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Tightly bound sulpholipids in chloroplast CF_0-CF_1

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Highly purified preparations of CF_0-CF_1 from chloroplasts contain a small amount of tightly bound lipids. Extraction and analysis of these lipids show that they are almost exclusively sulpholipids. The calculated amount of bound sulpholipids in spinach and in *Dunaliella salina* CF_0-CF_1 preparations are 5 and 20 mols/mol enzyme, respectively. Attempts to exchange the bound lipids with other lipids or with detergents have failed, indicating a very strong association with CF_0-CF_1.

Introduction

Lipid-protein interactions in biological and artificial membranes have been investigated by a number of different approaches including specific lipid requirements for catalytical activation of purified membrane enzymes and the use of labeled lipid analogues in ESR and NMR studies. The latter have demonstrated that some lipids within the membrane are immobilized by intrinsic proteins and gives rise to the concept of boundary lipids [1]. The lipid requirements of different membrane enzymes varies quite remarkably. The sarcoplasmic reticulum Ca-ATPase is non-specifically activated by most phospholipids as well as by detergents [1,2]. The Na/K-ATPase requires negatively charged phospholipids for activation [3], while cytochrome oxidase is very specifically activated and associated with cardiolipin [4].

A specific association of a particular lipid with membrane proteins is often reflected by the presence of tightly bound lipids in highly purified preparations of the enzyme as, for example, in cytochrome oxidase which contains bound cardiolipin [4].

As yet very little is known about lipid-protein interactions in the chloroplast thylakoid membrane which is unique in its high content of glycolipids [5]. Recently, we demonstrated that reconstitution of CF_0-CF_1 with chloroplast glycolipids activates ATP hydrolysis better than phospholipids. In the work presented in this communication we have analyzed the amount and composition of bound lipids in CF_0-CF_1 preparations isolated and purified from spinach and from the halotolerant alga *Dunaliella salina*. In both organisms we find that the enzyme preparations contain almost exclusively sulpholipids which are minor lipid components in the thylakoid membrane suggesting that this acidic glycolipid is firmly bound to the CF_0-CF_1 complex.

Materials and Methods

Isolation of CF_0-CF_1 from spinach

CF_0-CF_1 was isolated from spinach leaves (*Spinaca olerace*) and purified on a sucrose gradi-

Abbreviations: CF_0-CF_1, chloroplast ATP synthase; crude CF_0-CF_1, partially purified CF_0-CF_1 obtained by precipitation between 37% and 45% ammonium sulphate; purified CF_0-CF_1, CF_0-CF_1 purified on a sucrose gradient in the presence of 0.1% Triton X-100; MGDG, monogalactosyldiacylglycerol, DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SQ, sulphoquinovosyldiacylglycerol; P_i, inorganic phosphate.

ent containing 0.1% Triton X-100 as previously described [7].

Isolation of CF_0-CF_1 from Dunaliella salina

D. salina cells were grown under continuous illumination in the presence of 1 M NaCl and harvested at the late exponential growth phase as previously described [8]. Separate cultures were grown in the presence of 10 mM [^{14}C]bicarbonate (1 μCi/μmol) or 100 μM [^{35}S]sulphate (3.5 μCi/μmol) or 50 μM [^{32}P]phosphate (2 μCi/μmol) for 48 h in order to obtain optimal labeling of total lipids, sulpholipids and phospholipids, respectively. CF_0-CF_1 was solubilized and purified on a sucrose gradient in the presence of 0.1% Triton X-100 as previously described [9].

Extraction and analysis of lipids

Lipids were extracted essentially according to the method of Bligh and Dyer [10]. The enzyme samples were incubated in 0.8 ml water, 2 ml methanol and 1 ml chloroform for 30 min at 40°C and insoluble material was removed by centrifugation. One ml chloroform and 1 ml of 0.5 M NaCl were added. The mixture was vigorously mixed and centrifuged (3 min, 300 × *g*) to obtain phase separation. The water phase was removed and the organic phase dried under nitrogen and dissolved in 50 μl chloroform. Lipids were separated on a thin layer of chromatography plates on silica gel G (Merck, Darmstadt, F.R.G.) using the solvent system: chloroform/ methanol/ acetic acid/ water (90 : 20 : 12 : 4) and analysed either by iodine staining or by autoradiography (7 days exposure, Curix RP2, Agfa film). Quantification of lipids scraped and extracted from individual spots on the plates was carried out either by gas-liquid chromatography of their fatty acid methyl esters [11] for spinach or calculated from the radioactivity in each spot for *D. salina* lipids.

Other analytical methods

Protein was determined according to Lowry, and ATPase activity of CF_0-CF_1 was measured in the presence of 25% ethanol was previously described [9].

Isotopes

[^{14}C]Sodium bicarbonate and [^{35}S]sodium sulphate were obtained from CEN, Saclay, France and [^{32}P]phosphate from the Radiochemical Center, Negev, Israel.

Results

The purification of CF_0-CF_1 from spinach thylakoid membranes involves an extensive delipidationi of the enzyme. From about 80% of the total thylakoid polar lipids, which are extracted from the membrane during the solubilization of the enzyme, only 3% are precipitated in the 37%–45% saturated ammonium sulphate fraction (crude CF_0-CF_1) and only 0.2% of the total lipids are copurified with the enzyme in the final purification step on a sucrose-gradient in the presence of Triton X-100.

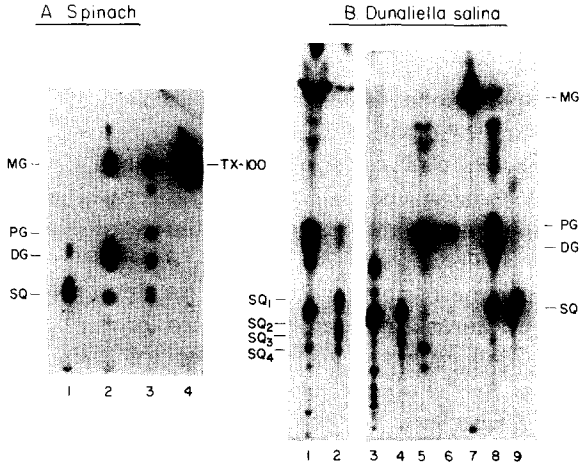

Fig. 1. Lipid analysis of CF_0-CF_1 preparations from spinach and from *D. salina* by thin layer chromatography. (A) Spinach lipids stained with iodine. Lanes 1, 2 – markers, lane 3 – crude CF_0-CF_1, lane 4 – purified CF_0-CF_1. TX-100, Triton X-100. (B) *D. salina* lipids analysed by autoradiography. *D. salina* were cultured separately in the presence of either [^{14}C]bicarbonate or [^{35}S]sulphate or [^{32}P]phosphate. CF_0-CF_1 was solubilized and purified on sucrose gradients. Lipids were extracted from fractions 4 (free lipids, lanes 1, 3, 5) and from fractions 10 (CF_0-CF_1, lanes 2, 4, 6) of the gradients, applied to TLC plates and analysed by autoradiography. [^{14}C] spots – lanes 1, 2 (total lipids), [^{35}S] spots – lanes 3, 4 (sulpholipids), [$^{32}P_i$] spots – lanes 5, 6 (phospholipids). Lanes 7, 8, 9 are markers. MG-MGDG, DG-DGDG, SQ_1, SQ_2, SQ_3, SQ_4 – the different sulpholipids identified in the CF_0-CF_1 fractions.

TABLE I

ANALYSIS OF LIPID COMPOSITION OF SPINACH CF_0-CF_1 PREPARATIONS

Preparations	Lipid/protein (%)	Relative amount of lipid (mol %)			
		MGDG	DGDG	SQ	PG
Thylakoid membranes	50	50	25	8	13.5
Crude CF_0-CF_1	8	40.5	13	28	17
Purified CF_0-CF_1	1	–	10 (0.5) [a]	90 (5) [a]	–

[a] Lipids were extracted from individual spots on the TLC plates (Fig. 1) and their content was determined either by gas liquid chromatography of their fatty acid esters (spinach, Table I) or from the radioactivity of [^{14}C], [^{35}S] or [^{32}P] in lanes 2, 4 and 6, respectively, in Fig. 1B (*D. salina*, Table II). The lipid-to-protein ratio was calculated by assuming a molecular weight of $5 \cdot 10^5$ for CF_0-CF_1.

These bound lipids make up about 1% (by weight) of the CF_0-CF_1 preparation of spinach (Table I) and about 4% in CF_0-CF_1 preparation of the halotolerant alga *Dunaliella salina* (Table II).

Analysis of the lipid composition of spinach CF_0-CF_1 preparations shows a progressive relative enrichment in sulphoquinovosyldiacylglycerol from 28% in the 'crude' preparation to about 90% in the purified preparation – namely a 10-fold enrichment in comparison to the composition of polar lipids in the thylakoid membrane (Fig. 1A, Table I). The heavy spot evident in the lipid extract of purified CF_0-CF_1 (Fig. 1A, lane 4) is due to Triton-X-100 carried over from the sucrose gradient purification which migrates similar to MGDG in this particular solvent system The absence of MGDG in this location was confirmed by analysis of esterified fatty acids and by a prolonged dialysis which removed most of the detergent from the preparation (not shown).

A similar result is obtained from the analysis of bound lipids in CF_0-CF_1 from *D. salina* cultured in the presence of [^{14}C]bicarbonate, [^{35}S]sulphate and [^{32}P]phosphate to label total lipids, sulpholipids and phospholipids, respectively. Lipid extraction of samples from the sucrose-gradient purification, demonstrated in Fig. 2, shows a clear enrichment of the enzyme-containing fractions in sulpholipids and a depletion in phospholipids. Analysis of the bound lipids by autoradiography of the separated lipids on TLC plates in shown Fig. 1B and summarized in Table II. Sulpholipids make up about 75% of the total bound lipids, but their composition in the case of the *D. salina* CF_0-CF_1 is heterogenous in contrast to spinach enzyme-bound sulpholipids. In addition to the major spot (65%), which appears to be identical to *D. salina* sulphoquinovosyldiacylglycerol (SQ_1), there are several more polar minor sulpholipids (SQ_{2-4}) which may be either other molecular species or breakdown products of SQ. By assuming a molecular weight of $5 \cdot 10^5$ for CF_0-CF_1 we

TABLE II

LIPID COMPOSITION OF *D. SALINA* PURIFIED CF_0-CF_1

Analysed Lipid	Lipid/protein (%)	(mol/mol) [a]	Amount of bound lipid [a] (mol lipid/mol CF_0-CF_1)				
			MGDG	DGDG	SQ_1	SQ_{2-4}	PG
[^{14}C] total lipids	4.2	26	2	2	12.2	7.3	2
[^{35}S] sulpholipids	2.7	17	–	–	11.6	5.4	–
[^{32}P] phospholipids	0.2	1	–	–	–	–	1

[a] Lipids were extracted from individual spots on the TLC plates (Fig. 1) and their content was determined either by gas liquid chromatography of their fatty acid esters (spinach, Table I) or from the radioactivity of [^{14}C], [^{35}S] or [^{32}P] in lanes 2, 4 and 6, respectively, in Fig. 1B (*D. salina*, Table II). The lipid-to-protein ratio was calculated by assuming a molecular weight of $5 \cdot 10^5$ for CF_0-CF_1.

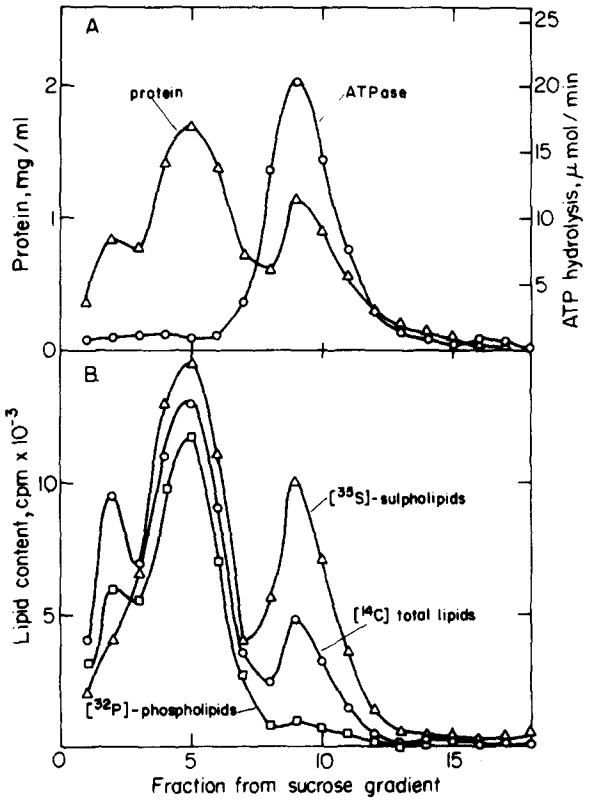

Fig. 2. Lipid and protein distribution on a sucrose gradient used to purify CF_0-CF_1. Analysis of protein (A), solvent activated ATPase (A) and lipid content (B) of fractions from the sucrose-gradient purification stage of CF_0-CF_1 from *D. salina* which was cultured in the presence of [^{14}C] bicarbonate or [^{35}S] sulphate or [^{32}P] phosphate.

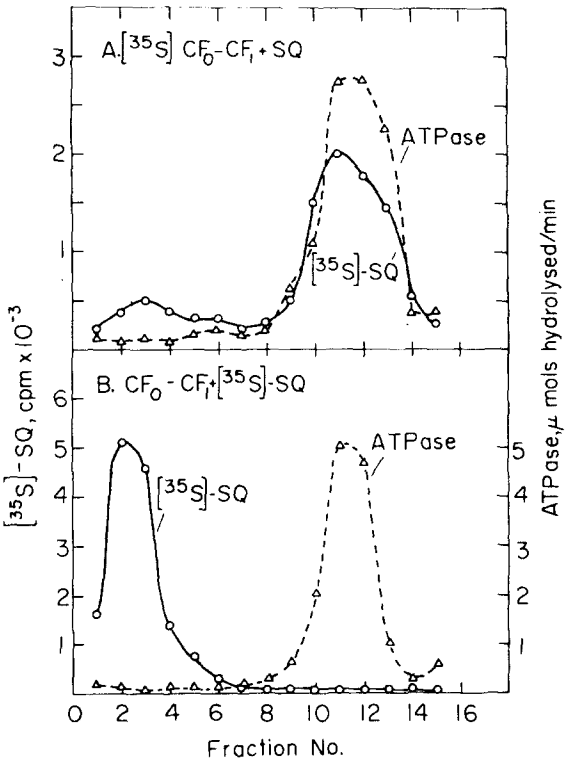

Fig. 3. Lack of exchange of bound SQ with reconstituted lipids. (A) CF_0CF_1 (40 μg) was purified from *D. salina* which was cultured in the presence of [^{35}S] sulphate and reconstituted with the following glycolipid mixture: MGDG (1.2 mg)/DGDG (0.6 mg)/SQ (0.2 mg) as in Ref. 6. The proteoliposomes where solubilized in 2 mg Triton X-100 and analysed on a 5–30% sucrose gradient. (B) Spinach CF_0-CF_1 (55 μg) was reconstituted with a glycolipid mixture containing [^{35}S]-SQ (10^5 cpm) and analysed as in (A). Lipids were extracted from 100 μl samples (of 600 μl fractions) from the sucrose gradients. Mg-ATPase activity was measured as in Ref. 6.

calculated 5 and 20 bound SQ molecules per mol CF_0-CF_1 of spinach and *D. salina*, respectively.

In order to understand the role of bound SQ in CF_0-CF_1 we tried to resolve the lipid from the enzyme by several approaches.

(1) An attempt to exchange the bound sulpholipids with phospholipids during the purification on a sucrose gradient was made by addition of 0.1% PC or PS (sonicated with 0.2% Triton X-100) to the gradients [7]. However,the presence of phospholipids did not decrease the amount of bound SQ indicating that under these conditions there is no exchange between phospholipids and bound SQ.

(2) An attempt to exchange bound SQ with glycolipids was made by reconstitution of CF_0-CF_1 with a mixture of glycolipids [6] followed by solubilization in Triton X-100 and analysis on a sucrose gradient to resolve the enzyme from the free lipids. Fig. 3 demonstrates that in spite of the 20-fold excess of free over bound sulpholipids under these conditions there is neither an appreciable exchange of bound [^{35}S]SQ with reconstituted lipids (3A) nor is there an exchange of free [^{35}S]SQ into bound sulpholipids (3B).

(3) Attempts to exchange bound SQ with different detergents (5 days of dialysis against Triton X-100, sodium cholate or octylglucoside) resulted in loss of only 10–30% of the bound SQ and was accompanied by an inhibition of ATPase activity

which could not be restored by reconstitution with glycolipids (data not shown).

These results suggest that the association between sulpholipids and CF_0-CF_1 is extremely strong and is essential for the integrity of the enzyme.

Discussion

Very little is known about the role of sulpholipids in chloroplast thylakoid membranes. Anderson suggested that sulphoquinovosyldiacyl glycerol may be good candidate for a fixed-boundary lipid within the chloroplast [12]. Indeed, there is some experimental evidence for a structural role of sulpholipids in the organization of chlorophyll in thylakoid membranes (reviewed in Ref. 13). A specific role of SQ in CO_2 fixation was suggested from the observation that substitution of sulphate for cysteine in the growth medium of the alga *Chlorella* resulted in depletion of SQ in chloroplasts and was correlated with inhibition of CO_2 fixation [14].

The results described in this communication suggest that purified CF_0-CF_1 from chloroplasts of higher plants and algae is a lipoprotein complex which contains tightly bound sulpholipids.

Although the possibility that the presence of bound sulpholipids in CF_0-CF_1 preparations is an artificat of the purification procedure cannot be ruled out it seems unlikely in view of the following observations. (a) Chloroplasts sulpholipids are more easily solubilized by the detergents used for purification of CF_0-CF_1 than MGDG (unpublished results) making it very unlikely that the presence of bound sulpholipids in CF_0-CF_1 results from a preferential enrichment due to poor solubilization. (b) The possibility that the bound sulpholipids are trapped between aggregated CF_0-CF_1 molecules seems unlikely due to the complete solubilization of the enzyme in detergents prior to reconstitution and the uniform distribution of intramembranal CF_0 particles in reconstituted proteoliposomes [15]. (c) Solubilization and purification of CF_0-CF_1 in the presence of a different detergent (sodium cholate) yields a similar amount of bound sulpholipid per enzyme as well as traces of MGDG. Dialysis removes completely the MGDG, but none of the SQ from this preparation indicating that unlike SQ, MGDG seems to be loosely associated with the enzyme. (d) Sulphoquinovosyldiacylglycerol is required for the catalytic activation of ATP hydrolysis as will be described in another publication.

It is interesting to note that Livne and Racker [16] have reported that sulpholipids specifically protect CF_1 against heat inactivation. These results also suggest a specific association between CF_0-CF_1 and sulpholipids, and is consistent with our results.

We have recently demonstrated that reconstitution of CF_0-CF_1 with chloroplast glycolipids stimulates ATPase activity of the enzyme and that this activation is probably due to MGDG [6]. In the present study we did not find evidence for tightly bound MGDG in CF_0-CF_1 preparations possibly suggesting that the affinity of the enzyme for MGDG is not as high for SQ. The interrelationship of the MGDG and SQ activation of CF_0-CF_1 will be discussed in another paper. It is interesting, however, to note that a correlation may exist between the interactions of cardiolipin with mitochondrial enzymes and SQ with CF_0-CF_1. Cardiolipin, the major negatively charged lipid in mitochondrial membranes, is required for the catalytic activation of cytochrome oxidase [4] and F_0-F_1 ATP synthase [17]. It has been found to be the only tightly-bound lipid in purified cytochrome oxidase [18] and was shown to be specifically associated with this enzyme in recontsituted phospholipid vesicles [19]. These results, together with our finding, may suggest that cardiolipin and sulphoquinovosyldiacylglycerol share a common property of playing a special role in the mechanism of energy coupling in both respiratory and photosynthetic systems, respectively.

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