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Change in the molecular organization of monogalactosyldiacylglycerol between resting and functioning thylakoid membranes. Involvement of the CF_0/CF_1-ATP synthetase

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The purpose of this investigation was to determine whether the molecular organization of thylakoid lipids, particularly that of monogalactosyldiacylglycerol (MGDG), was different in thylakoid membranes incubated under resting and functioning conditions. The transmembrane distribution and the initial hydrolysis rate of MGDG, both determined using the lipase from *Rhizopus arrhizus*, were selected as indicators for changes occurring in the lipid organization of spinach thylakoids. The transmembrane distribution of MGDG was 62% outside:38% inside in resting membranes (darkness) as well as in membranes incubated under conditions of coupled ($ADP + P_i$) or uncoupled (NH_4Cl) non-cyclic H_2O to ferricyanide electron flow in the presence of bovine serum albumin to remove breakdown products. Complete MGDG depletion in the outer monolayer (corresponding to about 34% of the total thylakoid lipids) did not affect greatly the electron flow activities. Compared to the control in the dark, the initial hydrolysis rate of MGDG by the lipase did not change when thylakoid membranes were incubated in the light under conditions of basal, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-inhibited H_2O to methylviologen electron flow. On the other hand, MGDG hydrolysis was markedly accelerated when the membranes were allowed to work under photophosphorylating ($ADP + P_i$) conditions. Pretreatment of thylakoids with 2 M NaBr (CF_1 removal), *N,N'*-dicyclohexylcarbodiimide (DCCD) (CF_0 block) and phlorizin (CF_1 block) strongly decreased the hydrolysis rate of MGDG below that of the control in the dark. Under coupled conditions of electron flow, the fast hydrolysis rate of MGDG could be reduced within 5 s by switching off the light or by adding DCMU or FCCP. The hydrolytic activity of the lipase from *Rh. arrhizus* toward MGDG was also shown to be decreased either by cholesterol enrichment or by progressive catalytic hydrogenation of thylakoid membranes. It is concluded that the hydrolysis rate of MGDG by the lipase likely reflects the lateral packing pressure of this lipid, e.g., increased hydrolysis rate is associated with a decreasing packing. The above results are therefore interpreted and discussed in terms of reversible changes in MGDG packing in the outer monolayer of thylakoid membranes that are dependent on the functional status of the coupling factor CF_0/CF_1.

Introduction

During each day of their life span in plants, thylakoid membranes are facing two alternate conditions they must adapt to. In the dark, these membranes are at rest and in this respect, they do not differ from any other biological membrane. In the light, however, thylakoids become energy-transducing membranes and, as such, they house a number of functional processes and structural changes. The functional events which follow light

Abbreviations: CF_0 and CF_1, membrane sector and catalytic moiety of the thylakoid coupling factor; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; MGDG, monogalactosyldiacylglycerol; Mops, 4-morpholinepropane sulphonic acid; P_i, inorganic phosphate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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irradiation of thylakoid membranes have been extensively studied and constitute by far the major body of our present knowledge on photosynthesis. On the other hand, what we know about the molecular organization of thylakoid membranes comes mainly from experiments carried out in the dark, so that our understanding of the structural changes occurring in the thylakoid membrane at work is still very limited.

This is especially true for acyl lipids. Presently, only very indirect evidence for possible light-induced changes in their molecular organization has been reported [1–3], but the molecular basis of such changes is still unknown. However, if such changes effectively occur, one would expect MGDG to be primarily concerned. Indeed, its polymorphic behaviour (in the presence of other thylakoid lipids) can be modulated by several factors [4,5], among which the proton and divalent cation levels are of immediate interest because of their light-induced concentration changes on both membrane sides [6]. The particular role attributed to MGDG in the packaging of membrane proteins [7], together with the demonstration that MGDG is essential for ATP-synthetase and ATP-P_i exchange activities [8–10], also suggest that if particular lipid–protein interactions become altered upon switching on electron flow and related activities, changes in the molecular organization of MGDG may then occur.

Consequently, we have come to the problem of the light-induced changes in MGDG organization by asking the following questions: (1) What technique can be used to allow us to interpret the results on a molecular basis? (2) How much of the total MGDG content of thylakoid membranes can take part in such changes? (3) Is there any factor or condition which is (more) specifically involved in the putative light-induced changes in MGDG organization? (4) Can we estimate the kinetics of such changes?

The adopted method was to incubate resting or working membranes in the presence of the lipase from *R. arrhizus* and to determine whether and how (a) the transmembrane distribution of MGDG and (b) the initial hydrolysis rate of MGDG were affected by the functional state of thylakoid membranes. Our results show that changes in the molecular organization of MGDG do occur in the working thylakoid membrane. These changes can be interpreted in terms of packing of MGDG molecules and are primarily dependent on the functional state of the CF_0/CF_1-ATP synthetase. The physiological implications of these changes are discussed with respect to the lateral diffusion processes which take place in the working thylakoid membrane.

Materials and Methods

The lipase from *R. arrhizus* (LRa) (EC 3.1.1.3) was purchased from Boehringer (Mannheim, F.R.G.) and used without further purification. Bovine serum albumin was defatted as described elsewhere [11]. All other chemicals were high-purity products from Fluka (Buchs, Switzerland). Silicagel 60-coated glass plates were from Merck (Darmstadt, F.R.G.). The palladium-di(sodium alizarine monosulphonate) catalyst was obtained from Molecular Probes, OR, U.S.A.

Preparation of thylakoid membranes. Spinach (grown in phytotron) leaves (50–80 g) were homogenized with 200–320 ml of ice-cold 300 mM sorbitol/1 mM $MgCl_2$/0.1% (w/v) bovine serum albumin/25 mM Tricine-KOH (pH 8) in a Waring blender. The resulting mixture was passed through four cheesecloth layers and one fine mesh nylon gauze. The filtrate was centrifuged for 2 min at $1800 \times g$ in a Sorvall HL-4 swinging bucket rotor. The pellets (consisting essentially of intact chloroplasts) were carefully resuspended in the homogenizing medium and centrifuged as above. The chloroplasts pellets were then suspended in distilled water (15–20 s) then further diluted with an equal volume of a double-strength medium (600 mM sorbitol/0.2% bovine serum albumin/50 mM Tricine-KOH (pH 8)). Thylakoid membranes were pelleted for 1.5 min at $6000 \times g$ and washed once more in SMT buffer (300 mM sorbitol/5 mM $MgCl_2$/10 mM Tricine-NaOH, pH 8).

Preparation of CF_1-depleted membranes. The procedure described by Nelson (1980) was slightly modified [12]. To thylakoid membranes (2 mg chlorophyll/ml) in 400 mM sucrose/10 mM NaCl/10 mM Tricine-NaOH (pH 8), an equal volume of 4 M NaBr was added slowly and the mixture was incubated on melting ice for 30 min in the dark. After 1 : 1 dilution with cold water, the suspension was centrifuged for 10 min at $20\,000 \times g$. Pellets were washed once more with SMT, then resuspended in SMT at 1 mg chlorophyll/ml.

Preparation of DCCD-treated membranes. The procedure used was derived from that of McCarty [13]. To thylakoid membranes (2 mg chlorophyll/ml) in SMT, DCCD (from a 100 mM stock in ethanol) was slowly added to obtain a final concentration of 2 mM. Following 15 min of incubation under slow stirring on melting ice, the suspension was diluted ten times with SMT and centrifuged for 5 min at $8000 \times g$. DCCD-treated membranes were then resuspended in SMT at 1 mg chlorophyll/ml.

Transmembrane distribution of MGDG under various functional conditions. The incubation medium (25 ml), containing 200 mM sorbitol/50 mM KCl/5 mM $MgCl_2$/1 mM $CaCl_2$/10 mM KH_2PO_4/10 mM $K_3Fe(CN)_6$/bovine serum albumin (20 mg/ml)/25 mM Tricine-NaOH (pH 8) and freshly isolated thylakoid membranes (0.1 mg chlorophyll/ml), was introduced and deoxygenated by N_2 bubbling in a magnetically stirring, thermostated cuvette (15 °C) which was fitted with a Clark O_2 electrode. After 3 min of equilibration in the dark, red light (30 mW/cm^2) was provided and

30 s later, the lipase was added (100 U/mg chlorophyll). O_2 evolution was simultaneously recorded. Three conditions were studied: darkness (which served as a control), uncoupled and coupled H_2O to ferricyanide electron flows. For the last two conditions, the incubation medium was supplemented with 10 mM NH_4Cl (uncoupled) or with 6 mM ADP (coupled), respectively.

Samples (1.6 ml) were withdrawn from the incubation medium every 60 s over a 12 min period and injected into extraction tubes containing 4 ml chloroform/methanol (53 : 37) and 0.64 ml 3 M KCl. It was checked that solvent extraction immediately stopped the lipase action [14]. After vigorous vortexing, the tubes were centrifuged 5 min at $1500 \times g$.

From the upper phase, 0.8 ml were taken, diluted with 2.4 ml water and the absorbance of residual ferricyanide was determined at 420 nm. This additional way of determining electron flow activity was necessary because the recording capacity of the Clark O_2 electrode system was exhausted already after 5–6 min of incubation.

The lower phase was quantitatively removed from the extraction tube with a syringe, evaporated to dryness under N_2 and lipids were dissolved in 300 μl chloroform/methanol (8 : 2) containing 0.01% (w/v) butylated hydroxytoluene as antioxidant. A 10 μl aliquot was removed for chlorophyll determination and the remaining 290 μl were quantitatively applied to TLC plates, using a Linomat IV automatic sample applicator (Camag, Muttenz, Switzerland).

Initial hydrolysis rate of MGDG under various functional conditions. The basic incubation medium was 300 mM sorbitol/3.5 mM $MgCl_2$/5 mM NaN_3/0.15 mM methylviologen/10 mM Tricine-NaOH (pH 8) and thylakoid membranes (0.5 mg chlorophyll/ml). When required, this medium was supplemented with either 3 mM ADP + 3 mM KH_2PO_4, 10 μM FCCP, 20 μM DCMU, 3 mM phlorizin, 3 mM ATP or cholesterol (2 μmol/mg chlorophyll). In this latter case, cholesterol was injected in the medium according to the method of Batzri and Korn [15] prior to the addition of membranes.

Incubations took place in the above cuvette (20 °C). After a 3 min equilibration period in the dark, white light was provided on three sides of the cuvette (270 mW/cm^2) and 30 s later, the lipase was added (16 U/mg chlorophyll). Samples (0.84 ml) were taken every 10 s over a 1 min period and injected into tubes containing 1.5 ml chloroform/methanol (53 : 37). After vigorous vortexing, the tubes were centrifuged as above. From the lower phase, a 30 μl aliquot was removed for chlorophyll determination and 500 μl were spotted on TLC plates.

Lipid separation and analyses. TLC plates were developed in acetone/hexane/acetic acid (80 : 60 : 4), sprayed lightly with 0.01% primuline in acetone/H_2O (1 : 1) and viewed under UV light. MGDG zones were scraped into tubes and assayed for galactose content [14]. Results were normalized to the chlorophyll content of each sample. Chlorophyll was determined according to Bruinsma [16]. Cholesterol was determined according to Rudel and Morris [17].

Continuous spectrophotometric assay for lipid hydrolysis. Fixed-time assays such as those described above are not very suitable for the estimation of rapid changes of hydrolysis rates. We have developed a simple continuous spectrophotometric procedure to follow lipid hydrolysis in liposomes and in membranes, which will be described in detail elsewhere (manuscript in preparation). It is based upon the metachromatic properties of the cationic dye safranine, leading to absorbance changes when the dye associates electrostatically with vicinal negative charges [18]. Briefly, 2.5–3 ml of a mixture consisting of 300 mM sorbitol/1 mM $CaCl_2$/50–70 μM safranine/20 mM Tricine-NaOH (pH 8) and thylakoid membranes (15–40 μg chlorophyll/ml) were equilibrated in the dark in the thermostated (20 °C) compartment of an Aminco DW-2a dual-wavelength spectrophotometer (see legends of the figures for additional details). When required, actinic light could be provided from the side illumination accessory through a Kodak 70 red filter (44 mW/cm^2 at the cuvette level) and the photomultiplier was shielded by a suitable combination of Schott green filters. When the lipase was added to the stirred content of the cuvette (16–20 U/mg chlorophyll), safranine reacted with the free fatty acids formed so that the change in ΔA (520 versus 554 nm) reflected the apparent initial rate of lipid hydrolysis (mostly that of MGDG). Similar experiments were carried out with thylakoid membranes hydrogenated in situ at 0 °C using the homogenous catalyst, palladium-di(sodium alizarine monosulphonate) [19].

Results

Transmembrane distribution of MGDG under various functional conditions

The experiments described here had to meet two requirements: (1) the hydrolysis pattern of MGDG should provide the necessary information from which MGDG asymmetry will be determined and (2) MGDG degradation should be carried out under conditions which will preserve electron transport activities during the incubation period necessary to fulfill the first requirement. To this end, we have used a high lipase/membrane ratio (100 U/mg chlorophyll) so as to shorten the time required in condition (1), and a high bovine serum albumin/membrane ratio (up to 200 mg/mg chlorophyll) so as to remove hydrolysis products (free fatty acids and lyso-galactolipids) with the maximum efficiency, therefore satisfying condition (2).

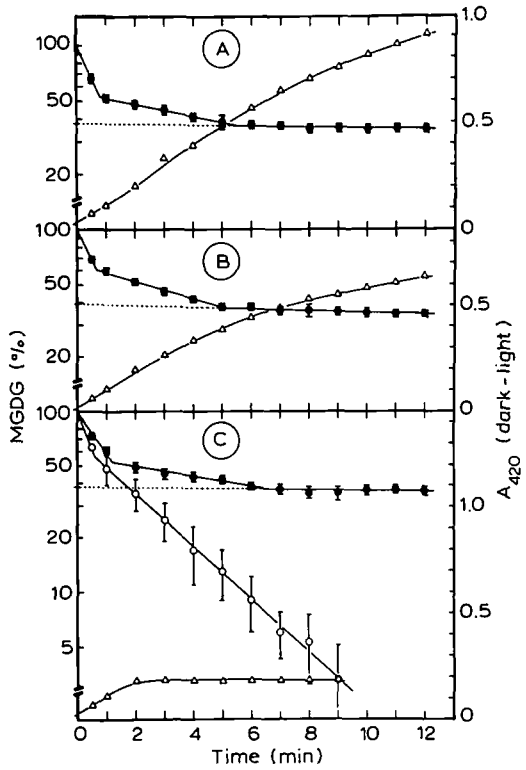

Fig. 1. Simultaneous time-courses of MGDG hydrolysis and of ferricyanide photoreduction in thylakoid membranes treated with the lipase from *R. arrhizus*. MGDG hydrolysis (%) was measured in the presence (●) or in the absence (○) of bovine serum albumin. Ferricyanide photoreduction (△) was expressed here as the absorbance difference between controls in the dark and illuminated samples. Incubations were carried out in the light under (A) coupled ($n = 4$) and (B) uncoupled ($n = 2$) conditions. In (C), MGDG hydrolysis was carried out in the dark in the presence of bovine serum albumin (●) ($n = 4$) and served as a control for (A) and (B); moreover, MGDG hydrolysis data from dark ($n = 3$) and light ($n = 3$) samples in the absence of bovine serum albumin were averaged (○). Coupled electron flow (△) was also determined in the absence of bovine serum albumin. Note the logarithmic scale on the left ordinate (MGDG hydrolysis) and the linear scale on the right one (electron flow activity).

The hydrolysis patterns of MGDG by the lipase from *R. arrhizus* in thylakoid membranes incubated at 15°C in the dark or under conditions of coupled and uncoupled H_2O/ferricyanide electron transports, together with the corresponding progress curves of electron flow activity are presented in Fig. 1. The hydrolytic behaviour of MGDG was characterized by three phases of decreasing slopes. The extrapolation of the last (quasi-horizontal) phase to zero-time has been shown to correspond to the outer/inner distribution ratio [14]. The present results clearly indicate that the transversal distribution of MGDG in thylakoid membranes working under conditions of coupled (Fig. 1A) or uncoupled (Fig. 1B) electron transport was the same as in resting membranes (Fig. 1C), and corresponded to about 62 : 38. The importance of bovine serum albumin in the above incubation conditions is illustrated in Fig. 1C. At the high lipase/membrane ratio used here, the absence of albumin during the lipolytic treatment led to an essentially complete degradation of MGDG. In this case, the hydrolysis pattern was almost monophasic, suggesting that the fast accumulation of large amounts of breakdown products drastically altered the membrane structure.

Since MGDG accounts for about 55% of the total thylakoid acyl lipids [14], it follows that the non-lytic hydrolysis of 62% of the total MGDG under the conditions of Fig. 1 corresponds to a 34% depletion of thylakoid membrane lipids. The influence of such a MGDG depletion on electron transport activities is also shown in Fig. 1. The initial electron flow rates were about 100–140 μmol O_2 evolved/mg chlorophyll per h. Both coupled and uncoupled electron transport were fully active up to the beginning of the hydrolysis plateaus (i.e., up to 5–6 min of lipase treatment) and only then began to decrease slowly during the next 6–7 min. However, the coupled activities (Fig. 1A) were always much less affected by MGDG depletion than the uncoupled ones (Fig. 1B) although the extents of MGDG degradation were similar in both cases. On the other hand, electron flow activity was completely abolished after 2 min of lipase treatment when bovine serum albumin was omitted from the incubation medium (Fig. 1C, open symbols). It should be pointed out that during these short incubations in the presence of albumin, MGDG was the only lipid to be significantly hydrolyzed, whilst the degradation of other thylakoid lipids (digalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, phosphatidylglycerol and phosphatidylcholine) was very small (data not shown).

Hydrolysis rate of MGDG under various functional conditions

Earlier works have suggested that upon switching on the light, several changes in the general thylakoid membrane organization occurred, among which membrane thinning [1,2] may be of great interest. Indeed, it is generally considered that thinning of a bilayer is associated with lateral expansion, therefore leading to a decreased packing of lipid molecules in this bilayer [20]. The sensitivity of many lipolytic enzymes toward the packing pressure of their lipid substrate is well-documented [21,22]. Provided that the lipase from *R. arrhizus* is also sensitive to lipid packing, this enzyme can be used to probe relative changes in the packing pressure of its main substrate MGDG in thylakoid membranes. Figs. 2 and 3 show that the hydrolysis rates of MGDG by this lipase were markedly diminished in thylakoid membranes which were either catalytically hydrogenated according to Vigh et al. [19] (Fig. 2) or enriched in cholesterol (Fig. 3) before being submitted to the lipolytic treatment. Results similar to those of Fig. 3 were obtained when the safranine assay was used instead

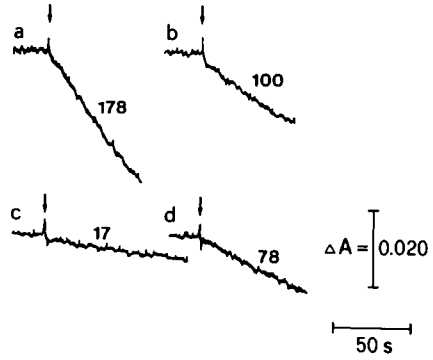

Fig. 2. Effect of catalytic hydrogenation of thylakoid membranes on the apparent hydrolysis rate of MGDG by the lipase from *R. arrhizus*. Catalytically hydrogenated membranes were prepared as follows. Thylakoids (60 μg chlorophyll/ml) were suspended in 300 mM sorbitol/10 mM NaCl/10 mM Tricine-KOH (pH 7.5) and hydrogenated for 2 min at 0 °C in the dark under 1 atm H_2 [19]. Membranes were then washed in the same medium and submitted to the lipolytic treatment. The assay cuvette contained 300 mM sorbitol/1 mM $CaCl_2$/20 mM Tricine-NaOH (pH 8)/50 μM safranine/17 μg chlorophyll/ml in a total vol. of 3 ml; the lipase was added (see arrows) at 20 U/mg chlorophyll. (a) control membranes; (b) control membranes with inactive catalyst (150 μg/ml); (c) membranes hydrogenated in the presence of activated catalyst at 150 μg/ml and (d) at 75 μg/ml. Linolenic acid, which represents about 75% of the total thylakoid acyl chains, was reduced by 90% in (c) and by 40% in (d), as indicated by gas-chromatographic analysis. The slopes of the absorbance change are expressed in % with respect to that of condition (b), taken as 100%.

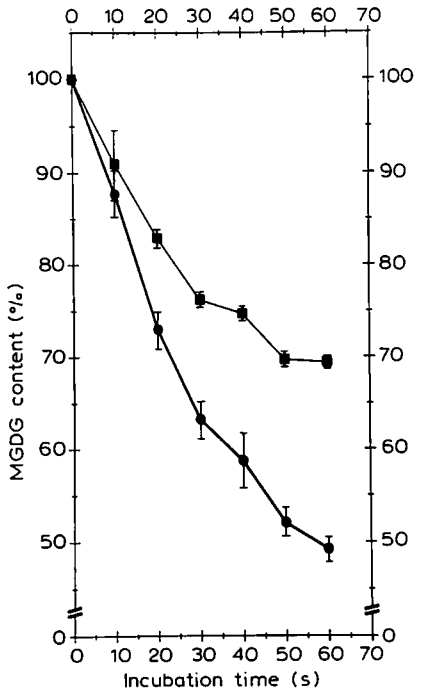

Fig. 3. Effect of cholesterol incorporation into thylakoid membranes on the initial rate of MGDG hydrolysis by the lipase from *R. arrhizus*. Preincubation of thylakoid membranes with cholesterol (2 μmol/mg chlorophyll) resulted in a cholesterol incorporation of about 1 μmol/mg chlorophyll at the membrane level. Thylakoids were treated with the lipase under coupled conditions (light, ADP+P_i) in the presence (■) ($n = 2$) and in the absence (●) ($n = 7$) of cholesterol.

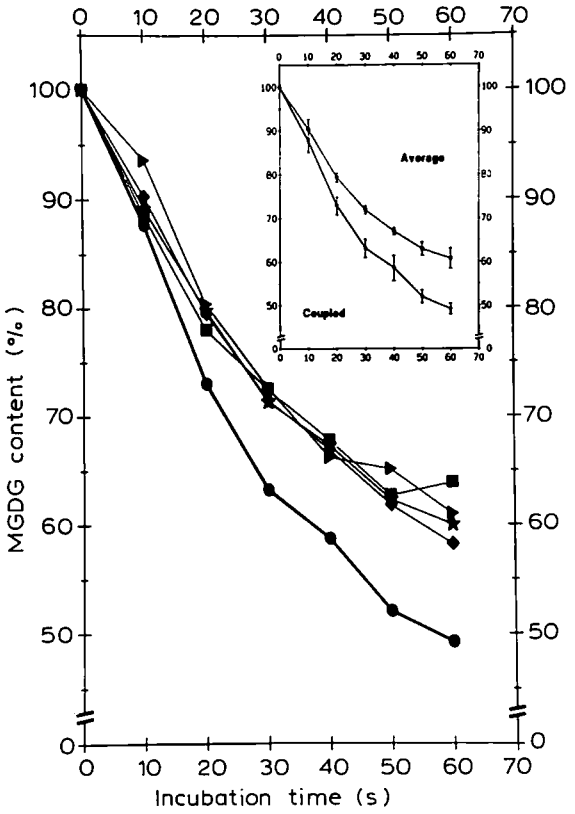

Fig. 4. Hydrolysis of MGDG by the lipase from *R. arrhizus* in thylakoid membranes submitted to various resting and working conditions. (★), dark control ($n = 4$); (■), light + DCMU ($n = 4$); (◆), basal electron flow ($n = 8$); (▶), FCCP-uncoupled electron flow ($n = 3$); (●), coupled electron flow (ADP + P_i) ($n = 7$). For sake of clarity, error bars have been omitted from the individual curves but are represented, together with the average of the first four conditions ($n = 19$) in the inset. See Materials and Methods for additional details.

of the thin-layer chromatographic analysis (data not shown).

The hydrolysis of MGDG by the lipase of *R. arrhizus* in thylakoid membranes submitted to two 'resting' conditions (darkness, light + DCMU) and to three 'working' conditions (basal, FCCP-uncoupled and coupled non-cyclic H_2O to methylviologen electron flows) is depicted in Fig. 4. It can be seen that when thylakoid membranes were incubated in the light under conditions of inhibited electron transport (DCMU) or of basal and FCCP-uncoupled electron transport, their respective MGDG hydrolysis patterns were identical (within experimental error) to that of dark-incubated membranes. About 40% of the total MGDG was degraded in 1 min in each case and the average initial hydrolysis rate was 900 nmol/min. On the other hand, MGDG hydrolysis was significantly increased in photophosphorylating thylakoids, as shown by its extent (50%) and its initial rate (1230 nmol/min). Under all these conditions, it has been ascertained that thylakoid functions were essentially unaltered after the 1 min incubation periods (data not shown, but see Fig. 1C). When the hydrolysis was carried out either in the light (basal

electron flow) or in the dark, but both in the presence of exogenous ATP (3 mM), the degradation extents of MGDG were similar to those measured in the absence of ATP. In this case, the residual MGDG after 1 min of hydrolysis varied from 58.2 to 60.6%.

Altogether, these results point to a possible involvement of the coupling factor (CF_0/CF_1) in enhancing the extent and the rate of MGDG hydrolysis. As shown by Kamienietzky and Nelson [23], NaBr treatment of thylakoids leads to a complete removal of CF_1 from the membranes whilst still preserving their electron flow capacity. Our preparations of NaBr-treated membranes were characterized by an unchanged MGDG content (as compared to fresh membranes) and when submitted to the lipolytic treatment in the light and in the presence of ADP + P_i, the resulting hydrolysis rate of MGDG (Fig. 5) was markedly decreased as compared to that of untreated membranes, be it under conditions of coupled electron transport or of darkness (Fig. 4). An even slower degradation of MGDG by the lipase from *R. arrhizus* was observed when fresh membranes were treated with either DCCD or phlorizin (Fig. 5), two compounds which block the proton channel (CF_0) and the ATP synthesis machinery (at the level of CF_1), respectively [13].

The observed decrease in MGDG hydrolysis upon treatment of membranes with DCCD and phlorizin may

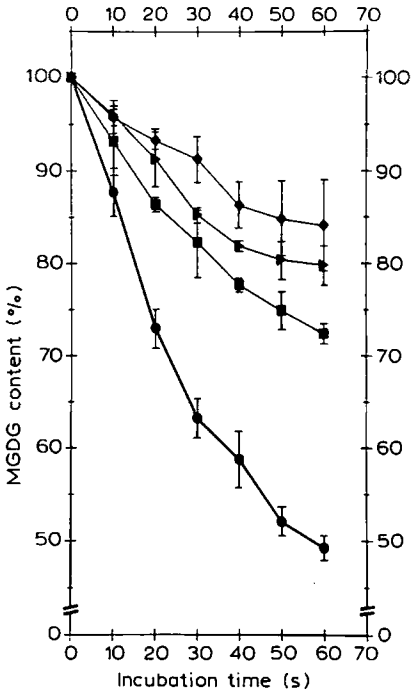

Fig. 5. Hydrolysis of MGDG by the lipase from *R. arrhizus* in thylakoid membranes submitted to treatments which specifically affect the coupling factor. In all cases, incubation was carried out in the light and in the presence of ADP + P_i. (●), control, photophosphorylating membranes ($n = 7$); (■), NaBr-treated membranes ($n = 3$); (▶), DCCD-treated membranes ($n = 3$); (◆), phlorizin-treated membranes ($n = 3$). See Materials and Methods for additional details.

TABLE I

Effect of FCCP, DCMU, DCCD and phlorizin on the activity of the lipase from Rhizopus arrhizus incubated with liposomes

Total lipids (including pigments) were extracted from fresh thylakoid membranes; the organic solvents were evaporated with N_2 gas and lipids were taken up in ethanol. Chlorophyll concentration was adjusted to 4 mg/ml and 1 ml of this solution was injected [15] into 99 ml of 300 mM sorbitol, 10 mM Tricine/NaOH (pH 8) and 50 μM safranine so as to obtain 40 μg chlorophyll/ml and 1% ethanol, FCCP, DCMU, DCCD or phlorizin (in ethanol) was added to 2.5 ml of the above medium to reach the following ratios (μmol compound per mg chlorophyll): 0.02, 0.04, 1 and 6, respectively. The temperature was 20 °C and the experiment was carried out in the dark. The lipase was then added (16 units/mg chlorophyll) and the change in A (520 vs. 554 nm) was recorded. The (apparent) initial rate (ΔA/min) and the amplitude (ΔA) of the hydrolysis signal after 1 min were calculated from the recordings. Values are the average of 3 assays ± S.D.

Addition	Initial rate	Amplitude
None	0.398 ± 0.056	0.205 ± 0.014
FCCP	0.388 ± 0.036	0.204 ± 0.009
DCMU	0.426 ± 0.062	0.208 ± 0.014
DCCD	0.421 ± 0.060	0.251 ± 0.012
Phlorizin	0.371 ± 0.010	0.197 ± 0.007

be due to a direct inhibitory effect of these compounds on the lipase itself and/or to a direct interaction of these rather hydrophobic substances with membrane lipids, thereby decreasing their affinity for the lipase. This has been checked by incubating liposomes made of total thylakoid lipids (including pigments) with DCCD, phlorizin, and also FCCP and DCMU at a compound/chlorophyll ratio identical to those used for Figs. 4 and 5. The apparent hydrolysis rate was determined by the spectrophotometric assay with safranine. Table I clearly shows that none of these four compounds has any effect on both lipid hydrolysis rate and extent. This indicates that the decreased MGDG hydrolysis in Fig. 5 can be assigned to more specific membrane alterations.

Light-induced swelling of thylakoids under photophosphorylation conditions could be a trivial explanation of results in Fig. 4. Although shrinkage rather than swelling was observed under these conditions [24], we have preequilibrated thylakoid membranes in sorbitol concentrations ranging from 0 to 600 mM, then determined the apparent hydrolysis rate of MGDG using the safranine assay. At low sorbitol concentrations (0–20 mM), the rate was slow, but increased 2–3 fold to reach a maximum and essentially constant value between 150 and 600 mM sorbitol (data not shown).

In all the experiments above, actinic light was provided 30 s before addition of the lipase (see Materials and Methods), so that thylakoid membranes were in a steady-state with respect to electron transport activity. Any change in MGDG organization (e.g., from dark to coupled conditions, see Fig. 4) must have therefore taken place during this 30 s period of time. How fast

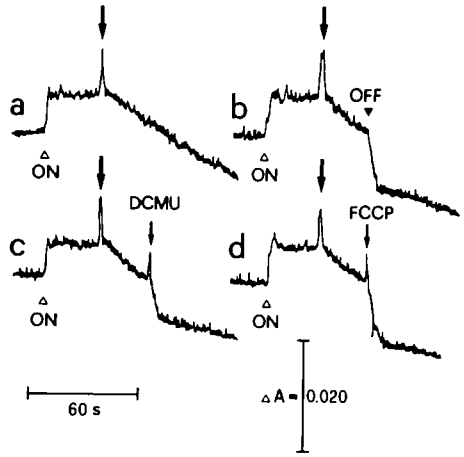

Fig. 6. Apparent hydrolysis rates of MGDG by the lipase from *R. arrhizus* in thylakoid membranes submitted to a working-to-resting transition and the corresponding effects of DCMU and FCCP. The safranine assay cuvette contained 300 mM sorbitol, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM ADP, 75 μM methylviologen, 75 μM safranine, 20 mM Tricine-NaOH (pH 8) and 40 μg chlorophyll/ml in a total volume of 2.5 ml. Temperature was 20 °C. The lipase was added (see large arrows) at 16 units/mg chlorophyll. Perturbations were introduced 25 s after addition of the lipase. (a), control; (b), light-to-dark transition; (c) effect of DCMU (1.6 μM); (d), effect of FCCP (0.8 μM). Special care was taken to avoid any influence of the actinic light on the photomultiplier. See Materials and Methods for additional details.

actually are these changes? We have attempted to answer this question by measuring the change in MGDG hydrolysis rate upon a light-to-dark transition or after addition of DCMU or FCCP to photophosphorylating membranes, using the safranine assay. The results are shown in Fig. 6. When thylakoid membranes, incubated in the presence of ADP + P_i, were illuminated, a rapid absorbance rise first occurred, which immediately stabilized (Fig. 6A). This light-induced absorbance change is likely attributable to secondary ion redistribution [6], because it can be fully reversed by DCMU, FCCP or darkness. After addition of the lipase from *R. arrhizus,* the signal immediately began to decrease, reflecting the apparent hydrolysis of MGDG in coupled membranes (Fig. 6A). Under the same conditions, switching off the light (Fig. 6B), adding DCMU (Fig. 6C) or FCCP (Fig. 6D) while the light was still on, resulted in a rapid deflection (corresponding to ion reequilibration), followed by a MGDG hydrolysis rate which was markedly slower than that observed upon addition of the lipase. Although the rapid absorbance variation due to ion reequilibration obscured somewhat the beginning of the slower hydrolytic phase, it can be concluded that the changes in MGDG organization reported here occurred within about 5 s (upper limit).

Discussion

The first part of this study deals with the transmembrane distribution of MGDG in resting and functioning thylakoid membranes. This is in no way a trivial point. Indeed, changes in phospholipid accessibility to phospholipases were reported to occur upon energization of *Acholeplasma laidlawii* cells by glucose [25]. More recently, an accumulation of phosphatidylserine in the outer leaflet of the erythrocyte membrane was obtained after ATP depletion together with disturbance of the interaction between cytoskeleton and the inner monolayer [26]. If such changes in lipid accessibility and/or asymmetry take place in these relatively simple membrane systems by altering the adenylate energy charge, it was sensible to look for possible changes in lipid asymmetry between the resting and the energized state of thylakoid membranes. It is clear, however, that the transmembrane MGDG distribution in thylakoids functioning under both coupled or uncoupled conditions of electron transport (Figs. 1A and B) was the same as in resting membranes (Fig. 1C), with an outside/inside molar ratio of 62 : 38, in agreement with earlier findings [14,27]. Although it can be speculated that the stability of MGDG asymmetry is an essential requirement for several membrane functions [7,10], it is worth recalling that, in prothylakoids, MGDG asymmetry is already established and that it is similar to that found in mature thylakoids, despite the marked qualitative and quantitative changes occurring in membrane proteins during the development process [28]. However, an important feature of the results of Fig. 1 is that the coupled and, to a lesser extent, the uncoupled electron flow activities are largely preserved despite the complete MGDG depletion in the outer monolayer (resulting from MGDG hydrolysis followed by the removal of breakdown products by bovine serum albumin). This finding emphasizes the crucial role of the inner MGDG in sustaining electron transport and should be related to the similar property exhibited by inner phospholipids in thylakoid [11,29,30] as well as in erythrocyte membranes [31]. Therefore, as far as (un)coupled electron flow is concerned, the critical factor is perhaps not MGDG asymmetry per se but rather the presence of an intact MGDG population in the inner monolayer of the thylakoid membrane.

The sensitivity of lipolytic enzymes to the packing pressure of their lipid substrate(s) has been best demonstrated using monolayer techniques [21]. These techniques being unavailable to us, we have applied to indirect and independent methods which are known to alter the packing pressure of membrane lipids. First, the double-bond content of thylakoid acyl lipids has been reduced by in situ catalytic hydrogenation of membranes. This method is a powerful tool to decrease membrane fluidity in a highly controlled way [19]. Second, cholesterol incorporation into thylakoid membranes has been shown to lead to membrane rigidification and to an increase in the order parameter of acyl chains [32]. From the decreased MGDG hydrolysis

rates in both hydrogenated (Fig. 2) and cholesterol-enriched membranes (Fig. 3), we conclude that the lipase from *R. arrhizus* is sensitive to the packing changes which affect MGDG in thylakoid membranes.

Although other factors may be involved in the increased digestion of MGDG under coupled conditions, the above results (Figs. 2 and 3) as well as other reports [21,22] favour the interpretation of the data of Figs. 4 and 5 in terms of changes in MGDG packing. The most striking feature of these results is the close relationship between MGDG susceptibility to digestion (MGDG packing) and the functional state of the coupling factor. Indeed, MGDG packing was clearly independent of the rate of electron transport (low in basal and high in uncoupled conditions), of the amplitude of the protonic potential (maximal in basal and zero in uncoupled conditions) (Fig. 4) and of the presence of ATP, either in the light or in the dark. In addition, compared to the control in the dark, no change was observed in DCMU-treated samples. These results also indicate that the activity of the lipase is not affected by the alterations of the surface charge density and pH which occur under some of these conditions. In contrast, the only change in MGDG packing, i.e., a packing decrease (Fig. 4), was observed when thylakoid membranes were incubated under photophosphorylating conditions, suggesting that the functional coupling factor, driving net ATP synthesis, is closely associated with this process. This conclusion is further supported by experiments in which specific alterations of the coupling factor were provoked by the use of the energy-transfer inhibitors, DCCD and phlorizin, or by the selective removal of the CF_1 moiety following NaBr treatment. As soon as the functionality of the coupling factor was suppressed by any of the abovementioned treatments, the hydrolysis rate of MGDG decreased strongly (Fig. 5), reflecting a packing increase of MGDG molecules. This increase could not be explained by either a simple inhibitory effect of DCCD and phlorizin on the lipase itself nor by any interaction between these compounds and MGDG molecules which might have decreased the affinity of the lipase toward its substrate (Table I).

The trivial explanation that thylakoid swelling would account for the increased hydrolysis rate observed under photophosphorylating conditions can be dismissed on the basis of our finding that the hydrolysis of MGDG was slower in hypotonic media than in iso- or hypertonic ones (see Results).

It is also of interest to evaluate the extent to which thylakoid membranes should be hydrogenated in order to obtain a reduction in MGDG hydrolysis rate similar to that observed between the coupled and the dark conditions in Fig. 4. From Fig. 2, it can be calculated that a 20–30% decrease in the linolenic acid content (with concomitant increase in linoleic, oleic and stearic acids) of thylakoid membranes would be required. This already represents a significant change in fluidity [19]. This comparison provides a more physical illustration of the difference in hydrolysis rates described in Fig. 4.

Since the packing changes, as expressed by MGDG hydrolysis rates, were already observable at a low level of MGDG degradation (see Figs. 4 and 5), it is very likely that they concern those MGDG molecules which are localized in the outer monolayer of the thylakoid membrane. Our results suggest that these molecules can adopt at least three degrees of packing, only two of which correspond to normal physiological states. In resting membranes, the mere presence of the intact (but inactive) coupling factor already imposes on MGDG molecules a degree of packing which is lower than that observed when the coupling factor is altered (compare Figs. 4 and 5). In photophosphorylating membranes, the intact (but now functioning) coupling factor imposes a further decrease on MGDG packing (Fig. 4). It must be emphasized here that only the resting (dark) and the photophosphorylating (light + ADP + P_i) conditions are of physiological significance in thylakoid membranes, and that blocked, basal or uncoupled electron transports represent essentially artificial processes which are not encountered in chloroplasts in vivo.

The experiments described in Figs. 4 and 5 only deal with the light-induced change in MGDG packing following a dark-to-light transition. The full demonstration that such a change is of physiological relevance requires that it is reversible. For technical reasons, we have used the safranine assay (Fig. 6) rather than the thin-layer chromatographic analysis to study the hydrolytic behaviour of MGDG during the light-to-dark transition. Clearly, as soon as the photophosphorylating condition is suppressed, whether by switching off the light, by inhibiting electron transport or by uncoupling, the fast hydrolysis rate is rapidly reduced within about 5 s. These results, together with those of Fig. 4, suggest that changes in MGDG packing are effectively reversible and, as such, may well reflect a part of the rapid adaptive response of the thylakoid membrane structure to external factors.

We come now to the nature of the mechanisms by which the functional state of the coupling factor may affect the molecular organization of MGDG in thylakoid membranes. These mechanisms should account for the rather high degree of specificity with respect to MGDG as well as for the long-range interaction capability. The latter is required because of the significant amount of MGDG engaged in the packing change (40–50% of the total MGDG) and because of the restricted localization of the coupling factor to the stroma-exposed regions of thylakoid membranes [33,34]. Several authors have reported on the interactions existing between the coupling factor and thylakoid lipids, mostly using reconstitution procedures. Evidence has been provided for highly ordered [35,36] and highly viscous [37] lipid domains

around CF_0. A requirement for galactolipids has been recognized for inducing a DCCD-sensitive proton channel in CF_0 [38,39]. Taken together, these data suggest that the most probable change which can occur in the lipids surrounding CF_0 should be a decrease in galactolipid order and viscosity, as illustrated in the present report. Pick and co-workers have presented detailed studies showing that the Mg^{2+}-ATPase and the ATP-P_i exchange activities of the coupling factor depend essentially on its surrounding lipids, native (H_{II}-forming) MGDG being particularly important in this respect [8–10]. For comparative purposes, it is instructive to recall that both the F_0/F_1 complex of mitochondria [40] and the CF_0-CF_1 complex of thermophilic cyanobacteria [41] also require a H_{II}-forming lipid for optimal activity, in agreement with our earlier proposal [27].

The interactions between MGDG and the CF_0 moiety of the coupling factor are thus sufficiently well-documented to envisage that any conformational change in CF_0 may be transmitted to the vicinal MGDG molecules. We propose the following, very general hypothesis: a transmembrane protein is commonly viewed as being formed of several hydrophobic sequences which are embedded in the lipid core of the membrane and interconnected by hydrophilic sequences, which are localized alternatively at the outer and inner side of the membrane. Assuming that the lateral spacing of the hydrophobic segments varies according to the functional state of the protein, a conformational change occurring in the protein upon a resting-to-working transition allows the hydrophobic segments to come into closer contact with each other, thus leaving more space for bulk lipids. This would result in decreased lipid packing. Conversely, the opposite change occurring after a working-to-resting transition would result in an increase of the distance between the hydrophobic segments of the protein, which would thus promote lateral compression of lipids. This pleated model for functional membrane proteins, however, requires further experimental basis.

It is more difficult to imagine how the conformational changes known to occur in the active CF_1 moiety [42] could be transmitted to membrane lipids. An obvious possibility is that conformational changes occur sequentially, first appearing in CF_1, then transmitted to CF_0 and finally to lipids. Support in favour of such a sequential mechanism may be found in a recent work by Li et al. [43]. On the other hand, direct interactions of CF_1 subunits with membrane lipids have been advocated [44–46] or more directly shown (Ref. 47; Pick, personal communication), thus again suggesting that a change in CF_1 conformation can be effectively transmitted on the surrounding membrane lipids.

Long-range interactions probably need to be invoked to explain why and how as much as 40–50% of the total MGDG could be influenced by changes occurring in the coupling factor, which is considered to be localized only in stroma-exposed regions of thylakoid membranes [33,34]. Since the isolated CF_0-CF_1 complex is able to bind large amounts of lipids [10], and since an average value of 1.3 nmol coupling factor per mg chlorophyll has been reported [48], it can be calculated that on the basis of 1 mg chlorophyll, the coupling factor complexes of thylakoid membranes may 'bind' about 1000 nmol MGDG, which is close to the amount of MGDG present in the outer monolayer. Although this calculation is based on the assumption that the MGDG binding capacity of the coupling factor is similar in thylakoid membranes and in artificial lipid vesicles, it can be taken as an indication that the outer monolayer as a whole might be able to sense the conformational changes occurring in the coupling factor during a resting-to-working or working-to-resting transition.

Finally, the physiological implications of these reversible changes in MGDG packing must be considered. It has been shown in several ways that fluidity is a determinant factor for the photosynthetic competence of thylakoid membranes [19,49,50]. Along the same line, Horvath et al. [51] have shown that decreasing the fluidity of thylakoids by catalytic hydrogenation progressively shifted these membranes from a coupled to an uncoupled state. However, the available data on thylakoid fluidity have been determined in resting membranes only and, to our knowledge, no attempt has ever been made to investigate the possible fluidity changes which may occur in photophosphorylating membranes. Our results represent a first step in this direction. Indeed, the decreased MGDG packing generated in photophosphorylating thylakoid membranes may be associated with an increase in membrane fluidity. A direct and important consequence is that the long-range diffusional processes, such as those involving plastoquinones or the light-harvesting chlorophyll-protein complexes, would be facilitated. A definite improvement of the whole photosynthetic capability of thylakoid membranes would therefore be expected.

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