmental changes [24–26] and longer-term effects [62–64]. (B) The thylakoids are made up of a bilayer of mixed amphiphatic lipid molecules. (C) Around the thylakoid margins a high degree of curvature is imposed upon the bilayer, which then becomes differentiated into an inner-facing concave surface and an outer-facing convex surface. Packing constraints favour the presence of cone-shaped lipids, such as monogalactosyldiacylglycerol, on the concave face and wedge-shaped lipids such as digalactosyldiacylglycerol on the convex face.

Destacking of thylakoid membranes in vitro has been shown to result in a uniform distribution of membrane proteins [23]. The extent of stacking in vivo is highly variable, depending upon such factors as species, developmental stage, nutrient status, and incident radiation. There is also evidence that short-term changes in stacking occur in thylakoid membranes of both algae [24] and higher plants [25,26] during state 1–state 2 fluorescence transitions. Taken together, these data imply that the two types of thylakoid membrane are contiguous. In fact, they are merely different regions of a single membrane, differing only in their proximity to adjacent thylakoids. Thus, in fig. 1 the thylakoids are represented as a series of partially-overlapping flattened vesicles.

When thylakoids are visualised by electron microscopy, a cross-section of the flattened vesicles is obtained (fig. 1). The planar (i.e., non-curved) length (L) of these thylakoids, as determined by electron microscopy, is highly variable but generally ranges from 700–1400 nm. The total thickness (t) is of the order of 25 nm and the average bilayer thickness (x) is 5 nm. Both x and t are assumed to be constant.

Assuming that the thylakoids are essentially flattened sphereoids, it is possible to derive an expression for the amount of curved membrane surface area on both the outer and inner membrane edges; i.e., the 'marginal' membranes. The surface area occupied by the marginal membranes as a percentage of the total thylakoid surface area is as follows:

Inner monolayer margins:

$$\% \text{ Total area} = \frac{L(t-2x)\frac{\pi^2}{2}}{L(t-2x)\frac{\pi^2}{2} + \left(\frac{L}{2}\right)^2 \pi} \times 100 \quad (1)$$

Outer monolayer margins:

$$\% \text{ Total area} = \frac{Lt\frac{\pi^2}{2}}{Lt\frac{\pi^2}{2} + \left(\frac{L}{2}\right)^2 \pi} \times 100 \quad (2)$$

Using eq. (1) and (2), and taking values of t = 25 nm, x = 5 nm, it is possible to investigate the effect of changing the thylakoid planar length (L)

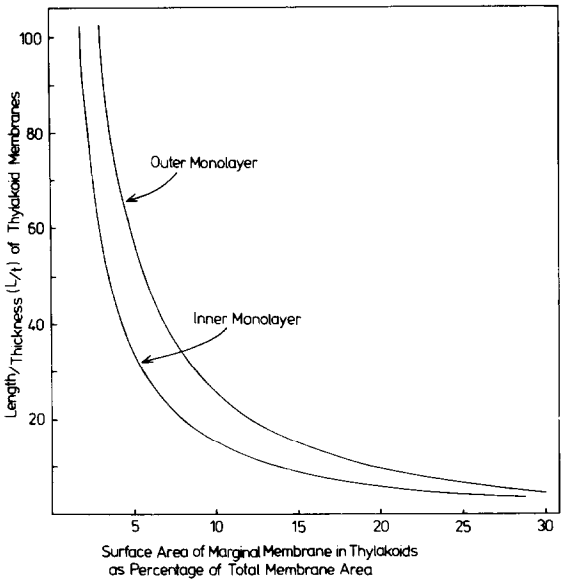

Fig. 2. Proportion of marginal membrane in thylakoids of different lengths. The curve is derived from (1) and (2) in section 2 of the text and shows the increasing proportion of marginal membrane present in thylakoids as their length is decreased while maintaining a constant thickness.

on the proportion of membrane occupied by the tightly curved margins. The ratio L/t is plotted against the percentage of marginal membrane in fig. 2. Over the observed range of thylakoid lengths in normal mature chloroplasts (700–1400 nm) the L/t ratio ranges from 28–56. The resulting percentage of marginal membrane ranges from 6.3–11.9% for the inner monolayer and 10.1–18.3% for the outer monolayer. Taking an average value of $L/t = 40$ (i.e. $L = 1000$ nm) we obtain the result that 8.6% of the inner monolayer and 13.6% of the outer monolayer surface area is derived from marginal membrane.

When $L/t = 40$ the surface area of the outer monolayer exceeds that of the inner monolayer by 2.15%. Some simple arithmetic then gives final values for the contribution of marginal membrane to that of the entire thylakoid surface area, both inside and outside.

Non-marginal membrane (inner + outer)	88.8%
Inner margins	4.1%
Outer margins	7.0%

This figure of over 11% as the total contribution of the margins to the thylakoid surface area is likely to be an underestimate for three principal reasons:

(1) So far it has been assumed, for the sake of simplicity, that the thylakoids are flattened spherical vesicles. Since spheres have a minimum surface area:volume ratio, any deviation from this shape would result in a greater edge for the same volume and hence a larger proportion of marginal membrane. Conventional representations of thylakoids visualise them as flattened oblate sphereoids. If the long axis of such a figure exceeds its short axis 3-fold, then the relative amount of marginal membrane will increase by ~70%.

(2) The flat surfaces of the thylakoids contain up to 6000 protein particles.μm^{-2} of size range 4–20 nm [27,28]. These membrane proteins are estimated to occupy 50% of the unstacked (stromal) and 70% of the stacked (granal) thylakoid surface. Since the ratio of stacked: unstacked thylakoids in mature tissue averages 65:35 [19,29–31], the overall proportion of membrane occupied by protein is ~63%. Lipids therefore only occupy 37% of the total flat thylakoid surface area.

(3) The thylakoid membrane proteins are almost certainly restricted to the flat membrane areas. For example the LHCP complex, which alone accounts for 50% of the total thylakoid protein [29,30], is specifically localised in the stacked regions of the thylakoid, which must perforce be planar. It is also most unlikely due to both thermodynamic and geometric packing considerations that the large protein molecules (4–20 nm in diameter) would occupy the sterically restricted and tightly curved marginal regions, whose average radius of curvature is only 10 nm. Therefore the marginal membranes will tend to be exclusively lipidic.

Taken together, these three factors lead to the conclusion that the proportion of lipid-occupied membrane in:

(i) Outer margins = 21.3%
(ii) Inner margins = 12.5%
(iii) Flat regions = 66.2%

3. TRANSBILAYER ASYMMETRY OF LIPID DISTRIBUTION AND MEMBRANE TOPOLOGY

Biological membranes consist of a mixture of lipid classes differing both in polar groups and fatty acid composition. Each lipid molecular species will differ in its relative hydrophobic and hydrophilic natures in any given aqueous ionic environment. Lipids will also have different geometric packing properties due to the relative effective sizes of the polar and hydrophobic regions.

In studies of multi-component vesicles it is found that lipids with relatively small polar groups (i.e., cone-shaped), such as phosphatidylethanolamine, are predominantly found in the inner (i.e., concave) monolayer of the vesicles [32–34]. Lipids with larger head groups, such as phosphatidylcholine or sphingomyelin, tend to occupy the outer (i.e., convex) monolayer. This transbilayer asymmetry arises spontaneously and is thermodynamically stable. The final asymmetric lipid distribution is determined by the interplay between polar–polar, polar–hydrophobic, and hydrophobic–hydrophobic group interactions, molecular geometry and thermodynamic forces [35,36].

The importance of such an asymmetric lipid distribution has been strikingly demonstrated in the case of photosynthetic membranes from the bacterium *Rhodopseudomonas sphaeroides* [37]. These photosynthetic membranes are formed from vesicular intracellular invaginations of the plasmalemma membrane. French-pressed cell lysates contain vesicles derived from these photosynthetic membranes that have been pinched off at the narrow neck connecting them with the plasmalemma. The vesicles are therefore topologically 'inside-out' since, although they preserve their normal orientation, the isthmus that normally connects the periplasmic or outer-facing surface with the outside of the cell is now severed, and this periplasmic surface now faces the inside of the vesicles although it preserves its normal concave curvature. In this case the lipid phosphatidylethanolamine, which is normally found on cytoplasmic-facing membrane surfaces, is mainly found on the periplasmic membrane surface. If topologically 'right-sided' vesicles are prepared by osmotic lysis then there is a rapid unidirectional transbilayer movement of phosphatidylethanolamine from the periplasmic surface (now facing out and hence with a convex curvature) to the inner-facing (now concave) cytoplasmic surface.

Similarly, rat liver microsomal vesicles are only able to synthesise phosphatidylethanolamine on their external membrane surface [38]. But this synthesis is followed by a transbilayer movement of the newly-formed phosphatidylethanolamine so that it eventually accumulates on the inner (concave) membrane. The conclusion is that it is the membrane topography which is the principal determinant of phosphatidylethanolamine distribution, with this lipid tending to partition into any concave-shaped membrane region.

4. STABILISING ROLE OF GALACTOLIPIDS IN THYLAKOID MEMBRANE STRUCTURE

A similar argument to that outlined above should apply to photosynthetic membranes from chloroplasts. Those chloroplast lipids which more readily pack into regions of high curvature will tend to partition into the deformed bilayer region around protein molecules and also into the thylakoid margins. It can be readily appreciated that since 63% of the 'flat' (i.e., non-marginal) thylakoid membrane is occupied by protein there must be a considerable deformation in the planar lipid bilayer configuration. Even if the perturbation only applies to those lipid molecules immediately adjacent to the proteins, a large proportion of the available lipid will nevertheless be in the perturbed region.

In the light of these deductions it is of con-

Table 1

The principal lipids (mol % total lipid) of chloroplast thylakoid membranes [14]

Lipid	*Spinacia oleracea*	Glycine max	*Zea mays* (mesophyll)
Monogalactosyl-diacylglycerol	38	39	40
Digalactosyl-diacylglycerol	29	28	30
6-Sulphoquinovosyl-diacylglycerol	18	14	14
Phosphatidyl-glycerol	11	11	10

siderable interest that almost half of the total thylakoid acyl lipid is made up of the galacto lipid, monogalactosyldiacylglycerol (table 1). This lipid does not form lamellar bilayer phases under physiological conditions, but forms hexagonal$_{II}$-type structures instead [39,40]. It is found that aqueous dispersions of 2:1 monogalactosyldiacylglycerol:digalactosyldiacylglycerol (the same ratio as is found in thylakoids) form mixed lamellar and inverted micellar phases [40,42].

To date there is no evidence of inverted micelles in thylakoids, although individual spherical inverted micelles would be much more difficult to detect in such a protein-rich membrane compared to the relatively easy task of finding cylindrical inverted micelles in pure lipid systems. However, there are inverted micellar regions, consisting predominantly of phosphatidylethanolamine molecules, in rabbit liver microsomes [43]. These inverted micelles probably accomodate and stabilise the cytochrome P450 enzyme complex in the interior of the bilayer membrane.

The monogalactosyldiacylglycerol molecule has a single neutral galactose residue on its polar headgroup. Measurements of ^{13}C-longitudinal relaxation times indicate that the head-group motions of the thylakoid lipids digalactosyldiacylglycerol, sulphoquinovosyldiacylglycerol, and phosphatidylglycerol are similar, but that of monogalactosyldiacylglycerol is considerably faster [44]. This implies that the monogalactosyldiacylglycerol head-group is significantly smaller than that of the other lipids. Surface pressure *vs* area isotherms of galactolipid monolayers show that the digalactosyldiacylglycerol film is more expanded than the monogalactosyldiacylglycerol film and that the area of the latter film appears to be governed by acyl chain interactions [39,45]. Again this implies that the monogalactosyldiacylglycerol has a less bulky head-group. Altogether these data show that, like phosphatidylethanolamine, the major thylakoid lipid, monogalactosyldiacylglycerol, has a small polar group and a relatively bulky hydrophobic region and hence is also roughly cone-shaped (see fig. 1).

Such a cone-shaped molecule will tend to pack into a membrane region exhibiting a concave curvature or into an inverted micellar structure. In this respect its packing properties resemble those of phosphatidylethanolamine in extra-chloroplastic membrane systems [37,38,43] or monoglucosyldiacylglycerol in cell-wall-less procaryotes such as *Acholeplasma laidlawii* [46]. The presence of a lipid domain of more or less pure monogalactosyldiacylglycerol in the tightly concave inner thylakoid margin would considerably stabilise such a structure. This would also account for about one-third of the total thylakoid monogalactosyldiacylglycerol. It is further proposed that the rest of the monogalactosyldiacylglycerol plays a role in stabilising concave deformities induced by the presence of proteins in the non-marginal membrane or in packaging intrinsic membrane proteins inside the bilayer by means of spheroidal inverted micelles (see fig. 3). This is in agreement with the suggestion in [47].

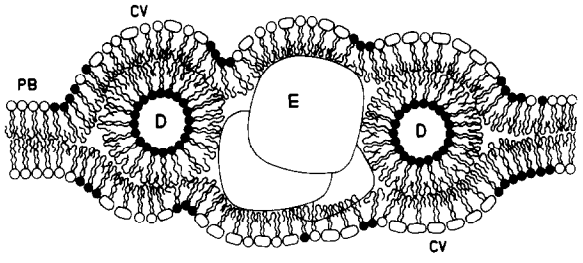

Fig. 3. Schematic representation of the deformations in the bilayer structure of thylakoids induced by inverted micelles: CV, lipid region of convex membrane curvature; PB, planar bilayer region of lipid; D, inverted micelles of monogalactosyldiacylglycerol; E, hydrophobic protein inside lipid bilayer/inverted micellar region. Lipid classes are represented as follows: monogalactosyldiacylglycerol, shaded circular polar groups; sulpholipid/phosphatidylglycerol, larger non-shaded polar groups; digalactosyldiacylglycerol, large non-circular polar groups. Large hydrophobic integral membrane proteins, such as E, may be stabilised within a completely hydrophobic structure formed from inverted micelles sandwiched between the two halves of the lipid bilayer. The inverted micelles consist of cone-shaped lipids, such as mongalactosyldiacylglycerol. Note that the lipid bilayer is extensively deformed into regions of convex curvature, which would be stabilised by wedge-shaped lipids such as digalactosyldiacylglycerol.

5. THE ROLE OF GALACTOLIPIDS IN THYLAKOID BIOGENESIS

These considerations also have implications for thylakoid biogenesis. The membranes in the

etioplasts of dark-grown plants and in the immature chloroplasts from the basal intercalary meristem of light-grown monocotyledons are often found in the form of structures termed pro-lamellar bodies [48]. The pro-lamellar bodies are organised in para-crystalline arrays that have been compared with the cubic mesophase structures sometimes adopted by lipids [49]. The cubic mesophase has been suggested as consisting of open, flat-edged polyhedral faces [50]. This kind of a figure would contain fewer regions of concave curvature since these would only be found at the intersection of adjacent faces. Plastids containing pro-lamellar bodies do have reduced proportions of monogalactosyldiacylglycerol and relatively more phospholipid when compared to chloroplasts containing only mature thylakoids [51,52]. The greatly accelerated biosynthesis of polyunsaturated monogalactosyldiacylglycerol, which accompanies both greening of etiolated plastids and normal light-grown plastid maturation [51,52], would disrupt the prolamellar body structure.

It is possible that the addition of newly-synthesized monogalactosyldiacylglycerol to pro-lamellar bodies is the primary cause of their metamorphosis into planar bilayer sacs with tightly curved margins. The galactolipids then stabilise these tightly curved marginal membranes and hence facilitate the assembly of the protein complexes associated with photosynthesis and photophosphorylation on a continuous relatively flat membrane bilayer. A prediction arising from these proposals is that there should be an excess of monogalactosyldiacylglycerol on the inner surface of the thylakoid bilayer. This has been experimentally confirmed by studies employing specific antibodies to the thylakoid lipids [53] and also using lipolytic enzymes [54,55]. These studies reveal a general transbilayer lipid asymmetry in thylakoids, but it is likely that more sophisticated experimental techniques will be needed before anything other than qualitative conclusions can be drawn from them.

The smaller the radius of curvature of the margins the greater is the number of flat membranes that can be stacked on top of one another. In this regard it is of interest that the diameter of the inner (concave) monolayer of the thylakoid margin is of the order of 20 nm or less. Estimates for the size of monogalactosyldiacylglycerol-containing inverted micelles in aqueous or lipid mixtures range from 10–20 nm [42]. Therefore the tightness of the curvature in this region approaches the limit possible for monogalactosyldiacylglycerol; i.e., the thylakoid membranes are probably about as tightly packed as is possible for their particular lipid composition.

6. STABILISATION OF CONVEX SURFACES

While monogalactosyldiacylglycerol is unequivocally the best thylakoid lipid to stabilise concave surfaces, the stabilisation of convex surfaces, such as the outer thylakoid margins may be achieved by either of the other three thylakoid acyl lipids or some mixture of them. Digalactosyldiacylglycerol has a relatively bulky polar group but also has bulky polyunsaturated acyl groups. The sulpholipid and phospholipid of the thylakoid have smaller polar groups but they also carry one negative charge each. In the alkaline environment (during illumination) of the stroma-facing outer thylakoid surface their effective polar group size would increase, due to an increased electrostatic repulsion. This would be analagous to the observed migration of phosphatidylserine to the outer surface of mixed lipid vesicles upon raising the pH of the medium [33]. These anionic lipids also have less bulky acyl chains than the galactolipids. However, both the phospholipid and sulpholipid of thylakoids have also been implicated in possible specific protein:lipid interactions [11,56,57], although it is not yet clear whether these interactions also obtain in vivo or are merely artefacts of detergent-containing in vitro reconstitution systems. The total proportion of lipid found in the convex marginal surfaces of thylakoids (21.3%) would account for most of the digalactosyldiacylglycerol in the thylakoid if this were the only lipid present in these regions. But a possible contribution of the sulpholipid and phospholipid in stabilising regions of convex membrane curvature can not yet be positively ruled out.

7. SUMMARY

It is proposed that the partially-overlapping flattened vesicles that constitute thylakoid membranes contain considerable regions of high surface curvature. This deviation from a planar bilayer con-

figuration may take principal forms:
(i) The tightly curved thylakoid margins;
(ii) Bilayer deformities induced by the presence of proteins and other structures in the membrane;
(iii) Inverted micelles involved in packaging and stabilising hydrophobic proteins within the bilayer.

The major thylakoid lipid, monogalactosyldiacylglycerol, is roughly cone-shaped and can not form complete lamellar bilayer phases under physiological conditions, even in combination with other thylakoid lipids. This galactolipid is therefore believed to play a crucial role in the stabilisation of the extensive regions of concave curvature found in thylakoid membranes. The other thylakoid acyl lipids are involved in planar bilayer formation and in stabilising the regions of convex membrane curvature. Specific interactions between the anionic lipids and membrane proteins are not ruled out in this model.

Among the more important corollaries to this hypothesis are the asymmetries that must exist amongst lipid components both across and along the thylakoid plane. The membrane is viewed as a composite heterogenous lipid–protein mixture in all dimensions – a true mosaic structure. The existence of extensive heterogeneous lipid domains, in the lateral bilayer plane, some of which would be greatly enriched in anionic lipids, has important implications for the numerous recent attempts to model granal stacking and light-induced charge separation that have been based upon consideration of surface charges [8,58–61].

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