Biochimica et Biophysica Acta 891 (1987) 28–39
Elsevier

BBA 42500

The role of different thylakoid glycolipids in the function of reconstituted chloroplast ATP synthase

Uri Pick [a], Meira Weiss [a], Kleoniki Gounaris [b] and Jim Barber [b]

[a] *Biochemistry Department, Weizmann Institute of Science, Rehovot (Israel) and* [b] *Department of Pure and Applied Biology, Imperial College of Science and Technology, London (U.K.)*

(Received 12 December 1986)

Key words: Chloroplast glycolipid; Galactolipid; Monogalactosyldiacylglycerol; ATPase activation; Proton conductance

ATPase activity of CF_0CF_1 from spinach chloroplasts is specifically stimulated by chloroplast lipids (Pick, U., Gounaris, K., Admon, A. and Barber, J. (1984) Biochim. Biophys. Acta 765, 12–20). The association of CF_0-CF_1 with isolated lipids and their mixtures has been examined by analyzing the stimulation of ATPase and ATP–P_i exchange activities, by binding studies and by measurement of proton conductance of reconstituted proteoliposomes. Monogalactosyldiacylglycerol is the only chloroplast lipid which by itself activates ATP hydrolysis. A mild saturation of the fatty acids of the lipid partially inhibits the activation. CF_0-CF_1 has a higher binding capacity for monogalactosyldiacylglycerol (1.5 mg/mg protein) than for other thylakoid glycolipids. However, ATPase activation is not correlated with the amount of bound lipid but rather with its type. For the same amount of bound lipid, monogalactosyldiacylglycerol best activates ATP hydrolysis, while the acidic lipids phosphatidylglycerol and sulphoquinovosyldiacylglycerol inhibit ATPase activity. Optimal activation of ATP–P_i exchange requires, in addition to monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol at a ratio of 6:3:1, respectively. Correlations between proton conductance, ATP–P_i exchange and uncoupler stimulation of ATPase activity indicate that sulphoquinovosyldiacylglycerol reduces the permeability of the proteoliposomes to protons. The results suggest that: (a) association of CF_0-CF_1 with polyunsaturated monogalactosyldiacylglycerol greatly stimulates ATPase activity; (b) reconstitution of coupled CF_0-CF_1 proteoliposomes requires a careful balance of the natural glycolipids of thylakoid membranes in similar proportions to their occurrence in chloroplasts, and (c) sulphoquinovosyldiacylglycerol may control the permeability of chloroplast membranes to protons.

Introduction

Different biological membranes are characterized by various lipid compositions in order to meet their special functions. The thylakoid membrane of plant and algal chloroplasts is unique in its unusually high content of glycolipids, amounting to over 70% of the total polar lipid content [1,2]. The reason for this unusual lipid composition of thylakoid membranes is not known, but it has been demonstrated that several proteins and

Abbreviations: CF_0-CF_1, chloroplast ATP synthase complex; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol; pyranine, 8-hydroxyl-1,3,6-pyrenetrisulphonic acid; SF-6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalonitrile; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Correspondence: U. Pick, Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel.

enzymatic systems in the thylakoid membrane require specific chloroplast lipids for their function. They include chlorophyll *a/b* complexes of Photosystem II (3), oxygen-evolving thylakoid fragments [4], cytochrome *b*-559 [5] and chlorophyllase [6].

We have recently demonstrated that chloroplast thylakoid lipids specifically stimulate ATPase activity of spinach CF_0-CF_1 and modify the kinetic properties of the enzyme [7]. Similar observations were reported recently for the lipid requirement of CF_0-CF_1 from the thermophilic cyanobacterium *Synechococcus* by Van Walraven et al. [8]. Both enzymes seem to specifically require mixtures of MGDG, the major glycolipid component of thylakoids, together with other lipids for optimal reconstitution of catalytically active proteoliposomes.

In the present study we attempted to determine the role of specific chloroplast glycolipids in the activation of the enzyme by measuring in parallel the activation of different partial reactions, proton conductance of proteoliposomes and binding of individual lipids and of lipid mixtures to the enzyme. Our hope was that these studies might help to assign specific roles to different thylakoid lipids not only in the activation of CF_0-CF_1 but also in the more general aspects of organization of the thylakoid membrane. The results indeed allow assignation of specific roles to different chloroplast glycolipids, both in CF_0-CF_1 activation and in controlling the permeability of the thylakoid membrane to protons.

Materials and Methods

Isolation of the CF_0-CF_1 ATP synthase. CF_0-CF_1 was isolated from spinach leaves (*Spinacia oleracea*) as previously described [9]. Purified and delipidated preparations of CF_0-CF_1 were prepared by two purification steps on Triton-X-100 (0.1%) and on sodium cholate (0.2%) containing sucrose gradients in the absence of exogenous lipids [9]. Most studies unless otherwise indicated) were carried out with the enzyme fraction precipitated between 37.5 and 45% saturation ammonium sulphate (crude preparation). The lipid contents of the crude and the purified preparations are about 8% and 1.5%, respectively [10].

Isolation of chloroplast lipids. Double-washed broken chloroplast thylakoids were prepared from spinach as previously described [11]. Lipids were extracted according to Bligh and Dyer [12]. Pigments and neutral lipids were removed from the extract on silicic acid columns (5% acetone in chloroform) and partially enriched fractions of monogalactosyldiacylglycerol (MGDG), digalactosylglycerol (DGDG), sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) were sequentially eluted from the columns with 50% acetone, 80% acetone, 10% methanol and 80% methanol in chloroform, respectively. Total chloroplast lipids were eluted in two steps with 50% and 100% methanol and combined. Further purification was achieved by preparative thin-layer chromatography (TLC). The purity of the lipids was verified by TLC on ammonium-impregnated plates as previously described [13]. Saturation of lipids was achieved by time-controlled catalytic hydrogenation in the presence of Adams' catalyst as described in Ref. 14. Quantification of lipids and their degree of unsaturation was determined by gas-liquid chromatography of their fatty-acid methyl esters as previously described [15]. Lipids labelled with ^{14}C or ^{32}P or ^{35}S were prepared from *Dunaliella salina* cultured in the presence of [^{14}C]bicarbonate, [^{32}P]phosphate or [^{35}S]sulphate as described in Ref. 10.

Reconstitution and assays. Reconstitution of CF_0-CF_1 ATP synthase with chloroplast lipids was performed by a modification of the cholate-dilution technique [16] as follows. Lipid mixtures dissolved in chloroform were dried under a stream of nitrogen, redissolved in diethyl ether and dried again and disspersed by sonication (4 min) at a final concentration of 2 mg/ml in a buffer containing 20 mM Na-Tricine (pH 8), 0.2 mM Na-EDTA and 0.2% sodium cholate. $MgCl_2$ (2 mM) and CF_0-CF_1 ATP synthase (0.5–1 mg/ml) were added to the lipid mixture and the suspension was incubated for 20–30 min on ice. 50 μl or 100 μl samples of the protein/lipid/cholate mixtures were diluted into 1 ml reaction mixtures containing either [γ-^{32}P]ATP (ATPase assay) or ATP + [^{32}P]P_i (ATP–P_i exchange assay) and assayed as previously described [7].

Lipid-binding measurements. Lipids containing ^{14}C or ^{35}S labelled tracers (about 0.1 μCi/mg)

were dried under nitrogen as above and dissolved at a final concentration of 1 mg/ml in 20 mM Na-Tricine (pH 8) 0.2 mM EDTA, 0.2% sodium cholate and sonicated for 5 min in a bath-type sonicator. Undispersed lipid was removed by a 5 min spin in an Eppendorf microfuge (12000 × *g*). 150 μl samples of sonicated lipid/cholate mixtures were incubated in the presence of 2 mM $MgCl_2$ either with or without CF_0-CF_1 (0.5–1 mg/ml) for 30 min on ice. The suspensions were diluted with an equal volume of 20 mM Tricine (pH 8) and centrifuged for 10 min in a Beckman airfuge at 120000 × *g*. Samples from the supernatants were counted to detrmine the residual lipid content.

Permeability to protons. Permeability to protons was measured by following fluorescence changes of trapped pyranine, a membrane-impermeable pH-indicator [17], essentially according to Admon et al. [18]. Pyranine trapping was performed by preincuabtion of protein/lipid/detergent mixtures in the presence of 50 μM pyranine. 1 ml of the measuring buffer containing 50 μM pyranine was added to a 0.6 × 25 cm Sephadex G-50 (course) column preequilibrated with the measuring buffer (20 mM Na Tricine (pH 8)/30 mM KCl/3 mM $MgCl_2$/10 nM valinomycin). 100 μl of the pyranine-containing enzyme/lipid/detergent mixtures were applied to the column and eluted at a flow rate of 2 ml/min at 21°C. The turbid liposome suspension which was freed of untrapped pyranine was collected in 3 ml cuvettes. pH changes were induced by addition of HCl. Fluorescence measurements were made in a Perkin Elmer MPF-44A spectrofluorimeter. The exitation and emission wavelengths were 465 nm and 510 nm, respectively.

Materials. Soybean phospholipids, brain phosphatidylserine and other chemicals and detergents were obtained from Sigma Chemicals. [^{14}C]bicarbonate and [^{35}S]sulphate were obtained from CEN, Saclay, France, and [^{32}P]phosphate from the Radiochemical Centre, Negev, Israel.

Results

Optimization of the reconstitution conditions of CF_0-CF_1 with chloroplast lipids

Compared with previous work [7], two technical improvements in the reconstitution of CF_0-CF_1 with chloroplast glycolipids were achieved by eliminating Mg^{2+} during the presonication of lipids and by lowering the lipid/detergent ratio (Fig. 1). Elimination of Mg^{2+} during the sonication of glycolipids facilitated their dispersion and improved the reconstitution of tightly-coupled CF_0-CF_1 proteoliposomes (data not shown).

Reconstitution of catalytically active CF_0-CF_1 proteoliposomes with chloroplast lipids is optimal at much lower lipid concentrations than with phospholipids. Conversely – an excess of chloroplast lipids very often inhibits the reconstitution of both ATP–P_i exchange and ATPase activities (Fig. 1). Optimal reconstitution conditions with

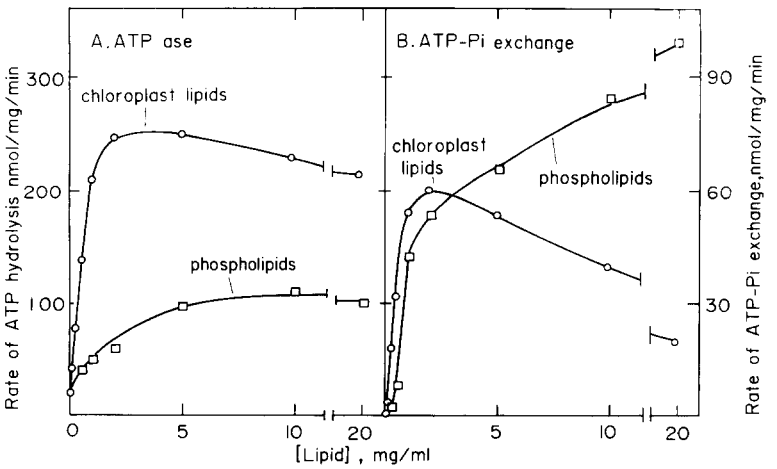

Fig. 1. Comparison of the lipid-concentration dependence for reconstitution of ATPase and of ATP–P_i exchange between chloroplast lipids and phospholipids. Chloroplast lipids or soybean phospholipids at the indicated concentrations were sonicated for 5 min in 0.2% sodium cholate, 20 mM Na-Tricine and 0.2 mM Na-EDTA, reconstituted with CF_0-CF_1 and assayed as described under Materials and Methods.

chloroplast lipids are obtained with a lipid : cholate : protein ratio of 3 : 4 : 1, whereas with phospholipids the optimal ratio is 10 : 3 : 1. The possibility of reconstituting CF_0-CF_1 with a relatively low lipid/protein ratio is interesting because it resembles more closely the situation of the enzyme in the native thylakoid membrane, in which the ratio of lipid to total protein is below 1 : 1.

The effect of individual glycolipids and of glycolipid mixtures on the catalytic activities of CF_0-CF_1

We have previously demonstrated that depletion of chloroplast lipids from MGDG inhibits the reconstitution of catalytically active CF_0-CF_1 proteoliposomes which suggested that MGDG plays a specific role in the activation of the enzyme [7]. In order to test more directly the role of individual chloroplast lipids in the activation of CF_0-CF_1 we have reconstituted the purified enzyme with each of the four major chloroplast lipids. A summary of the catalytic properties of the reconstituted preparations is presented in Table I. Only MGDG by itself stimulates the rate of ATP hydrolysis, while the acidic lipids SQDG and phosphatidylglycerol actually inhibit ATP hydrolysis with respect to the unreconstituted enzyme. Our previous failure to reconstitute CF_0-CF_1 with MGDG alone [7] was probably due to inappropriate reconstitution conditions (presence of Mg^{2+} and high lipid concentrations). However, as is also evident from Table I, none of the individual chloroplast lipids catalyses high rates of ATP–P_i exchange when reconstituted with CF_0-CF_1. The reconstitution of ATP–P_i exchange can be improved by using mixtures of MGDG/DGDG rather than individual glycolipids, as demonstrated in Fig. 2. Optimal activation of ATP–P_i exchange is achieved by reconstitution with a 2 : 1 mixture of MGDG/DGDG. A further stimulation of ATP–P_i exchange is obtained by an addition of the acidic phospholipid, phosphatidylserine. The effect of phosphatidylserine is to reduce the permeability of chloroplast lipid proteoliposomes to protons and thus improve the coupling between ATP synthesis and proton translocation in the reconstituted system [7]. In order to test whether this stimulation is a general effect of acidic lipids, we compared phosphatidylserine

TABLE I

ACTIVATION OF CF_0-CF_1 BY INDIVIDUAL CHLOROPLAST LIPIDS

Lipid added during reconstition	Rate of reaction (nmol/mg protein per min)	
	ATP hydrolysis	ATP–P_i exchange
(I) Crude preparation		
None	75	2
Total chloroplast lipids	310	65
MGDG	370	11
DGDG	110	3
SQDG	45	0
Phosphatidylglycerol	30	0
(II) Purified delipidated preparation		
None	42	0
Total chlorplast lipids	480	45
MGDG	465	10
DGDG	160	0
SQDG/PG (1 : 1)	25	0

with the two natural acidic chloroplast lipids SQDG and phosphatidylglycerol with respect to their effect on reconstitution of catalytically active CF_0-CF_1 proteolliposomes with 2 : 1 mixtures of MGDG/DGDG (fig. 3). SQDG but not phos-

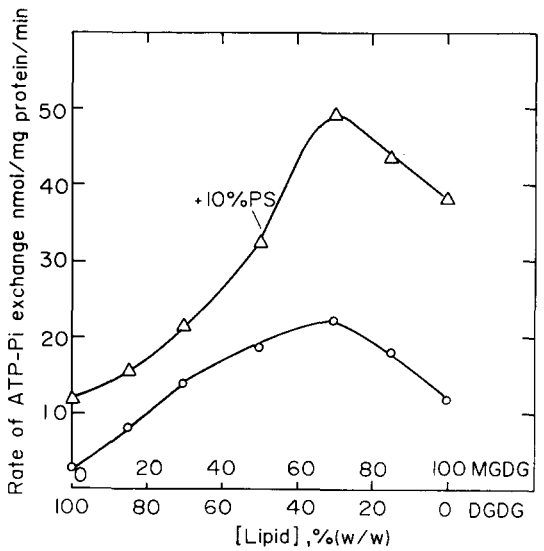

Fig. 2. Optimization of the MGDG/DGDG ratio for reconstitution of ATP–P_i exchange. CF_0-CF_1 was reconstituted with different lipid mixtures (total concentration – 2 mg/ml) and assayed for ATP–P_i exchange as described in Materials and Methods.

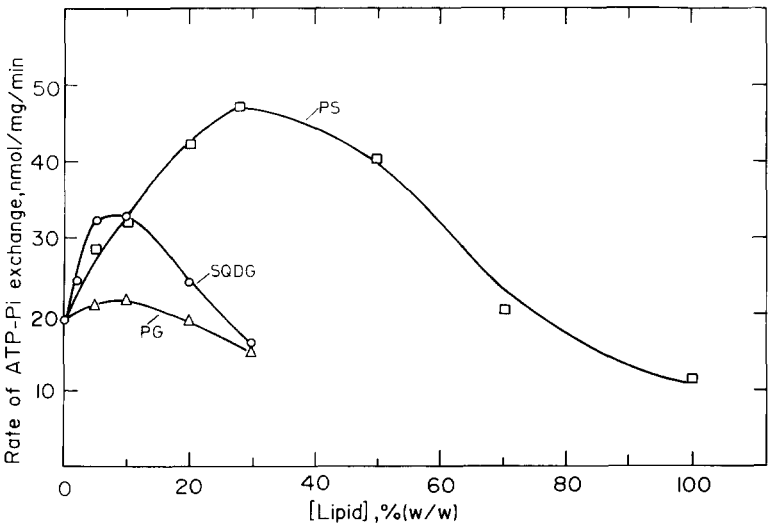

Fig. 3. The effect of acidic lipids on reconstitution of ATP–P_i exchange. CF_0-CF_1 was reconstituted with mixtures of MGDG/DGDG (2:1) with PG, PS or SQDG at the indicated ratios (total lipid concentration, 2 mg/ml) and assayed for ATP–P_i exchange activity.

phatidylglycerol also stimulates the reconstitution of ATP–P_i exchange, but the optimal SQDG fractional concentration is lower than for PS (10% compared to 30%). Excess of all acidic lipids inhibits reconstitution.

The effect of fatty acid hydrogenation on the reconstitution of CF_0-CF_1 with chloroplast lipids

In a previous work we have demonstrated that hydrogenation of MGDG inhibits its capacity to reconstitute catalytically active CF_0-CF_1 proteoliposomes [7]. Since complete hydrogenation of MGDG changes very markedly its physical properties [14] we have more carefully analysed the effect of controlled hydrogenation levels of MGDG fatty acids on their capacity to reconstitute ATP–P_i exchange. As is demonstrated in Fig. 4, very mild hydrogenation (probably of only one double bond) is sufficient to inhibit the reconstitution of ATP–P_i exchange by approx. 50% and a similar inhibition was obtained also by a mild hydrogenation of total chloroplast lipids. ATP hydrolysis is also similarly inhibited by mild hydrogenation of MGDG, but to a smaller extent (20–30%, not shown).

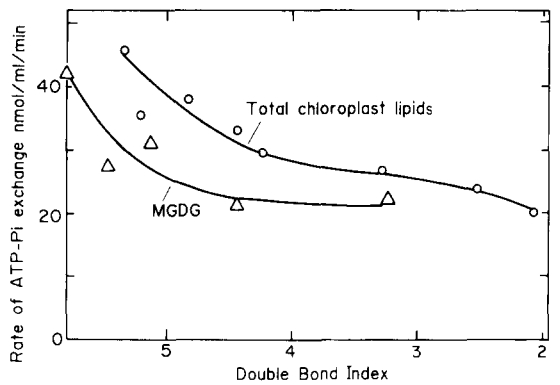

Fig. 4. The effect of partial hydrogenation of MGDG and of total chloroplast lipids on reconstitution of ATP–P_i exchange. Controlled catalytic hydrogenation of MGDG or of total chloroplast lipids was performed by incubation of lipids for different time intervals with Adams' catalyst [14]. CF_0-CF_1 was reconstituted either with 6:3:1 mixtures of MGDG/DGDG/SQDG, or with total chloroplast lipids (2 mg/ml) and assayed for ATP–P_i exchange activity. The double bond index represents the average number of double bonds per lipid molecule as determined by gas-liquid chromatography.

Binding of chloroplast lipids to CF_0-CF_1

The binding of thylakoid lipids to CF_0-CF_1 was measured by sucrose gradient centrifugation and by a differential proteoliposome precipitation in an airfuge under the same reconstitution conditions as used for kinetic measurements.

In brief, lipids containing radioactive tracers were presonicated with detergents (usually 0.2% sodium cholate), incubated with or without CF_0-CF_1 (30 min on ice) and either layered on top of sucrose gradients or centrifuged in an airfuge.

Fig. 5 demonstrates a comparison of MGDG distribution on a 5–25% sucrose gradient in the presence and absence of enzyme. In the absence of

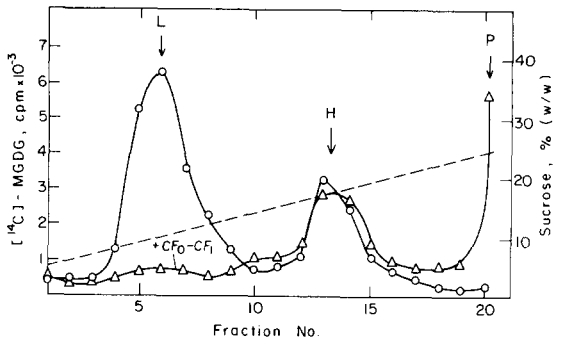

Fig. 5. Analysis of the association between MGDG and CF_0-CF_1 by a sucrose density gradient. MGDG (1 mg/ml) containing [^{14}C]MGDG was sonicated for 5 min in 0.2% sodium cholate buffer, centrifuged for 5 min in an Eppendorf microfuge to remove undispersed lipid and incubated with 2 mM $MgCl_2$ in the presence or absence of CF_0-CF_1 (0.8 mg/ml) for 30 min on ice. 0.5 ml samples were applied to 9.5 ml 7–25% sucrose gradient containing 20 mM Na-Tricine (pH 8) and 2 mM $MgCl_2$ and centrifuged for 8 h at 40000 rpm in SW-41 rotors. Fractions from the gradients were analyzed for lipid ([^{14}C] counts) and for ATPase activity. ○, MGDG without enzyme. △, MGDG + CF_0-CF_1. L, H and P indicate light, heavy and pellet lipid fractions, respectively.

TABLE II

EFFECT OF DIFFERENT DETERGENTS ON THE ASSOCIATION OF MGDG WITH CF_0-CF_1

[^{14}C]MGDG (2 mg/ml) was sonicated for 5 min in the presence of the indicated detergents, undispersed lipid (0–15%) was removed by centrifugation in an Eppendorf microfuge and the suspension was incubated with or without CF_0-CF_1 (0.8 mg/ml) in the presence of Mg^{2+} for 30 min on ice. After centrifugation of 175 μl samples in a Beckman airfuge (10 min at 120000 × *g*) samples of the supernatant were taken to measure the residual radioactivity. The specifically bound MGDG designates the percentage of lipid precipitated by the enzyme after subtraction of nonspecifically precipitated lipids in the absence of enzyme. Other details are described under Materials and Methods.

Detergent	Amount of precipitated MGDG (%)		Specifically bound MGDG (%)
	− CF_0-CF_1	+ CF_0-CF_1	
0.2% sodium cholate	28	90	86
0.6% sodium cholate	15	58	50
0.1 octylglucoside	26	92	90
0.1% Triton X-100	30	39	13

CF_0-CF_1 the lipid is resolved into a major light fraction (L) and a minor heavy fraction (H). In the presence of enzyme the L fraction essentially disappears and is substituted by a new lipid peak at the bottom of the gradient, while the heavier lipid fraction is unaffected. Since all the ATPase activity is recovered at the bottom of the gradient (P), it appears that the L fraction preferentially binds to the enzyme during reconstitution and comigrates with it. The H fraction may reflect incompletely dispersed lipids which are inacessible to the enzyme, since it can be greatly reduced or eliminated by a brief centrifugation (10 min in an Eppendorf microfuge) before the incubation with the enzyme without affecting the efficiency of reconstitution. Based on this observation, we have developed a simple assay to separate protein-associated lipids from free lipids by high-speed centrifugation using an airfuge ultracentrifuge. Basically, the lipid-detergent or lipid-detergent-enzyme complexes are incubated for 30 min in the presence of Mg^{2+} and centrifuged for 10 min at 120000 × *g* followed by analysis of the residual lipids in the supernatant.

Table II summarizes the effect of three different detergents on the association of MGDG with CF_0-CF_1 by this procedure. CF_0-CF_1 increases the amount of precipitated lipids in the presence of 0.2% sodium cholate or 0.1% octyl glucoside from about 25–30% to 80–90%. Essentially all the enzyme is recovered in the pellet, indicating that the precipitated lipid is associated with the enzyme. This differential precipitation of MGDG by CF_0-CF_1 is hardly affected by the centrifugal force in the range 70000–180000 × *g* and is not increased by longer centrifugation. However, in the presence of Triton X-100 or of higher sodium cholate concentrations less lipid is coprecipitated with the enzyme, probably due to competition between detergent micelles and the enzyme for binding of lipids. The major drawback in this analysis is the lipid fraction which precipitates in the absence of enzyme. It amounts to 0–40% of the total lipid, depending on the affectiveness of the sonication and on the type of lipid (Table III). It can be significantly reduced by a low-speed centrifugation of presonicated lipid-detergent suspensions (10 min in an Eppendorf microcentrifuge). It appears, therefore, that this lipid fraction may represent undispersed lipid. This assumption is con-

TABLE III

BINDING OF PURIFIED CHLOROPLAST LIPIDS TO CF_0-CF_1 ANALYSED BY SUCROSE GRADIENT FRACTIONATION AND BY DIFFERENTIAL CENTRIFUGATION

A. SUCROSE GRADIENT FRACTION

Analysis of lipid binding by sucrose gradient fractionation was performed as in Fig. 5. Fraction 3 to 10 (light), 11 to 18 (heavy) and the pellets were combined and counted. Fractions L, H and P stand for light, heavy and pellet fractions, respectively. (−) and (+) signs stand for absence or presence of CF_0-CF_1. The fraction of bound lipid was calculated by two different methods. Other details are given in the text.

Fraction	% of total lipid in fraction						Bound lipid (% of total)	
	$-CF_0CF_1$			$+CF_0CF_1$				
	L^-	H^-	P^-	L^+	H^+	P^+	$\frac{L^- L^+}{L^-}$	$\frac{P^+}{L^+ + P^+}$
MGDG	70	25	3	12	30	42	83	78
DGDG	56	40	4	29	42	20	48	41
SQDG	58	12	2	72	15	13	15	15
Phosphatidylglycerol	90	7	0	33	11	55	63	62

B. AIRFUGE

Lipid binding by the airfuge method in the presence of 0.2% Na-cholate was performed as in Table II. S and P stand for supernatant and pellet fractions, respectively.

Fraction	% of total lipid in fraction				Bound lipid (% of total)
	$-CF_0CF_1$		$+CF_0CF_1$		
	S	P	S	P	
MGDG	84	16	22	78	74
DGDG	70	30	52	48	26
SQDG	95	5	78	22	18
PG	100	0	45	55	55

sistent with the H fraction in the sucrose-gradient fractionation (Fig. 5). Moreover, comparisons of different lipid types and of different sonicated batches show a good correlation between the H fraction and the lipid precipitated in the absence of CF_0-CF_1 (Table IIIA and B). Since the sucrose gradient analysis suggests that this lipid fraction does not associate with CF_0-CF_1, it was treated as 'background' in the binding analysis and simply subtracted from the lipid precipitated by CF_0-CF_1 to obtain the specifically bound lipid.

A comparison of the binding of different chloroplast lipids to CF_0-CF_1, as measured by the airfuge technique and by sucrose gradient centrifugation, is summarised in Table III. The binding of MGDG and of phosphatidylglycerol to CF_0-CF_1 is significantly higher than the binding of DGDG and SQDG in both of these techniques. The same

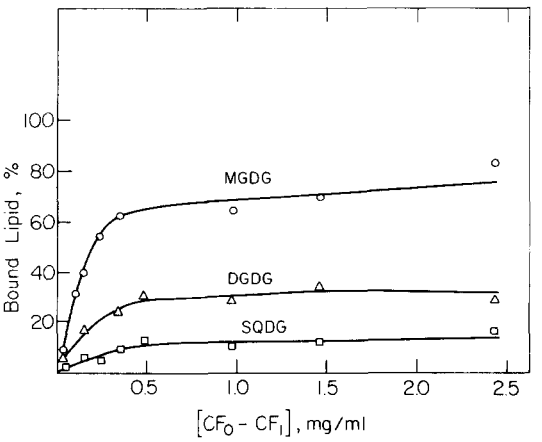

Fig. 6. Binding of chloroplast glycolipids to CF_0-CF_1. Sonicated glycolipids (1 mg/ml) containing tracers were incubated with CF_0-CF_1 at the concentrations indicated in the figure. The separation of free from bound lipids and the calculation of specifically bound lipids was as in Table II.

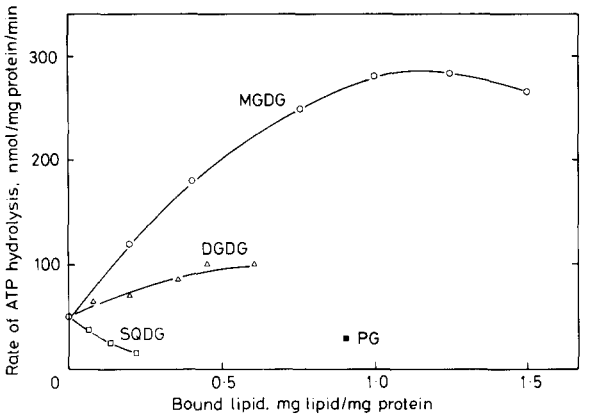

Fig. 7. Correlation between ATPase activity and lipid binding. CF_0-CF_1 was reconstituted without or with 0.2–2 mg/ml MGDG, DGDG, SQDG or PG and 0.2% sodium cholate and analysed for binding by the airfuge technique or for ATPase activity.

result is obained also from enzyme titration curves as demonstrated in Fig. 6. Calculations yield values of 1.5, 0.75 and 0.2 mg lipid/mg protein for MGDG, DGDG and for SQDG, respectively.

The correlation between ATPase activation and the amount of bound lipid for purified chloroplast lipids was estimated from the lipid concentration dependence of ATPase activity and binding and is summarised in Fig. 7. The figure clearly demonstrates that for the same amount of bound lipid, MGDG induces the highest ATPase activation and DGDG induces a mild activation, while SQDG and phosphatidylglycerol inhibit ATPase activity. Therefore, the differences in ATPase activity cannot be accounted for by the differences in the binding capacity of CF_0-CF_1 for individual lipids.

In view of these marked differences in the binding of individual purified chloroplast lipids to CF_0-CF_1 it seemed of interest to find out whether preferential binding of MGDG by CF_0-CF_1 is manifested also in lipid mixtures. Incubation of CF_0-CF_1 with the optimal reconstitution mixture of MGDG/DGDG/SQDG at a 6 : 3 : 1 ratio results in a similar fractional bindingo f each lipid species, as shown in Table IV. Similar results were also obtained at low enzyme or at low lipid concentrations. These results suggest that the lipid mixture is homogeneous and that there is no phase separation or exclusion of a particular glycolipid during reconstitution with the enzyme.

Correlations between catalytical activities and permeability of CF_0-CF_1 proteoliposomes to protons

We have shown in a previous paper that there is a correlation between ATP–P_i exchange activity and the permeability to protons of CF_0-CF_1 proteoliposomes reconstituted with different lipid mixtures [7]. In this earlier work the permeability to protons was estimated from the decay of artificially induced pH-gradient by following fluorescence changes of 9-aminoacridine. Since this

TABLE IV

BINDING OF GLYCOLIPID MIXTURES TO CF_0-CF_1

Binding of purified or mixtures of glycolipids was measured as in Tables II, III. Saturating and limiting enzyme concentrations are 1 and 0.3 mg protein/ml, and high and low lipid concentrations are 2 and 0.5 mg lipid/ml, respectively.

Lipid type	Bound lipid (% of total)		
	saturating [enzyme] – high [lipids]	low [enzyme] – high [lipids]	saturating [enzyme] – low [lipids]
MGDG	61	40	85
DGDG	17.5	15	32
SQDG	18	8	21
MGDG [a], DGDG, SQDG (6 : 3 : 1)	53 [b]	33 [b]	72 [b]
MGDG, DGDG [a], SQDG (6 : 3 : 1)	46 [b]	36 [b]	75 [b]
MGDG, DGDG, SQDG [a] (6 : 3 : 1)	43 [b]	35 [b]	66 [b]

[a] Represents the radioactive labelled lipid species in the mixture.
[b] Fractional amount of radiolabelled lipid species bound.

method requires the induction of relatively large pH gradients (at least 3 pH units) and exposure of the enzyme to acidic pH and since the mechanism of response of 9-aminoacridine to ΔpH is controversial [19], we have extended these studies by using an alternative procedure which consists of trapping the impermeable fluorescent pH-indicator, pyranine, inside CF_0-CF_1 proteoliposomes prepared by a modified reconstitution procedure in which sodium cholate is removed by gel filtration [18]. The ATPase and ATP-P_i exchange activities of proteoliposomes prepared by this reconstitution technique are identical to those prepared by cholate dilution [7] and are unaffected by pyranine.

Fig. 8 demonstrates a comparison of fluorescnece traces induced by a pH shift (8.3–7.2) between phospholipids and chloroplast lipids CF_0-CF_1 proteoliposomes. Acidification induces a biphasic fluorescence quenching. A fast phase, probably reflecting the response of external (free or bound) probe and a slower phase. The latter most probably reflects pH equilibraiton inside the proteoliposomes, since it is accelerated by protonophores and ionophores such as nigericin and gramicidin as well as by different detergents. The comparison shows that chloroplast lipid proteoliposomes are much more permeable to protons than are phospholipid proteoliposomes, confirming our previous results with the 9-aminoacridine technqiue [7].

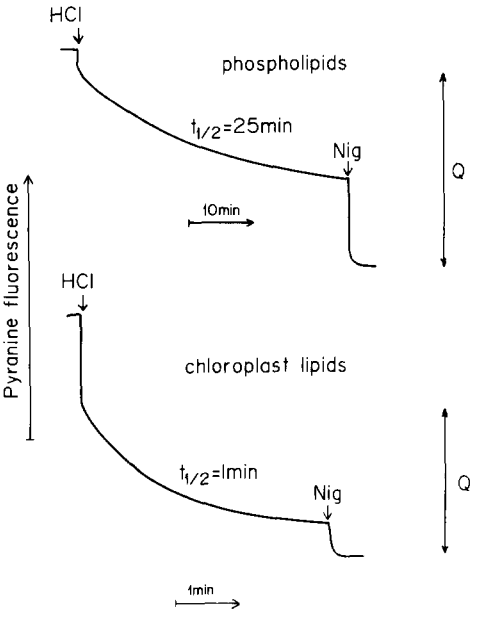

Fig. 8. Comparison of pH equilibration rates across phospholipid and glycolipid CF_0-CF_1 proteoliposome membranes measured with trapped pyranine. CF_0-CF_1 proteoliposomes were reconstituted with either soybean phospholipids or with chloroplast total lipid extracts in the presence of 50 μM pyranine and the untrapped pyranine was removed on a Sephadex G-50 column. The pH was adjusted to 8.3 and at the point indicated by an arrow HCl was injected to bring the pH to 7.2. The temperature was 12°C. Nig indicates addition of 1 μM nigericin. Q represents the total fluorescence quenching of trapped pyranine. Other details are described under Materials and Methods.

TABLE V

CORRELATIONS BETWEEN PROTON PERMEABILITY AND CATALYTIC ACTIVITY CF_0-CF_1 PROTEOLIPOSOMES MADE FROM DIFFERENT LIPIDS

CF_0-CF_1 proteoliposomes were formed by reconstitution with different lipids or lipid mixtures. $t_{1/2}$ is the half-time for pH equilibration at 10°C measured by fluorescence changes of trapped pyranine. ATPase activity was measured in the presence (UC) or absence (C) of 0.1 μM SF-6847.

Lipid mixture	$t_{1/2}$-pH equilibration (min)	Activity (nmol/mg per min)			ATPase / ATP-$P_i$exch	ATPase (UC) / ATPase (C)
		ATP-P_i exchange	ATPase (C)	ATPase (UC)		
Soybean phospholipids	75	82	120	192	1.46	1.60
Phosphatidylcholine	15	20	40	62	2.00	1.55
Chloroplast glycolipids	4	65	310	422	4.77	1.36
MGDG	1	20	275	294	13.70	1.07
DGDG	3	8	45	52	5.62	1.15
MGDG/DGDG (2:1)	2	35	330	390	9.43	1.18
MGDG/DGDG/SQDG (6:3:1)	5	60	285	385	4.75	1.35

Table V summarizes pH equilibration rates and catalytic parameters of CF_0-CF_1 proteoliposomes reconstituted with different lipids and lipid mixtures. The ratios of ATP hydrolysis to ATP–P_i exchange and of uncoupler-stimulated to basal ATPase activities were also calculated in order to express a rough estimate of the coupling between ATP hydrolysis and proton translocation. As expected, the results suggest an inverse correlation between proton conductance and coupling ratios, both between phospholipids and chloroplast lipids and beween different glycolipid mixtures. Phospholipid proteoliposomes have a lower proton conductance, a lower ATPase/ATP–P_i exchange and a greater stimulation of ATP hydrolysis by uncouplers in comparison to chloroplast lipid proteoliposomes, suggsting that the better coupling between ATP synthesis and hydrolysis in the former is due to their lower conductance to protons. Proteoliposomes made of MGDG as well as mixtures of MGDG/DGDG have an extremely high proton permeability and the worst coupling ratios, but addition of 10% SQDG significantly reduces their proton conductance and improves their coupling ratios, similar to total chloroplast lipids. These results are consistent with our earlier demonstration that phosphatidylserine, which also stimulates ATP–P_i exchange (Figs. 2, 3), reduces the permeability to protons of chloroplast lipid proteoliposomes. However, as is also evident from Table V, the turnover rates of the enzyme (ATPase + SF-6847) vary between different phospholipids and glycolipids irrespective of their prpoton conductance, suggesting that the rate of ATP synthesis catalyzed by CF_0-CF_1 is limited not just by ΔpH but also by associations with specific lipids.

Discussion

The purpose of the present work has been to characterise the interactions of CF_0-CF_1 with endogenous chloroplast lipids in an attempt to understand the role of individual lipids in the activation of the enzyme.

The results of this and our previous work disclose several clear differences in the reconstitution of CF_0-CF_1 between chloroplast lipids and phospholipids, which can be mostly ascribed to MGDG:

(a) Optimal reconstitution with chlorplast lipids requires a lower lipid/protein ratio – this can be the result of the different physical properties of glycolipids, which may allow a more efficient packaging of the protein within the lipid bilayer, or to a higher affinity of the enzyme for binding of glycolipids. It has been proposed before that MGDG, the major chloroplast glycolipid, may be important in the packaging of protein complexes in thylakoid membranes [14].

(b) Differences in the kinetic properties – chloroplast lipids specifically enhance ATPase activity of CF_0-CF_1. The V_{max} is increased and the K_m (ATP) is decreased in comparison to phospholipids [7]. This can be ascribed mainly to the activation by MGDG. Although CF-CF_1 does seem to have an exceptionally high binding capacity for MGDG, this does not seem to be the reason for the specific effect of this lipid on the kinetic properties of the enzyme (Fig. 7).

(c) Proteoliposomes made from chloroplast lipids are more permeable to protons in comparison to phospholipids. The high permeability of chloroplast lipid proteoliposomes to protons is reflected also in the poor coupling between ATP synthesis and hydrolysis in the reconstituted system. This result seems paradoxical for lipids derived from thylakoid membranes, which sustain pH gradients of 3–4 pH units in the light and catalyse high rates of ATP synthesis. Comparisons of the passive proton permeability beween chloroplast thylakoids and chloroplast lipid vesicles show that the latter are significantly more permeable to protons (Pick, U., unpublished data). It seems, therefore, that certain proteins in the natural thylakoid membrane may affect the organization of lipids in the membrane in such a way as to significantly decrease the permeability of the membrane to protons.

The observation that MGDG by itself fully activates ATP hydrolysis is surprising in view of the fact that MGDG does not form bilayers and is not expected to create closed vesicle [14]. Indeed, preliminary electron microscopic pictures of freeze-fractured MGDG-reconstituted CF_0-CF_1 show that the majority of the lipid is organized in non-bilayer structures (Gounaris, K., unpublished

data). Similarly, estimates of pyranine trapping capacity of reconstituted MGDG-CF_0-CF_1 suggest that the internal volume of the lipid-protein complexes is very small, consistent with the absence of vesicles (Pick, U., unpublished data). Also, the very low rate of ATP–P_i exchange and the poor stimulation of ATPase activity by uncouplers lead to the same conclusion. The possibility that endogenous lipids carried over in the enzyme preparation contribute to the organization of exogenous MGDG during reconstitution seems unlikely, since the amount of endogenous lipids in our CF_0-CF_1 preparation is only 8% and 1–2% (w/v) in crude and purified preparations, respectively, and most of this lipid is SQDG, which is tightly bound to the enzyme and does not exchange with exogenous lipids upon reconstitution [10].

The role of acidic lipids is of particular interest in view of the specific requirements for acidic lipids of other membranal enzymes such as cytochrome oxidase [21], that (Na + K)-ATPase [22] and the mitochondrial F_0-F_1-ATPase [23]. The effect of acidic lipids on CF_0-CF_1 is rather complex. Certain acidic lipids (SQDG, phosphatidylserine) in well-defined proportions seem to be required for optimal reconstitution of coupled CF_0-CF_1 proteoliposomes. We have demonstrated that this effect is due to a decrease in the permeability to protons of proteoliposomes composed of glycolipids. The same effect is obtained also in the absence of CF_0-CF_1, indicating that the general organization of the glycolipid bilayer has been affected. However, the observation that this effect is obtianed by SQDG and not by phosphatidylglycerol and that the optimal proportions of phosphatidylserine and SQDG are different suggests that this is not a general effect of all acidic lipids. Acidic lipids by themselves (Table I) or in access (Fig. 3) inhibit the activity of CF_0-CF_1. This inhibition is not correlated with the permeability of proteoliposomes to protons and was obtained with either acidic phospholipids (phosphatidylglycerol, phosphatidylserine) or with acidic glycolipids (SQDG), indicating a nonspecific inhibitory mechanism. The possibility that this inhibition is a result of the reconstitution method rather than an intrinsic property of the enzyme cannot be ruled out and could not be tested, since we have not succeeded in reconstitution of CF_0-CF_1 with glycolipids by other techniques. However, it may be noted that an excess of acidic lipids (phosphatidylserine, phosphatidic acid) in phospholipid mixtures inhibit the activity of CF_0-CF_1 also upon reconstitution by other techniques (freeze-thaw), suggesting that this inhibition may reflect a specific property of the enzyme. Purified delipidated PS II particles are similarly inhibited by access of acidic lipids [4], indicating that this may be a general property of enzyme complexes in thylakoid membranes. It is of great interest that SQDG seems to have a double role with respect to CF_0-CF_1: it is present as a tightly-bound non-exchangeable lipid in CF_0-CF_1 preparations [10], suggesting that it has a specific structural role and is also required to decrease the permeability to protons of glycolipid proteoliposomes. Interestingly, in thermophilic cyanobacteria phosphatidylglycerol rather than SQDG seems to fulfill the latter role [8]. This difference may be due either to the much lower degree of unsaturation of the cynaobacterial MGDG or to alterations in the enzymes.

Finally, the requirement for mixtures of bilayer and non bilayer forming lipids which we observed for optimal reconstitution of spinach CF_0-CF_1 was also found for thermophilic cyanobacterium CF_0-CF_1 [8] and for mitochondrial F_0-F_1 [24] and it seems to reflect, therefore, a general requirement of F_0-F_1 proton ATPases. It is also striking that the optimal balance of MGDG/DGDG/SQDG required for reconstitution of CF_0-CF_1 proteoliposomes is essentially identical to their natural abundance in thylakoids, suggesting that this specific composition is optimal for the normal functioning of the thylakoid membrane.

Acknowledgements

The authors are grateful to Prof. Aliza Dvir from the Department of Biochemistry of the Tel-Aviv University for her help and advice concerning the purification of chloroplast glycolipids. J.B. and K.G. acknowledge the financial support of the Agricultural and Food Research Council. This work was supported also by the U.S.-Israel Binational Science Foundation (to U.P., BSF 2891/82).

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