REVIEW ARTICLE

The thylakoid membranes of higher plant chloroplasts

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Introduction

“Grau teurer Freund, ist alle Theorie und gruen des Lebens goldner Baum”

Goethe

(All theory, dear friend, is grey; but the precious tree of life is green)

Although it is improbable that the great German writer had the biochemistry of thylakoids in mind when he wrote this, his words could not be more apt. Chloroplasts play a pivotal role in sustaining life on Earth by their dual property in carrying out the primary fixation of carbon and, also (by using water as source of reducing power) in releasing oxygen for use in respiration. A key factor in carbon fixation is the trapping of light energy by the green pigment chlorophyll and the extreme efficiency of the process undoubtedly relates to the unique nature of the thylakoid membrane. In the last few years great advances have been made in our understanding of the molecular arrangement of the thylakoid constituents, but in spite of this our theories about their exact functions remain to some extent ‘grey’. In this Review we intend to describe the unique features of thylakoid structure and composition, to emphasize the interactions of different constituents and to suggest and expand ideas for the possible functions of individual components.

Basic morphology

The light-harvesting and electron transport processes of photosynthesis are localized in lamellae which consist of about 50% protein, 40% acyl lipid and 10% pigment (by weight). Usually electron micrographs of chloroplast are taken in the longitudinal profile since that reveals best the stacking of individual membranes. The appearance of chloroplasts does differ between plants with, for example, C_4 plants often having ‘agranal’ chloroplasts in their bundle sheath cells and plastids with normal granal stacks in their mesophyll cells. However, this cannot be taken as a rule as C_4 plants (e.g. *Muhlenbergia racemosa*) are known where prominent grana are seen in the bundle sheath cells (Jensen, 1980). The morphology of chloroplasts changes markedly during normal development and changes are also observed during adaptation to high (‘sun’) or low (‘shade’) light or as a result of subtle changes in energy distribution between photosystems (State 1 and State 2 transitions: Punnett, 1970; Barber,

1983a) and protein phosphorylation (Telfer *et al.*, 1984). Thus the chloroplast membranes show a high degree of plasticity and adaptability to different situations, which must surely be important for their efficient functioning. Moreover, during the stacking process, there is a lateral re-arrangement of protein components so that the new membrane structure is not only morphologically but functionally quite distinct (Barber, 1985; Staehelin & Arntzen, 1983).

Analysis of chloroplast lamellae reveals about 40 different peptides of which 14 have been sequenced at the level of the gene (Cramer *et al.*, 1985; Dyer, 1985). Further proteins have been identified as components of photosystems 1 and 2, the cytochrome b_6-f complex and the CF_0 – CF_1 complex, but a large number of the total thylakoid proteins have yet to be given a particular role. Thylakoid lipid composition is unusual in that instead of the commonly encountered phosphoglycerides the major components are glycosylglycerides (cf. Harwood, 1980a). One uses the word ‘unusual’ advisedly since, because of the huge amounts of photosynthetic membranes in algae and plants, the glycosylglycerides are, in fact, the most common membrane lipids in the world. Thus, the thylakoid membrane is especially interesting to membrane biochemists from a number of standpoints. First, its acyl lipid composition is unique. Second, its protein content is dominated by large amounts of a few proteins having important functional properties and third, it contains pigments (bound to proteins) which are not only visually beautiful but offer particular challenges to the biochemist.

Synthesis of acyl components: organelle co-operation

The idea that the chloroplast is not self-autonomous with regard to synthesis of its protein components and the concept of co-operation of organelles and the signal hypothesis in the latter’s formation are well known. Less understood is the application of these concepts to lipid synthesis. The necessity for involvement of endoplasmic reticulum in the generation of thylakoid polyunsaturated fatty acids was suggested by Mazliak’s group (cf. Harwood, 1980b) and has been developed further by others. Roughan & Slack (1982) made a thorough review of proposed inter-relationships between chloroplasts and other cell compartments and also drew attention to the differences between ‘16:3-plants’ (which use a more primitive prokaryotic mechanism) and the ‘18:3-plants’ (which use a eukaryotic pathway). The overall generation of thylakoid acyl groups and complex lipids is

Abbreviations used: ACP, acyl carrier protein; CTP, cytidine triphosphate; CDP, cytidine diphosphate; MGDG, monogalactosyldiacylglycerol; DG, diacylglycerol; DGDG, diagalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol (the plant sulpholipid); PA, phosphatidic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; LHC-2, light-harvesting chlorophyll a/chlorophyll b complex; PS1, photosystem 1; PS2, photosystem 2.

summarized in Fig. 1. Since Roughan & Slack's excellent review, a number of significant advances in our knowledge have been made.

In leaf tissues, the chloroplast seems to be the only site of fatty acid synthesis *de novo* (Weiare & Kekwick, 1975; Ohlrogge *et al.*, 1979) and this synthesis appears to occur in soluble fractions; activity previously found in membrane fractions is thought to be due to loose associations of soluble components with the membrane (Walker & Harwood, 1985). Details of individual partial reactions of the Type II synthetase have been elucidated in avocado (Caughey & Kekwick, 1982), barley (Hoj & Mikkelsen, 1982) and in spinach (e.g. Shimakata & Stumpf, 1982a). A specific β -oxo-acyl-ACP synthetase utilizes palmitoyl-ACP as primer and results in stearate synthesis (Shimakata & Stumpf, 1982b). The desaturation of stearate to oleate, which is chloroplast-localized, has only been studied thoroughly in one tissue, namely developing safflower seeds, where it is soluble (McKeon & Stumpf, 1982). In other tissues this Δ^9 desaturase seems to be loosely associated with thylakoids (c.f. Wiare & Kekwick, 1975; Walker & Harwood, 1985) and, certainly, in addition to CoA and glycerol 3-phosphate (Roughan *et al.*, 1979) the presence of thylakoids has a marked effect on the nature of the products made during fatty acid synthesis. These products of fatty acid synthesis (mainly palmitate and oleate) are accumulated in chloroplast lipids via the action of acyltransferases (glycerol 3-phosphate and monoacylglycerol 3-phosphate acyltransferases). The former enzyme is in the stroma and uses oleoyl-ACP to esterify the C-1 position of glycerol (Frentzen *et al.*, 1983). In contrast, the envelope monoacylglycerol 3-phosphate acyltransferase prefers palmitoyl-ACP for the esterification of the C-2 position (Frentzen *et al.*, 1983). The PA produced by these enzymes is then hydrolysed by phosphatidate phosphohydrolase to yield DG which is required for synthesis of thylakoid lipids such as the galactosylglycerides or is reacted with CTP to yield the CDP-diacylglycerol needed for thylakoid phosphatidylglycerol (Moore, 1982).

Varying proportions of monogalactosyldiacylglycerol (MGDG) found in thylakoids contain a DG backbone which is derived from endoplasmic reticulum PC and is imported into the chloroplasts. This eukaryotic pathway provides DG with C_{18} fatty acids at both positions. In contrast, '16:3-plants' use a plastid-localized pathway where the DG is generated by chloroplastic phosphatidate phosphatase and contains combinations of C_{18} and C_{16} acids (Heinz & Roughan, 1982).

Movement of lipids in and out of the chloroplast is also involved in the further desaturation of oleate. The conversion of oleate to linoleate occurs in conjunction with PC, probably in the endoplasmic reticulum (Dubacq *et al.*, 1976; Stymne & Appelqvist, 1978; Roughan & Slack, 1982). PC can be transported to the chloroplasts by specific exchange proteins (Kader *et al.*, 1984) where it can be exchanged with PC in the outer chloroplast envelope (cf. Block *et al.*, 1983; Miquel *et al.*, 1984). It also provides linoleate for transfer to MGDG (Ohnishi & Yamada, 1982) which is the substrate for linolenate formation (Jones & Harwood, 1980). A newly-discovered galactolipid exchange protein (Yamada *et al.*, 1984) may be involved in movement of MGDG between different chloroplast membranes. Further details of galactolipid synthesis can be found in Douce & Joyard (1980) and Roughan & Slack (1982) and

recent studies on sulpholipid formation in Kleppinger-Sparace *et al.* (1985).

PG, the major thylakoid phospholipid, like the galactolipids also appears to be synthesized in the chloroplast envelope (Andrews & Mudd, 1984). This phospholipid would have to be inserted into thylakoids either by membrane flow or via a transport protein. Although a phospholipid transport protein has been isolated from spinach leaves (Kader *et al.*, 1984) it was not found in stromal fractions of the chloroplast (Schwitzguebel *et al.*, 1984).

In summary, although the chloroplast is capable of catalysing the majority of the reactions required for thylakoid lipid formation, co-operation with other cellular compartments is required for complete synthesis. Furthermore, the extent to which the chloroplast can code for the various enzymes required is, at present, unknown.

Synthesis of thylakoid membrane proteins and their function

As mentioned above, the main protein components of the thylakoid membrane are located in several different types of intrinsic supermolecular complexes which together facilitate the transfer of electrons and H^+ from water to $NADP^+$ and catalyse the synthesis of ATP from ADP (Barber, 1983b). The resulting products, NADPH and ATP, are then utilized by the Calvin cycle in the chloroplast stroma. In total there are five different membrane complexes involved in thylakoid functioning, diagrammatically depicted in Fig. 2, consisting of polypeptides which are either encoded for by the nuclear or the chloroplast genomes. In the case of the former all protein synthesis takes place on cytoplasmic 80 S ribosomes and the products are transported into the chloroplast before being inserted into the thylakoid membrane. In contrast, chloroplast-encoded proteins are synthesized within the organelle on 70 S ribosomes. With the exception of the light-harvesting chlorophyll *a*/chlorophyll *b* (LHC-2) complex, all the other complexes [photosystem 2 (PS2), photosystem 1 (PS1), cytochrome b_6-f and CF_0-CF_1], are composed of a mixture of nuclear- and chloroplast-encoded polypeptides. To date no sequences of the nuclear genes have been published except for the LHC-2 of pea (Cashmore, 1984) and *Lemna* (Tobin *et al.*, 1984). Many of the chloroplast genes encoding for thylakoid membrane proteins, however, have been located and sequenced (cf. Dyer, 1985).

The PS2 complex functions as a water: plastoquinone (PQ) oxidoreductase and consists of a hydrophobic reaction centre core involved in light absorption, primary charge separation and PQ reduction, and several hydrophilic proteins necessary for the water oxidation process (Murata & Miyao, 1985). The latter components, of which there are at least three (16, 23 and 33 kDa), are located on the inner membrane surface and are nuclear encoded. The reaction centre core, however, consists of at least six chloroplast-encoded polypeptides with genes denoted as: *psbA* for the herbicide-binding 32 kDa Q_b protein (often called the D1 protein); *psbB* for a 47 kDa polypeptide which binds chlorophyll *a*; *psbC* for a 43 kDa polypeptide which also binds chlorophyll *a*; *psbD* for another 32 kDa protein very similar to the D1 protein but designated D2; and two more genes (*psbE* and *F*) which code for the 10 and 4 kDa apoproteins of

Fig. 2. Schematic models of the known proteins which co-operate to capture light energy and promote electron flow from water to $NADP^+$ in the thylakoid membranes of higher plants and green algae

The shaded polypeptides are those which are thought to be nuclear encoded while the unshaded components are products of the chloroplast genome (see text). (a) The PS2 complex which acts as a water:plastoquinone (PQ) oxidoreductase and is shown associated with 'bound' LHC-2; (b) the PS1 complex which reduces soluble ferredoxin (Fd) and oxidizes plastocyanin (Pc); (c) the cytochrome b_6-f complex which can act either as a plastoquinol (PQH_2):plastocyanin (Pc) oxidoreductase or as a Fd:Pc oxidoreductase. In the latter case it is thought that a 37 kDa Fd reductase is bound to the complex. (d) ATP synthase (CF₀-CF₁ complex) which utilizes a transmembrane electrochemical potential gradient of H^+ to phosphorylate ADP to ATP.

cytochrome b_{559} . All these chloroplast genes have been located and sequenced for a variety of plants (see, e.g., Alt *et al.*, 1984; Widger *et al.*, 1985). The analyses show a sequence similarity between the 47 and 43 kDa chlorophyll-binding proteins and between the D1 and D2 proteins. It had been argued that the 47 kDa protein contained the binding sites for the reaction centre chlorophyll P680 and for the primary electron acceptors, phaeophytin and Q_A (Nakatani, 1984) although suggestions that the D1 and D2 proteins form the reaction centres of PS2 have been made (Trebst, 1986). Closely associated with the PS2 core complexes is a large pool of LHC-2 (see Fig. 2a) which constitutes approx. 50% of the total protein of the thylakoids (Thornber & Barber, 1979). This complex consists of at least two nuclear encoded polypeptides of molecular masses in the region

of 25–30 kDa and binds approximately equal levels of chlorophyll a and chlorophyll b.

The PS1 complex contains the reaction centre chlorophyll P700 and several Fe-S centres which act as electron acceptors (see Fig. 2b). The complex functions as a plastocyanin:ferredoxin oxidoreductase and has properties similar to the reaction centres of the green photosynthetic bacteria. The complete complex consists of at least 10 polypeptides whose size may vary between species. As with PS2, there is probably a reaction centre core complex consisting of two very similar polypeptides which are chloroplast-encoded (*psaA* genes), having molecular masses in the region of 60 kDa (Malkin, 1986). These proteins bind chlorophyll a molecules and one of them may contain P700; both gene sequences are known (Fish *et al.*, 1985). Closely associated with the two core

polypeptides are five other non-chlorophyll binding proteins (20, 16, 14, 10 and 8 kDa) some of which are chloroplast- and some nuclear-encoded (Dyer, 1985). Their removal from the core by SDS treatment indicates that one or two of them must correspond to bound ferredoxins A and B, while the nuclear-encoded 20 kDa component seems to be involved in the binding of plastocyanin to its site of oxidation. The remaining three polypeptides make up two peripheral light-harvesting assemblies, LHC-1a (23 and 22 kDa) and LHC-1b (20 kDa) which bind chlorophyll a and chlorophyll b in the ratio of about 5:1. This high ratio emphasises that the majority of chlorophyll b in the thylakoid membrane belongs to LHC-2. The LHC-1a and LHC-1b polypeptides are encoded on the nuclear *Cab* genes but have not yet been cloned and sequenced.

The cytochrome b_6-f complex catalyses the transport of reducing equivalents from PS2 to PS1 by acting as a plastoquinol:plastocyanin oxidoreductase. It can also function as a ferredoxin:plastocyanin oxidoreductase, in which case it promotes cyclic electron flow around PS1. Several of its constituent polypeptides are encoded by chloroplast genes; *petA* for 34 kDa cytochrome *f* polypeptide, *petB* for 23 kDa cytochrome b_6 polypeptide, *petD* for a 17 kDa polypeptide of unknown function. The *petC* gene for the 20 kDa Rieske Fe-S protein is, however, located in the nucleus. There is some evidence that a 37 kDa nuclear-encoded flavoprotein having ferredoxin:quinone oxidoreductase activity may be bound to the outer surface of some cytochrome b_6-f complexes (Clark & Hind, 1983) and that such a binding is facilitated by the 17 kDa *petD* gene product (Vallejos *et al.*, 1984). The *petA*, *petB* and *petD* genes have been sequenced (e.g. Phillips & Gray, 1984; Widger *et al.*, 1984; Willey *et al.*, 1984).

As with the other functionally active complexes, the ATP synthetase complex, with its intrinsic CF_0 and extrinsic CF_1 components, is made up of several subunits. It is generally thought that CF_1 consists of three α , three β , one γ , one δ and one ϵ subunits. The α and β polypeptides have molecular masses of about 58 kDa and are encoded for by the *atpA* and *atpB* genes of chloroplasts, respectively. The ϵ subunit (14 kDa) is also a chloroplast genome product (*atpE* gene) while the γ (39 kDa) and δ (19 kDa) polypeptides are coded for by the nuclear genes *atpC* and *atpD*. There is convincing evidence that ATP synthesis occurs within CF_1 , which also shows latent ATPase activity. The role of CF_0 is to provide a channel for H^+ to cross the membrane and consists of at least three subunits designated I, II and III. Subunits I (15 kDa) and III (8 kDa) are products of the chloroplast genes *atpF* and *atpH*, respectively, while subunit II (12 kDa) is coded by the *atpG* gene of the nucleus. Subunit III binds dicyclohexylcarbodi-imide and it seems that six of these polypeptides constitute a H^+ -conducting pore across the membrane. The chloroplast genes *atpA*, *atpB*, *atpE*, *atpF* and *atpH* have been sequenced and in some cases revealed a remarkable homology with related subunits found in mitochondrial and other ATP synthase systems (cf. Dyer, 1985).

Protein distribution and functional consequences

At the beginning of this article it was emphasized that the thylakoid membranes of higher plant chloroplasts consist of regions of stacked and unstacked membranes.

This structural organization can be understood in terms of heterogeneity of surface charge distribution, which gives a physical explanation as to why the outer surface of the thylakoid membrane is in tight appression or not (Barber, 1980, 1982). In short, the argument is that, unlike the non-appressed, the appressed membranes have surfaces which carry low levels of net electrical charge. As a consequence, coulombic repulsion between adjacent surfaces is minimized so that van der Waals' attractive forces can stabilize very short membrane-membrane distances. On the other hand, it is postulated that non-appressed regions have high surface charge densities which hinder close membrane-membrane interactions even under high salt conditions. This physicochemical description of thylakoid organization has gained support from a variety of experiments (Barber *et al.*, 1980) and complements biochemical studies stemming back to the pioneering work of Boardman & Anderson (1964) which indicated that PS2 and PS1 can be enriched to varying degrees in different membrane fragments. A more precise picture has emerged recently, however, based on separating different thylakoid membrane fragments by two-phase partition techniques (Akerlund *et al.*, 1976). Convincing evidence has been presented indicating that the LHC-2 and PS2 complexes are preferentially located in the appressed regions while PS1 and CF_0-CF_1 complexes are exclusively found in non-appressed exposed membranes (Andersson & Anderson, 1980). The cytochrome b_6-f complexes can be found in both membrane regions (Albertsson, 1985) although the binding of the 37 kDa ferredoxin:quinone oxidoreductase is likely to be limited to those which are in the non-appressed lamellae (Barber, 1985). This picture of extreme lateral separation of PS2 and PS1 is probably too simple and certainly does not allow for the dynamic properties of the membrane. For example, even though the non-appressed lamellae predominantly contain PS1 and CF_0-CF_1 complexes, some PS2 complexes with reduced antenna sizes known as β -units can often be found in this membrane region. The PS2 complexes in the appressed regions have larger antenna sizes and are known as α -units (Melis & Thielen, 1980).

The lateral separation of the protein complexes along the plane of the membrane raises the question of the functional consequences of such an arrangement. Clearly the various complexes must intercommunicate in order to bring about light-induced electron and H^+ flow from water to $NADP^+$ and at the same time use some of the energy available to synthesise ATP. As Fig. 3 shows there are three components which can mediate the transfer of reducing equivalents between the complexes; the hydrophobic redox agent plastoquinone, which is reduced by PS2 and oxidised by cytochrome b_6-f , and two water soluble proteins plastocyanin and ferredoxin. Plastocyanin is a Cu-containing protein of 10.5 kDa which is coded for by the nuclear genome. It is located on the inner thylakoid surface where it is reduced by cytochrome b_6-f complex and oxidized by PS1. Ferredoxin is a low molecular mass protein of about 11 kDa which contains a 2Fe-2S active centre and, like plastocyanin, is probably nuclear-encoded. It accepts electrons from the bound-ferredoxins A and B, at the outer surface of the PS1 complex and passes them either to $NADP^+$ via ferredoxin: $NADP^+$ oxidoreductase or back to the cytochrome b_6-f complex in order to facilitate a cyclic electron flow around PS1. As shown in

Fig. 3. Scheme to show how the intrinsic and extrinsic thylakoid proteins interact together to bring about the transfer of electrons from water to NADP^+ and at the same time pump protons from the outer to the inner surfaces so as to generate electrochemical potential energy for ATP synthesis at the $\text{CF}_0\text{-CF}_1$ complex

The model emphasizes the possible roles of plastoquinone/plastoquinol (PQ/PQH_2), plastocyanin (Pc) and ferredoxin (Fd) as long-range diffusional redox carriers.

Fig. 3, the flow of electrons between the various complexes gives rise to a vectorial transport of H^+ across the membrane so as to establish the electrochemical potential gradient necessary to drive ATP synthesis at the $\text{CF}_0\text{-CF}_1$ complex. It is still a matter of debate which of the diffusible components are involved in the long range movement of reducing equivalents. On balance, PQ is a more likely candidate because it exists as a pool which can communicate between several photosynthetic chains (Witt, 1971) and which can rapidly diffuse along the fluid mid-plane of the lipid bilayer (Millner & Barber, 1984). This conclusion is favoured by the flash-absorption studies by Haehnel (1984) but the simple picture of PQ being the only long range diffusible redox carrier is not in line with the finding that 50% of cytochrome $b_6\text{-f}$ complexes are in the appressed regions and therefore distant from PS1.

It is unlikely that the lateral separation of PS1 is beneficial for transferring reducing equivalents between complexes. It does, however, have advantages for regulating light distribution between PS2 and PS1. For non-cyclic electron flow to operate at maximum efficiency under light-limiting conditions, each photosystem must be equally excited. Yet the two photosystems have different absorption properties. It is these two conflicting characteristics which give rise to the Emerson enhancement effect (Myers, 1971). Interestingly, it has been found in algae (Bonaventura & Myers, 1969), and later in higher plants (Chow *et al.*, 1983), that a regulatory mechanism exists to reduce the enhancement phenomenon. It seems that all oxygen-evolving organisms have the ability to regulate the distribution of excitation

energy between PS2 and PS1 in order to compensate for spectral imbalances which may occur over short time periods throughout the day. The control process is known as State 1-State 2 transitions (Barber, 1976; Williams, 1979). Studies with intact and isolated higher plant systems have indicated that the regulation is achieved by a pool of LHC-2 which is able to migrate reversibly between the appressed and non-appressed membranes (cf. Barber, 1985; Staehelin & Arntzen, 1983). When there is excess PS2 light, the LHC-2 complexes, which make up the mobile pool, become phosphorylated on their surfaces at threonine residues close to the *N*-terminus (Bennett, 1983). It has been postulated that the change in the surface electrical charge density due to the phosphorylation increases coulombic repulsion between adjacent surfaces at the edges of the appressed regions so that phosphorylated LHC-2 diffuses amongst the PS1 complexes and increases their absorption cross-sections by energy transfer (Barber, 1985, 1986). In this way some of the excess energy originally absorbed by PS2 is made available to PS1, leading to a reduction of Emerson enhancement and an overall increase in quantum efficiency of photosynthesis (Canaani *et al.*, 1984). In excess PS1 light the kinase responsible for LHC-2 phosphorylation is switched off, due to oxidation of the intersystem plastoquinone pool, and a membrane-bound phosphatase catalyses the dephosphorylation process (see Fig. 4). Without the lateral separation of PS2 and PS1 complexes between the appressed and non-appressed membranes it would be difficult to visualize how the mobile antenna system would operate.

Fig. 4. The concept of a mobile pool of LHC-2 which can act as a light-harvesting antenna for PS1 when phosphorylated and for PS2 when non-phosphorylated

There is no evidence that the mobile LHC-2 becomes physically attached to either photosystem and presumably it acts as an additional antenna by energy transfer. The phosphorylation occurs when the inter-system plastoquinone pool is over-reduced by, for example, excess PS2 light and a membrane-bound kinase is activated. In excess PS1 light the plastoquinone pool is oxidized and the kinase inactivated so that a membrane-bound redox phosphatase catalyses the dephosphorylation of LHC-2 and promotes its lateral migration to the vicinity of PS2 complexes within the appressed region. The partitioning between the appressed and non-appressed membranes can be understood in terms of changes in the electrical characteristics of the LHC-2 surface accompanying the phosphorylation/dephosphorylation processes. Abbreviation: OEC, oxygen-evolving complex.

Acyl lipid distribution: lateral and transmembrane features

Thylakoid membranes contain about 45–50% of their acyl lipids as MGDG, 25–30% as digalactosyldiacylglycerol (DGDG) and up to 10% as sulpholipid (SQDG). The only significant phospholipid is PG (about 10% of total) (Harwood, 1980a). Some thylakoids (e.g. those from peas) may contain small though significant quantities of PC, but other phosphoglycerides are virtually absent. The two galactosylglycerides are characterised by an exceptionally high content of α -linolenic acid (up to 95% for MGDG). In the so-called '16:3 plants', hexadecatrienoate is also found in MGDG. The acyl composition of the two acidic lipids is also rich in linolenate but in addition SQDG contains about 35% palmitate and PG contains palmitate and a unique acid, *trans*- Δ^3 -hexadecenoic acid (Harwood, 1980a; Quinn & Williams, 1983). Thus, the thylakoid fatty acid composition, like the acyl lipid content, is both unique and relatively simple.

Experiments in several laboratories have provided data on both the lateral and transmembrane distribution of acyl lipids (Gounaris *et al.*, 1983a; Henry *et al.*, 1983; Murphy & Woodrow, 1983). As detailed above, lateral protein distribution in the thylakoids is known to be highly asymmetric, but the lipid components do not appear to follow a similar striking lateral distribution. When the distribution of acyl lipids was determined in appressed and non-appressed membranes obtained through phase-partition of thylakoid fragments (Gounaris *et al.*, 1983a), the appressed membranes appeared to be enriched in MGDG and in acidic lipids in comparison with the non-appressed membranes. Examination of LHC-2/PS2-enriched appressed membrane fragments derived by the action of detergents (Gounaris *et al.*, 1983b) showed a further enrichment, in particular that of

MGDG in comparison with DGDG. Results obtained from examination of granal and stromal lamellae prepared by mechanical methods, however, revealed no difference apart from an enrichment of MGDG in granal membranes (Chapman *et al.*, 1984) and it has been questioned whether detergent- or mechanically derived fragments are a representative sample of appressed membranes. Analysis of membranes obtained from plants grown under high or low light intensities (Chapman *et al.*, 1984) or from developing monocotyledons (Mackender & Gillanders, 1984) confirmed the absence of an extreme lateral asymmetry of the lipids. Thus although in the above cases large changes in the amount of appressed to non-appressed membranes occur, there are only small or no changes in the relative amounts of acyl lipids. Contrary to the suggestion that *trans*- Δ^3 -hexadecenoate in PG plays a role in granal stacking (Tuquet *et al.*, 1977), the acid's distribution is not markedly different between stromal and granal lamellae, nor does its content differ significantly between high-light and low-light grown plants (Chapman *et al.*, 1984) and it is present at normal levels in mutant plants with very low amounts of stacking (Bolton *et al.*, 1978). However, an important difference concerning the lipid content of granal and stromal lamellae is its ratio to protein. Thus, the stromal membranes contain a much higher lipid to protein molar ratio (about 4) than the grana (about 2.5) (cf. Ford *et al.*, 1982; Murphy & Woodrow, 1983; Chapman *et al.*, 1984).

Three approaches have been used to study the transmembrane distribution of lipids: chemical labelling procedures, antibody localization and enzymic hydrolysis (Table 1). Results from these experiments have not always been in very good agreement. In chloroplast thylakoids from a variety of plants, PG has been shown to be enriched (70–75%) in the outer (stromal) leaflet (Rawlyer & Siegenthaler, 1981; Unitt & Harwood, 1985).

Table 1. Transmembrane distribution of thylakoid lipids

Ranges are shown where different plant species were analysed. References: ¹Rawlyer & Siegenthaler (1985), ²Rawlyer *et al.* (1986), ³Sundby & Larsson (1985), ⁴Gounaris *et al.* (1984a), ⁵Radunz (1980), ⁶Rawlyer & Siegenthaler (1981), ⁷Unitt & Harwood (1985).

Lipid	Distribution			
	Analytical method...	Enzymic hydrolysis	Chemical labelling	Antibody localization
MGDG		65% outside ¹ 56–65% outside ²	59% outside ² 60% outside ³	Mainly outside ⁴ Mainly inside ⁵
DGDG		15% outside ¹ 12–22% outside ²	22% outside ² 60% outside ³	Mainly inside ⁵
SQDG		30% outside ²		Mainly outside ⁵
PG		70% outside ⁶ 66–82% outside ⁷	79% outside ⁷	Mainly outside ⁵

Interestingly, while palmitate in PG was evenly distributed, linolenate was enriched (63–82%) and *trans*- Δ^3 -hexadecenoate was exclusively located in the outer leaflet (Unitt & Harwood, 1985). These data are in accordance with those of Duval *et al.* (1980) who showed that *trans*- Δ^3 -hexadecenoate was hydrolysed particularly rapidly during studies of the effect of phospholipase A₂ treatment of spinach thylakoids on LHC-2 chlorophyll fluorescence.

In early studies with polyclonal antibodies, Radunz (1980) concluded that SQDG, in addition to PG, was concentrated on the outer surface while the galactosylglycerides were enriched at the inner surface. However, Gounaris *et al.* (1984a), through the use of monoclonal antibodies against individual thylakoid lipids, found more labelling of MGDG on the outer surface. Such an enrichment agrees with chemical labelling data (Sundby & Larsson, 1985; Rawlyer *et al.*, 1986) and acyl hydrolase studies (Rawlyer & Siegenthaler, 1985; Rawlyer *et al.*, 1986). So far as DGDG localization is concerned there is also disagreement. Sundby & Larsson (1985) found 62% of this lipid in the outer leaflet of spinach thylakoids by a technique involving oxidation of galactose with galactose oxidase or periodate followed by reduction with NaB³H₄. In contrast, similar experiments with lettuce thylakoids showed 22% of DGDG in the outer leaflet if only the head group radiolabel was measured. However, it was found that significant labelling of the acyl chains was present which could lead to an over-estimation of the accessible DGDG in right-side-out vesicles (Rawlyer *et al.*, 1986). Acyl hydrolase digestion gave figures of 10–22% of DGDG in the outer leaflet of thylakoids from different plants (Rawlyer & Siegenthaler, 1985; Rawlyer *et al.*, 1986).

The transmembrane distribution of SQDG in thylakoids is unknown. Radunz (1980) reported that antibodies to this lipid bound preferentially to the outer surface but noted that large portions of the total SQDG were not accessible to the antibodies. In digestion studies with a potato leaf acyl hydrolase (which has a high activity towards SQDG) only 10–12% of the total SQDG was digested in stacked lamellae. Again, large portions (> 30%) of the lipid were inaccessible to the lipase (Rawlyer *et al.*, 1986). There is agreement, therefore, that significant quantities of thylakoid SQDG are inaccessible to different probes. In addition, free flow particle

electrophoresis measurements on isolated thylakoid membranes and other studies including calcium binding and treatments with carbodi-imides (Nakatani *et al.*, 1978; Prochaska & Gross, 1975; Berg *et al.*, 1974) give evidence to support the notion that acidic lipids do not contribute to the electrical charge of at least the outer leaflet. Such evidence, together with emerging data on the association of acidic lipids with protein complexes (see a later section) has led to the suggestion that SQDG and possibly PG in thylakoid membranes interact closely with integral proteins and, thus, may be inaccessible to external probing (Barber & Gounaris, 1986).

In conclusion, up-to-date evidence on the thylakoid membrane has shown that (with the exception of *trans*- Δ^3 -16:1-PG) there is no absolute asymmetry of the lipid components in either lateral or transmembrane distribution. It has been suggested (Rothman & Lenard, 1977) that any lipid asymmetry observed in biomembranes could be an incidental consequence of the topological constraints resulting from lipid biogenesis. In addition, there seems to be no functional or structural rationale for expecting an absolute asymmetric distribution. It should, however, be noted that only very small amounts of lipids could be required to satisfy specific local requirements. Analyses of membranes or large fragments would not provide insight into such a local preferential distribution. Thus, it is possible that asymmetrically distributed protein complexes require small amounts of specific lipids for optimal activity or conformation. It has also been suggested (Rothman & Lenard, 1977; Israelachvili *et al.*, 1980) that areas of acute membrane curvature could be produced by local lipid asymmetry which would give the two leaflets different surface tensions and degrees of curvature.

Structural aspects of acyl lipids

While some membrane characteristics are due to the properties of lipids rather than proteins, the functional state of biological membranes is considered to be determined by the protein components. The latter are involved in a dynamic co-operation with the lipid matrix. In all systems studied, the only lipid organization found compatible with a functional biological membrane is the bilayer or lamellar phase. It has been shown, however, that for a number of isolated membrane lipids, in water mixtures and at physiological temperatures, this type of

organization is not thermodynamically stable. Isolated diacyl lipids extracted from biological membranes can adopt on hydration either the lamellar phase or what has been termed as the hexagonal type II phase. This latter type of organization was first described by Luzzati & Husson (1962) as consisting of water cylinders enclosed in a lipid matrix with the hydrocarbon chains radially orientated outwards from the central axis of the cylinders. A number of lipids of biological origin have since been found to prefer such a configuration and it appears that the hexagonal type II phase is a common lipid conformation.

Nearly every biomembrane contains substantial amounts of lipid species which when isolated and dispersed in water orient in the hexagonal type II organization, but the thylakoid membranes of higher plants are notable in that the major glycolipid (MGDG) comprising nearly half of the total lipid content would adopt such a non-bilayer configuration (Gounaris & Barber, 1983). In contrast, all other lipid classes present in thylakoid membranes form typical bilayer structures. This polymorphic phase behaviour of membrane lipids has been rationalized in terms of the dynamic molecular shape assumed by the individual components (Israelachvili *et al.*, 1977, 1980). Thus, the behaviour of a particular lipid system can be described by geometric expressions which relate the hydrocarbon chain volume and chain length and the hydrocarbon-water interfacial area to the aggregated structure. So the ability of the MGDG to adopt an hexagonal type II phase can be rationalized in terms of its molecular shape and it is strongly dependent on the degree of unsaturation of its fatty acyl chain. On reduction of the number of double bonds the hexagonal configuration is no longer observed, as judged by freeze-fracture electron microscopy (Gounaris *et al.*, 1983c). Saturated MGDG adopts a bilayer configuration in the form of open sheets rather than the conventional closed structures typical of liposomes. Such polymorphic phase transitions can be predicted by conformational analysis procedures which demonstrate that the organization of MGDG molecules depend on the degree of unsaturation of its acyl chains (Brasseur *et al.*, 1983).

The galactolipids MGDG and DGDG dominate the lipid composition of the thylakoid membranes. DGDG is a typical bilayer-forming lipid. MGDG/DGDG mixtures have been examined by biophysical methods at various ratios (Sen *et al.*, 1981a,b; Sprague & Staehelin, 1984). When mixtures at molar ratios similar to those occurring naturally are examined, a variety of non-bilayer organizations can be observed. A typical type of arrangement observed in freeze-fracture electron microscopy has been termed the 'lipidic particle' and was demonstrated to correspond in galactolipid mixtures to inverted micelles sandwiched within a bilayer (Sen *et al.*, 1981a). Particles of this type occur in binary mixtures of lipids extracted from other membrane systems and were first described by Verkleij *et al.* (1979) in aqueous dispersions of cardiolipin and phosphatidylcholine in the presence of Ca^{2+} . Other related, and probably interconvertible, structures have been observed in galactolipid mixtures and it has been shown that these structures are influenced by factors interfering with lipid-lipid, lipid-water and water-water interactions (Sen *et al.*, 1982).

The polymorphic phase behaviour of the two galactolipids has been shown to be strongly affected by

the presence, in the mixture, of the two acidic thylakoid lipids, namely PG and SQDG (Gounaris *et al.*, 1983d). Thus, total thylakoid lipid extracts adopt a bilayer configuration when dispersed in distilled water. This arrangement, however, is extremely sensitive to factors such as the pH of the suspending medium, the level of cations and temperature (Gounaris *et al.*, 1983d). Thus, in total thylakoid lipid extracts, dispersed in aqueous buffers similar to those used for membrane functional assays, a variety of non-bilayer configurations have been observed. These non-bilayer arrangements are dependent on the presence of MGDG in its fully unsaturated form when it is capable of adopting the hexagonal type II organization. Similar non-bilayer structures have been reported to occur in aqueous dispersions of total lipid mixtures from the mitochondrial inner (De Kruijff *et al.*, 1979), and rod outer segment (De Grip *et al.*, 1979) membranes.

The hexagonal type II organization of membrane lipids has never been observed in biomembranes under physiological conditions. In fact, non-bilayer configurations are difficult to reconcile with the need to maintain a stable semipermeable membrane system. Nevertheless, the very absence of these type of structures, so commonly observed in lipid extracts from biomembranes, has raised questions on the mode of interaction of membrane lipids with other components and has led to new ways of thinking about membrane architecture and function.

The role of acyl lipids in membrane function

It is generally assumed that the major role of lipids in membranes is the formation of a bilayer which will act as a structural matrix and will limit the transmembrane movement of most hydrophilic solutes. In addition the bilayer provides 'solution' thus preventing non-specific protein-protein aggregation and is required to be in a fluid state so as to allow protein diffusion and conformational changes to occur. The above requirements, however, could be easily fulfilled by a few lipids and the extreme diversity of lipid molecular species and membrane-specific lipid composition cannot be rationalized in these terms. A number of proposals have been made ascribing specific roles to the thylakoid lipids in the functioning of the membrane. Conclusions, however, are far from established.

There are three obvious differences between the lipids of thylakoids and those of other biomembranes. First, the composition is dominated by glycolipids rather than phospholipids. Studies on lipid monolayers (Bishop *et al.*, 1980) established that both MGDG and DGDG form monolayers which are much more condensed than those formed by phospholipids with comparable fatty acyl chains. They also demonstrated that the packing properties of total thylakoid lipids are determined by the packing characteristics of the two galactolipids. This has suggested that there are very strong head-group interactions between the galactolipid molecules. Proposals on lateral hydrogen bonding between lipid molecules have been previously made (Karlsson, 1982) but no such binding could be shown by i.r. spectroscopy of thylakoid galactolipids (A. A. Foley & J. L. Harwood, unpublished work). Indeed, the postulate has been questioned on the basis that competition from water would not allow such intermolecular bonding to occur. It remains, however, that strong head-group interactions exist between

galactolipids which would enhance the stability of the membrane and also increase its impermeability. The second characteristic of the lipids, which is an unusual property of the thylakoid, is their high content of α -linolenic acid (all *cis*-9,12,15-octadecatrienoic acid). An obvious explanation for this would be that such fatty acids ensure that the membrane remains in a highly fluid state (Ford & Barber, 1983). The necessity, however, of trienoic fatty acids in the thylakoid lipids to maintain fluidity has been questioned on the basis of monolayer and ^{13}C n.m.r. experiments (Bishop *et al.*, 1980; Coddington *et al.*, 1981). Nevertheless, lipid properties in biomembranes are greatly affected by the presence of intrinsic proteins and it is possible that such high levels of unsaturation are required in order to optimize the fluidity requirements of a membrane system with a high protein-to-lipid ratio such as the thylakoid. A highly fluid system would be required not just for the diffusion of lipophilic compounds, such as plastoquinone, but for the lateral diffusion of protein complexes and substrate-induced conformational changes. It has been reported (Millner *et al.*, 1984; Chapman *et al.*, 1983) that the fluidity of the thylakoid membrane is correlated with the integral protein-to-lipid ratio rather than changes in the unsaturation levels of the lipids in response to certain environmental parameters.

The occurrence of *trans*- Δ^3 -hexadecenoic fatty acid in PG molecules is a third lipid characteristic peculiar to the thylakoid membranes. The possible role of PG molecular species containing this unusual fatty acid is not fully elucidated but it has been implicated in two aspects, namely the supramolecular organization of the oligomeric form of LHC-2 and the phenomenon of chilling sensitivity in plants. A number of experiments (Remy *et al.*, 1982; Tremolieres *et al.*, 1984) have suggested that PG containing *trans*- Δ^3 -hexadecenoic acid is specifically associated with LHC-2 and is implicated in the formation of the oligomeric form of this protein complex. In addition, taking into consideration the complementarity of the fatty acid double bond with the double bond of the phytol of chlorophyll (Foley & Harwood, 1982) it has been suggested that this lipid molecular species could have an orientating effect on some chlorophyll molecules associated with the protein, thus permitting a more efficient excitation energy transfer between subunits (Tremolieres *et al.*, 1984).

The phenomenon of chilling sensitivity of some plant species has received considerable attention over the years. Lyons and Raison and their co-workers have suggested (for review see Quinn & Williams, 1978) that the differences in the fluidity of the chloroplast membranes between chilling-resistant and chilling-sensitive plants is one of the major factors preventing membrane damage in the resistant species. These suggestions, however, have been criticised (Bishop *et al.*, 1979) as far as the thylakoid membranes are concerned since no gel phase domain is formed at temperatures resulting in chilling injury in the sensitive plants. Recently, however, Murata and co-workers (Murata *et al.*, 1982; Murata, 1983; Murata & Yamaya, 1984) have correlated chilling sensitivity to the relative amounts of certain PG species. They have postulated (Murata & Kurisu, 1984) that chilling-sensitive plants contain higher proportions of PG containing the *trans*- Δ^3 -fatty acid and palmitate than do the resistant plants. Thus in the sensitive plants the PG molecular species induce phase

transitions which trigger the processes of chilling injury. This theory was supported by recent work by Roughan (1985) who examined a very large number of plants and found only a few exceptions to chilling sensitivity as predicted by thylakoid PG composition.

The levels of *trans*- Δ^3 -hexadecenoic acid (Burke *et al.*, 1982), the fatty acyl chain unsaturation (Blein, 1980; Pillai & St John, 1981; Burke *et al.*, 1982) and lipid class composition (Pillai & St John, 1981) of thylakoids have been also implicated with herbicide resistance of some plant species. Unequivocal conclusions have not been reached and more recently Chapman *et al.* (1984) and Rawlyer *et al.* (1986) have reported that no obvious differences were found between the lipid composition and characteristics of triazine-resistant or -susceptible species. It remains possible that the resistance to herbicides, as concluded by Chapman *et al.* (1984), reflects no changes in the lipids but only alterations in protein structure (Hirschberg & McIntosh, 1983). However, it should be noted that specific lipid requirements could be satisfied by very small quantities of lipids such that the whole lipid composition need not necessarily appear affected.

Lipid-protein interactions

In all biomembrane systems, a number of extrinsic proteins and, by definition, all intrinsic protein complexes must interact in some way with the lipid matrix, the interactions being either electrostatic, hydrophobic or steric.

The majority of the thylakoid lipids are electroneutral galactolipids and thus no electrostatic interactions between these lipids and proteins can be expected. The only lipids which could, if their charge was exposed to the surface, participate in such types of interactions are SQDG and PG, which are negatively charged at all physiological pH values. There is no evidence to date, however, to suggest that in the thylakoid membranes any extrinsic protein is associated with the membrane through direct interactions with these lipids.

Acidic lipids could however interact and thus stabilize exposed, charged, portions of intrinsic proteins. All intrinsic proteins interact with lipids and although some phenomena are related to membrane lipids in general, others are specific in terms of their lipid requirement. In the latter case, specificity could be expressed either in terms of polar head groups or fatty acyl chains.

A non-specific effect is the stabilization of protein conformation by lipids. This is by no means a new concept and it has been observed in a variety of membrane proteins (Sandermann, 1978; Cocucci & Ballarin-Denti, 1981; Jolliot *et al.*, 1982). The importance of a defined hydrophilic to lipophilic balance in the lipid molecules for the maintenance of protein conformation has been suggested (Bohnenberger & Sandermann, 1983).

As discussed in an earlier section, the ability of membrane lipids to adopt non-bilayer configurations *in vitro* contrasts with their function in biological membranes. There have been observations, however, that conditions which tend to destabilize lipid-protein interactions tend to induce the formation of such structures in the membrane (Gounaris *et al.*, 1983e; Gounaris *et al.*, 1984b; Machold *et al.*, 1977). Such observations have led to the suggestion that perhaps MGDG facilitates the incorporation and stabilization of proteins into the bilayer of the thylakoids (Quinn &

Williams, 1983; Williams *et al.*, 1984) so that its non-bilayer configuration is not observed in the membrane under physiological conditions.

Lipids have been found to be associated with protein complexes isolated from the thylakoid membranes by means of detergents (Rawlyer *et al.*, 1980; Tremolieres *et al.*, 1981; Remy *et al.*, 1982; Siefermann-Harms *et al.*, 1982; Gounaris & Barber, 1985). Although it remains possible that such associations reflect a specific detergent effect, insight could be provided into the association *in vivo* of these proteins with the lipids.

Immunological studies in which lipid antibodies have been used to inhibit functional processes have also been reported (Radunz, 1981; Radunz *et al.*, 1984). Another approach used to determine whether lipids are involved in the function of the thylakoids has been the treatment of the membranes with phospholipases (Rawlyer & Siegenthaler, 1981a,b; Siegenthaler *et al.*, 1984; Krupa, 1983; Jordon *et al.*, 1983). Such studies, however, have not been absolutely conclusive in assigning a functional role to a specific lipid.

Reconstitution of protein complexes, isolated from thylakoids with detergents, into lipid matrices has proved valuable for the identification of lipid requirement and specificity. It should, however, be emphasized that intrinsic membrane complexes copurify from the membrane with small amounts of endogenous lipids. Thus, although the reconstitution experiments are carried out with defined lipid compositions, the effect of the endogenous lipid remaining should be considered.

Reconstitution studies showed that restoration of energy transfer from purified LHC-2 to the PS2 complexes specifically requires the presence of MGDG in the lipid mixture (Siefermann-Harms *et al.*, 1982). Studies in which lipids were added to PS2-enriched membranes which had been delipidated by the action of the detergent Triton X-100 showed that addition of total lipid extract enhanced the oxygen-evolving capability of the membranes (Gounaris *et al.*, 1983b; Akabori *et al.*, 1984).

Restoration of the activity of the cytochrome b_6-f complex was achieved by reconstitution into thylakoid lipids (Hurt & Hauska, 1981). The restoration was dependent on thylakoid lipids with lecithins being ineffective, although no lipid class specificity was indicated. The potential of the cytochrome b_{559} of the thylakoids has been shown to depend on lipids. Reconstitution of preparations in which the cytochrome was in a low potential with thylakoid lipids restored it in a high potential (Butler & Matsuda, 1983; Whitford *et al.*, 1984).

Another intrinsic membrane protein which has been shown to exhibit specific lipid requirements for activity is the chlorophyllase enzyme (Terpstra & Lambers, 1983a,b; Lambers *et al.*, 1984).

Perhaps the most detailed examination of lipid requirements for thylakoid enzymes has been with the CF₀-CF₁ ATP synthase complex. In a series of experiments (Pick *et al.*, 1984, 1985; Gounaris *et al.*, 1984c) the differential effects of individual lipids on the activity of the complex was demonstrated. Effects were shown on the kinetics of both the ATP hydrolysis and P_i-ATP exchange. MGDG was essential for recovering the activity and it was required to be present in the reconstituting mixture in the naturally occurring ratio. In addition, its acyl unsaturation was critical. The presence

of small amounts of charged lipids (10%) in the lipid mixture was required for optimal activity, higher levels inhibiting the preparation. An important observation was the strong association of the enzyme complex with SQDG (Pick *et al.*, 1985). It was found that small amounts of this lipid co-purified with the enzyme and were not exchangeable. Removal of SQDG from the protein complex rendered it inactive. Thus it was proposed that SQDG is an integral part of the complex.

Conclusions

It will be apparent that lipids may play other roles in thylakoids besides the simple provision of a semi-permeable matrix. The unique and highly conserved lipid composition of thylakoid membrane has always suggested that specific roles may be found for the integration and organization of membrane protein complexes as well as their enzyme activity. Now that we are gaining knowledge of the detailed structure and location of many thylakoid proteins, further advances will come from experiments on specific lipid interactions with different membrane components. At present, our understanding of the molecular organization of the thylakoid is limited by a relative absence of experimental evidence concerning such interactions.

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