

# Determination of Glycolipids, Sulfolipid and Phospholipids in the Thylakoid Membrane

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Determination of binding of antibodies to lipids onto the surface of the thylakoid membrane, before and after the removal of the CF<sub>1</sub>-complex with sodium bromide, showed that in the immediate vicinity of CF<sub>1</sub> sulfolipid and monogalactolipid occur in higher concentration and are therefore arranged in domains. The molar ratio of the CF<sub>1</sub>-complex to glycolipids was determined in *Nicotiana tabacum* chloroplasts of different structure. Thus, in the chlorophyll-deficient tobacco mutants *N. tabacum* Su/su and Su/su var. Aurea, the molar ratio of CF<sub>1</sub>/monogalactolipid is the same and found to be 1:570. The structure of the lamellar system in these mutants is characterized by a higher ratio of stroma lamellae to grana stacks when compared to the green wild type. In the wild type the ratio CF<sub>1</sub>/monogalactolipid is 30 per cent larger (1:740). In contrast to this the molar ratio CF<sub>1</sub>/sulfolipid and CF<sub>1</sub>/digalactolipid is the same in the wild type and the Su/su mutant, whereas these ratios are twice as high in the yellow mutant *Nicotiana tabacum* Su/su var. Aurea.

The binding of glycolipids and phospholipids onto the subunits of CF<sub>1</sub> from *Spinacia oleracea* was determined in the Western blot procedure by using monospecific antisera. These experiments lead to the result that the two large subunits ( $\alpha$  and  $\beta$ ) are marked by antisera to monogalactosyldiglyceride, digalactosyldiglyceride and sulfoquinovosyldiglyceride. The antisera to phospholipids react differently: whereas the antiserum to phosphatidylinositol only reacts with the  $\alpha$ -subunit, the antiserum to phosphatidylcholine and that to phosphatidylglycerol react just as the antisera to glycolipids with both large subunits. It is observed that the antiserum to monogalactolipid occasionally marks the  $\gamma$ -subunit. This might mean that the glycolipids and the respective phospholipids are tightly bound onto the reacting  $\alpha$ - and  $\beta$ -subunits of the CF<sub>1</sub>-complex. Incubation of the subunit CF<sub>1</sub> with lipase from *Rhizopus arrhizus* and with phospholipase C from *Chlostridium perfringens* after their transfer to the nitrocellulose membrane abolishes the positive reaction of the peptides with the antisera to glycolipids and phospholipids.

## Introduction

Quantitative determinations of the maximal binding of antibodies to lipids onto the outer surface of the thylakoid membrane, before and after the removal of the CF<sub>1</sub>-complex, have led to the result that in the immediate vicinity of this complex the monogalactolipids occur in higher concentration and therefore are arranged in domains [1, 2]. This means that the thylakoid membrane spanning hydrophobic portion of the ATPase (*i.e.* CF<sub>0</sub>) is surrounded by monogalactolipids. The reason for this accumulation of monogalactolipid molecules is assumed to lie in the highly unsaturated character of the fatty acids of this lipid, which might facilitate, due to the higher fluidity of the

surrounding membrane regions, conformational changes of the complex. The hydrophilic CF<sub>1</sub>-portion of the ATPase is composed of 5 subunits with the stoichiometry  $\alpha_3, \beta_3, \gamma, \delta$  and  $\epsilon$  [3–5]. This portion protrudes out of the membrane to such an extent as to enable the binding of 8–10 antibody molecules, as shown by our determination of binding of homologous antibodies [6].

The influence of lipids on the enzymic activity of the CF<sub>0</sub> has been repeatedly described. Thus, the activity of the membrane-bound ATPase of the sarcoplasmic reticulum as well as that of erythrocytes is stimulated by phosphatidylserine [7, 8]. In activity measurements of the CF<sub>1</sub>/CF<sub>0</sub>-complex in proteo-liposomes Pick *et al.* [9] found that a high monogalactolipid portion (60% of total lipids) in a lipid mixture, which corresponds to the composition of the thylakoid membrane lipids, causes a high stimulation. Here, the unsaturated character of the fatty acids apparently plays the

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decisive role, as the use of monogalactolipids as well as that of phosphatidylserine with saturated fatty acids reduces the activity again (7–9). Pick *et al.* [10] isolated CF<sub>1</sub>/CF<sub>0</sub> complexes in the form of lipid-protein complexes which contained tightly bound sulfolipid from *Spinacia oleracea* and from *Dunaliella salina*. This lipid was not replaceable neither by phospholipids nor by other glycolipids. Thus, the sulfolipid is considered to be an integral component of the CF<sub>1</sub>/CF<sub>0</sub> complex. The aim of the publication is to determine *via* the binding of antibody molecules whether in the thylakoid membrane in the immediate vicinity of the coupling factor of photophosphorylation the sulfolipid also occurs in a domain-like arrangement as is the case for the monogalactolipid [1]. Moreover, a comparative analysis is made in *Nicotiana tabacum* chloroplasts of different lamellar structures [6, 11–13] in order to determine the correlation between CF<sub>1</sub>/CF<sub>0</sub>-complex present and the glycolipids in the thylakoid membrane. Furthermore, we analyze in the present paper by means of the Western blot technique, using monospecific lipid antisera, the binding of glycolipids and phospholipids onto the subunits of the coupling factor of photophosphorylation.

## Materials and Methods

### Antisera

Monospecific polyclonal antisera to the two galactolipids mono- and digalactosyl-diglyceride [14, 15], to the sulfolipid [16], to the entire coupling factor [6], to the tunnel protein C<sub>IV</sub>\* of the CF<sub>0</sub>-complex [17] and those to the phospholipids phosphatidylcholine, phosphatidylinositol and phosphatidylglycerol [1] were obtained by immunization of rabbits. The monospecificity of the phosphatidylcholine and -inositol antisera were demonstrated in the passive heme agglutination test, and for the antisera to galactolipids, sulfolipid and phosphatidylglycerol in the ELISA-test and Dot Blot procedure [18]. The demonstration of the monospecificity of the CF<sub>1</sub>-antisera was carried out by double diffusion tests and cross immunoelectrophoresis. When using the lipid antisera as reagents in the Western blot procedure, IgG prep-

arations, obtained by ammonium sulfate precipitation, were used.

### SDS polyacrylamide gel electrophoresis and Western blot procedure

The analysis of the polypeptides was carried out as described earlier by use of a 3.5% polyacrylamide collector gel and a 10% separation gel or by a 9–12% polyacrylamide gel gradient [18]. The protein samples (15  $\mu$ g CF<sub>1</sub>-complex per run) were incubated for 5 min at 100 °C with 60  $\mu$ l sample-buffer (0.02 M Tris buffer, containing 4% SDS and 4% mercapto ethanol). Thereafter, the samples were supplemented with a drop of bromide phenol blue and a drop of 60% sucrose. The electrophoresis was run at 4 °C for 16 h at 0.12 mA/cm<sup>2</sup>. The transfer of the analyzed peptides to nitrocellulose membranes (Schleicher and Schüll BA 85) was done according to Rennart *et al.* [19] by diffusion at 4 °C during 30 h. The incubation with antibodies as well as demonstration of antigen-antibody complex formation on the nitrocellulose membranes was carried out according to earlier described methods [18].

### Enzymic decomposition of glycolipids and phospholipids

For the enzymic decomposition of the lipid antigenic determinants the peptides of the CF<sub>1</sub>-complex were analyzed in the SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose (NC) membranes and incubated in this condition with the corresponding lipases. For the decomposition of the glycolipid antigenic determinants the NC-membranes were treated with lipase from *Rhizopus arrhizus* (Boehringer Mannheim, 50,000 units/ml). For this purpose the preparation was diluted 100-fold with buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>-buffer containing 0.15 M NaCl and 3 mM KCl) and incubated at room temperature for 30 min. For the splitting of phospholipids the NC-membranes were incubated with phospholipase from *Chlostridium perfringens* (Sigma, Heidelberg, preparation type 1, 10–20 units per mg protein). This enzyme preparation was diluted 20-fold with 50 mM Tris pH 7.35. Thereafter, the NC-membranes were incubated with lipid antisera with the further treatment according to an earlier publication [18].

\* The tunnel protein C<sub>IV</sub> of CF<sub>0</sub> was a gift from Dr. W. Sebald.

*Methanol treatment of the transferred polypeptides*

For the extraction experiments the subunits of  $CF_1$ -complexes were transferred to the Immobilion-P Transfer Membrane (Millipore Corporation Bedford, No. IPUH 20200). The membranes were washed with 5.5 mM phosphate puffer, containing 0.1 M NaCl and 2.6 mM KCl and then washed for 1 h under continuous shaking in methanol. The other detection reactions were carried out as described earlier.

*Quantitative determination of antibody binding onto the thylakoid membrane*

Stroma-freed chloroplasts from *Antirrhinum majus* were obtained according to earlier described methods [1]. These chloroplast preparations consist exclusively of intact lamellar systems, which are called stroma-freed chloroplasts. 30 mg chloroplasts were supplemented in parallel assay series with increasing amounts, 0.05, 0.1, 0.3 and 0.7 ml of antiserum and control serum (Table I). Each assay was adjusted to 1.5 ml with 0.06 M phosphate buffer pH 7.3 and incubated for 4 h at 20 °C and for 16 h at 4 °C. Thereafter the unfixed antibodies were removed by a first washing with the above described phosphate buffer. Thereafter the chloroplast preparation loaded with antibodies was washed five times with the same buffer and then subjected to a protein determination according to Lowry *et al.* [20]. The amount of bound antibodies was determined by subtracting from the protein value of the respective antiserum assay the value obtained by incubation with the control serum (Table I).

*Removal of the  $CF_1$ -complex*

For the removal of the  $CF_1$ -complex 100 mg stroma-freed chloroplasts were suspended in 10 ml 2 mM tricine buffer pH 8, containing 40 mM NaCl, 1 mM  $MgCl_2$ , 0.3 M sucrose and 2.0 M sodium bromide [21]. After a 30 min treatment by shaking the suspension in an ice cooled water bath, the chloroplasts were diluted (1:1 v/v) with distilled water and sedimented by centrifugation for 20 min at  $3000 \times g$ . The sediments were washed two more times in 0.06 M phosphate buffer and centrifuged each time before being used for the determination of the antibody binding. For control experiments stroma-freed chloroplasts were treated according

to the above described method with 0.06 M phosphate buffer only.

**Results***Distribution of sulfolipid in the outer surface of the thylakoid membrane*

The distribution of the sulfolipid molecules was determined, as in the case of monogalactosyldiglyceride, *via* an analysis of the maximal binding of antibodies onto the outer surface of the thylakoid membrane, before and after the removal of the  $CF_1$ -complex with sodium bromide. As chloroplast preparation we used so-called stroma-freed chloroplasts, from *Antirrhinum majus* sibling 50, since these chloroplasts have a fully intact lamellar system with a defined outer surface. As seen in Fig. 1 from the course of antibody binding before and after removal of the  $CF_1$ -complex, the amount of antibodies to sulfolipid bound in dependence on the amount of antiserum added reaches a saturation value. This means that even after the removal of the hydrophilic portion of the ATPase only a certain amount of antibodies can be bound. This amount corresponds either to the number of sulfolipid molecules on the surface or the amount of antibodies bound is limited by the steric relation of the sulfolipid molecules to each other. If one compares the relationship between size of the reacting antigen and antibody molecules, considering in particular the voluminous shape of antibody molecules, it becomes clear that by the binding of an

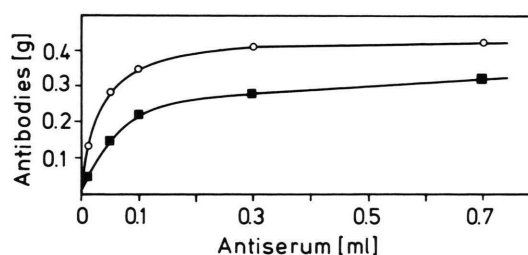


Fig. 1. Course of the binding of antibodies to sulfoquinovosyldiglyceride onto the outer surface of the thylakoid membrane of *Antirrhinum majus*, before and after treatment of stroma-freed chloroplasts with sodium bromide, in dependence on the amount of antiserum. ■-■ binding of antibodies of sulfolipid onto stroma-freed chloroplasts, washed with 0.1 M Tricine buffer pH 8.0 (control). ○-○ binding of antibodies to sulfolipid onto stroma-freed chloroplasts after removal of the  $CF_1$ -complex by washing with sodium bromide.

Table I. Binding of antibodies onto the outer surface of the thylakoid membrane of *Antirrhinum majus* chloroplasts.

Antiserum to	Treatment of stroma-freed chloroplasts	Number of antibody molecules bound ( $\times 10^{17}$ )
CF <sub>I</sub> -complex	control, untreated	14
CF <sub>I</sub> -complex	sodium bromide	8
Sulfolipid	control, untreated	10
Sulfolipid	sodium bromide	17
Monogalactolipid	control, untreated	6
Monogalactolipid	EDTA	15
Stroma-freed chloroplasts	control, untreated	42
Proteins of the thylakoid membrane	control, untreated	41

The number of antibody molecules refers to 1 g chloroplasts. The antibody binding values for monogalactolipid, proteins of the thylakoid membrane and for stroma-freed chloroplasts are taken from earlier publications [1, 2]. EDTA: ethylenediaminetetraacetic acid.

antibody to a sulfolipid molecule, a considerably larger area is covered than that occupied by the sulfolipid molecule itself. The results shown in Table I demonstrate that after partial removal of the CF<sub>I</sub>-complex twice the amount of sulfolipid molecules can be bound. This permits the conclusion that in the surface of the thylakoid membrane in the vicinity of the CF<sub>I</sub>-complex not only monogalactolipid molecules but also sulfolipid molecules are located in domains. The high binding value of antibodies to CF<sub>I</sub>, also after treatment of stroma-freed chloroplasts with sodium bromide, is due to the fact that a relatively small number of antigen molecules evenly distributed in the membrane surface permits for steric reasons the binding of a larger number of antibody molecules than a large number of antigen molecules lying closely together.

#### *Molar ratio between CF<sub>I</sub>-complex and glycolipids in chloroplasts of differing structures*

With immunological methods we were able to show that in the thylakoid membrane in the immediate vicinity of the CF<sub>I</sub>-complex not only monogalactolipids [1, 2] but also sulfolipids are arranged in a domain-like structure. Therefore, chloroplasts of *Nicotiana tabacum* species which differ with respect to the structure of their lamellar system [11, 13] were studied with the aim to see whether a stoichiometric relationship between the CF<sub>I</sub>-complex present and the mentioned glycolipids exists. Whereas chloroplasts of the wild type have a nor-

mal ratio between grana and stroma thylakoids, chloroplasts of the tobacco mutant Su/su var. Aurea have a lamellar system with extended single thylakoids and only occasional membrane doublings [13, 22, 23]. The chloroplasts of the Su/su mutant are structurally located in between these two chloroplast types [11, 12, 22]. However, also in this mutant stroma thylakoids prevail [6]. We were able to show with immunological methods that the CF<sub>I</sub>-content depends on the structure of the lamellar system of the respective chloroplasts. In the three types of chloroplasts studied the CF<sub>I</sub>-content increases by 40 per cent when stroma thylakoids increase [6]. In the same trend the content of monogalactolipids increases in these chloroplasts by 60 per cent and that of the sulfolipids by 13 per cent. The molar ratio of CF<sub>I</sub> to these glycolipids in these *Nicotiana tabacum* chloroplasts is given in Table II. The ratio of CF<sub>I</sub> to monogalactolipid is approximately constant in chloroplasts of the two Su/su mutants and found to be approx. 30% higher than in the wild type. Due to the fact that this ratio is constant in the mutants which have, as shown earlier, a higher CF<sub>I</sub>-content and a smaller light harvesting complex of photosystem II [12, 13, 22], it is concluded that the number of monogalactolipid molecules associated with the CF<sub>I</sub>-complex is constant. In the wild type this ratio is apparently hidden by the higher portion of the monogalactolipids associated with the light harvesting complex of photosystem II [25]. For the ratio of sulfolipid to CF<sub>I</sub> no clear proportionality is seen, as the ratio appears to be nearly the same in chloroplasts of

Table II. Molar ratio of CF<sub>1</sub>-complex to monogalactolipid, digalactolipid and sulfolipid in chloroplasts of different *Nicotiana tabacum* species.

	Chloroplast preparations		
	Wild type JWB	Su/su	Su/su var. Aurea
CF <sub>1</sub> /MGDG	1/741	1/576	1/567
CF <sub>1</sub> /DGDG	1/490	1/445	1/238
CF <sub>1</sub> /SL	1/155	1/139	1/85

The amount of CF<sub>1</sub>-complex was determined by immunological methods via rocket-immune electrophoresis [6]. In the three chloroplast types studied the CF<sub>1</sub>-content is increased in the Aurea chloroplasts in comparison to the wild type chloroplasts by 40% [6]. The lipid determination was carried out according to earlier described methods [14–16, 24].

the Su/su mutant and the wild type, but reduced to half in chloroplasts of the mutant *N. tabacum* Su/su var. Aurea. The situation for the digalactolipid appears to be the same as for the sulfolipid.

#### Binding of Glycolipids and Phospholipids onto the Subunits of the CF<sub>1</sub>-Complex

In order to determine the binding of lipid molecules onto subunits of the CF<sub>1</sub>-complex\*, the peptides of this complex were analyzed by SDS-polyacrylamide gel electrophoresis. The analysis of CF<sub>1</sub> from spinach yields 5 subunits (Fig. 2) with strong bands for the  $\alpha$  and  $\beta$  subunits which contribute to the structure of CF<sub>1</sub> with the 3-fold amount in comparison to the  $\gamma$ ,  $\delta$  and  $\epsilon$ -subunits. Besides these five bands in the SDS-polyacrylamide gel electrophoresis of the CF<sub>1</sub>-complex of spinach occasionally two more bands in the region of 96 and 43 kDa appear (Fig. 2). As these bands react in the Western-Blot-technique on nitrocellulose membranes after incubation with the homologous antiserum to the intact CF<sub>1</sub>-complex (Fig. 3), we conclude that they represent aggregates or not fully separated subunits of the CF<sub>1</sub>-complex. In order to verify the purity of the CF<sub>1</sub>-complex the analyzed and transferred subunits were incubated with an antiserum to the proton channel-forming 8 kDa peptide [26]. The reaction was negative, which means, that the CF<sub>1</sub> preparation used for the characterization of lipids did not contain im-

purities coming from the CF<sub>0</sub>-portion of the complex.

The analyzed subunits were transferred by diffusion to nitrocellulose membranes and incubated with monospecific antisera to the glycolipids monogalactolipid, digalactolipid and sulfolipid as well as to the phospholipids, phosphatidylinositol, phosphatidylcholine and phosphatidylglycerol. As seen in Fig. 3 an antiserum to the monogalacto-

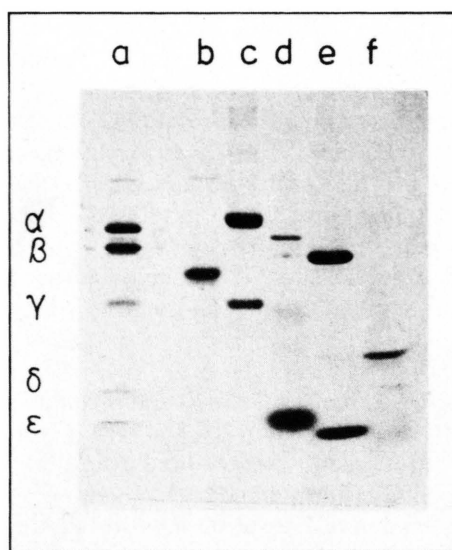


Fig. 2. SDS-polyacrylamide gel electrophoresis of the coupling factor of photophosphorylation from *Spinacia oleracea* (CF<sub>1</sub>). a) CF<sub>1</sub>-complex from spinach; b–f) marker proteins: b) phosphorylase (92 kDa) and ovalbumine (45 kDa); c) bovine serum albumine (67 kDa) and D-aminoacid oxidase (37 kDa); d) catalase (60 kDa) and myoglobine (17.5 kDa); e) glutamate dehydrogenase (56 kDa) and cytochrome c (12.3 kDa); f) hexokinase (25 kDa) and trypsin (24 kDa).

\* CF<sub>1</sub>-complex preparations are the gift of Professors Dr. S. Bickel-Sandkötter, Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität in Düsseldorf, and Dr. P. Gräber, Biologisches Institut der Universität Stuttgart.

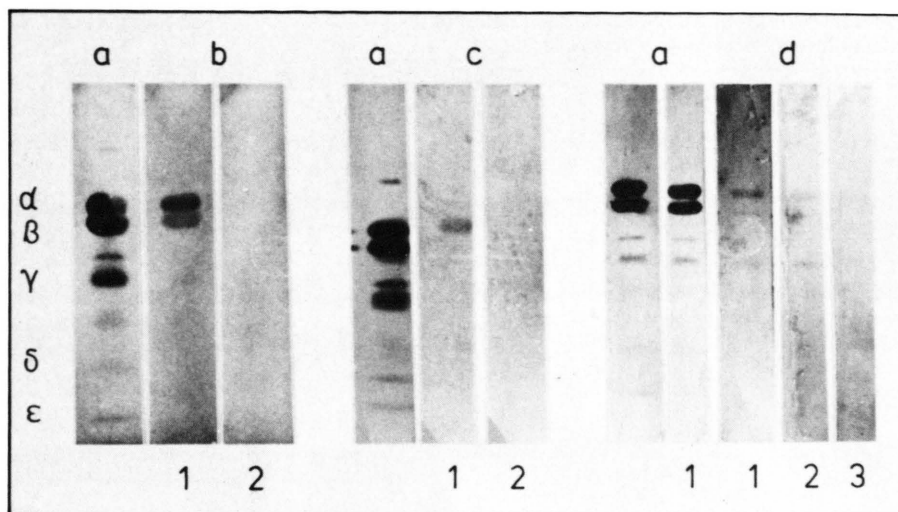


Fig. 3. Reaction of the polypeptides of the CF<sub>1</sub>-complex from spinach with the antisera to monogalactolipid and to the CF<sub>1</sub>-complex in the Western Blot procedure. Nitrocellulose membrane with the polypeptides after reaction with antisera to: a) CF<sub>1</sub>-complex (serum dilution 1:300); a<sub>1</sub>) CF<sub>1</sub>-complex (serum dilution 1:600); b<sub>1</sub>) monogalactolipid (89 H 5) (serum dilution 1:100); c<sub>1</sub>) monogalactolipid (83 H 2) (serum dilution 1:100); d<sub>1</sub>) monogalactolipid (89 H 5) (serum dilution 1:200); d<sub>2</sub>) monogalactolipid (89 H 5) (serum dilution 1:600); b<sub>2</sub>, c<sub>2</sub>, d<sub>3</sub>) control sera (serum dilution 1:100).

lipid (83 H) marks the two large subunits  $\alpha$  and  $\beta$  with very high intensity. With a second antiserum, in dependence on the serum dilution, differing marker intensities were observed. At a serum dilution with the factor 1:100 only the  $\alpha$ -subunit is marked whereas at the stronger dilutions of 1:300 to 1:600 the two large subunits and in addition the  $\gamma$ -subunit appears to be marked (Fig. 3, band d<sub>1,2</sub>). The corresponding control sera gave at the same dilution no reaction. For the detection of the digalactolipid the serum of only one animal was used, testing separately the serum from the original immunization and that of the first booster immunization. Whereas the serum of the first immunization marked at a dilution of 1:20 the  $\alpha$ - and  $\beta$ -subunits with a relatively strong intensity, the serum from the booster injection only marked (at the same dilution) the  $\alpha$ -subunit (Fig. 4, band b<sub>1,2</sub>).

For the detection of the sulfolipid also two different antisera were used. Both sera, just as in the case of the sera to galactolipids, behaved differently. Whereas one serum (29 H) at a dilution factor of 1:100 marked both the  $\alpha$ - and  $\beta$ -subunits with the same intensity (Fig. 4c<sub>1</sub>), the second antiserum at a dilution of 1:100 marked only the  $\alpha$ -subunit (Fig. 4d<sub>1</sub>). At any rate, from these positive marker experiments it can be concluded that the galacto-

lipids mono- and digalactosyldiglyceride and the anionic sulfolipid are bound onto the  $\alpha$ - and  $\beta$ -subunits of the CF<sub>1</sub>-complex from spinach chloroplasts.

The reactions with the phospholipid antisera are summarized in Fig. 5b, c and d. From this figure it is clearly seen, that the phospholipid antisera react differently. Whereas the antiserum to phosphatidylinositol marks only the  $\alpha$ -subunit, the antisera to phosphatidylcholine and phosphatidylglycerol mark both large subunits (Fig. 5b–d). The antiserum to phosphatidylinositol and that to phosphatidylglycerol are pooled sera from 3, respectively 2 different monospecific polyclonal antisera. The lecithin antiserum originates from the immunization of only one animal and had to be used in a 2-fold higher concentration. The experiments show that phosphatidylinositol is only bound to the  $\alpha$ -subunit whereas lecithin and the electronegative phosphatidylglycerol are localized on both large subunits. The  $\delta$ - and  $\epsilon$ -subunit were not marked under the described antigen-antibody ratio conditions, neither with the glycolipid, nor with the phospholipid antisera. As the molecular masses of these small subunits represent only 30 to 40% of the mass of the  $\alpha$ - and  $\beta$ -subunits which in addition contribute with three identical subunits in

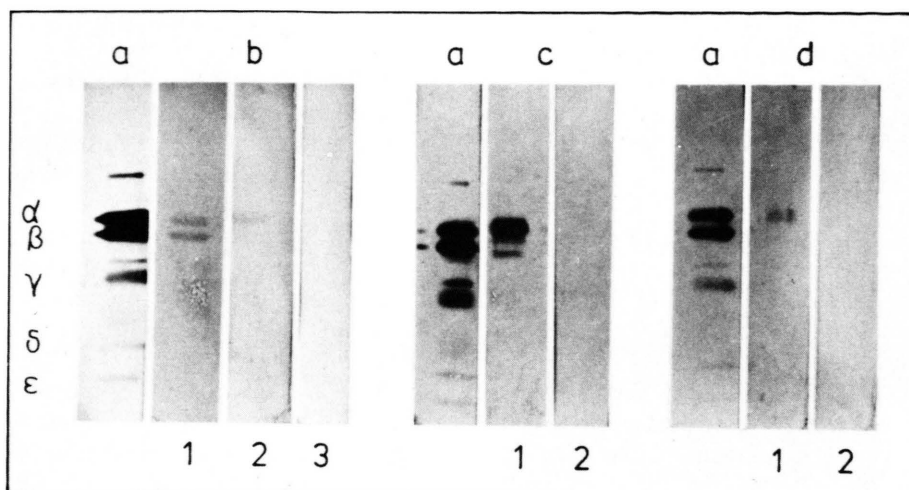


Fig. 4. Reaction of the polypeptide subunits of the CF<sub>1</sub>-complex from spinach with the antisera to digalactolipid, sulfolipid and CF<sub>1</sub>-complex in the Western Blot procedure. Nitrocellulose membrane with the polypeptides after the reaction with antisera to: a) CF<sub>1</sub>-complex (serum dilution 1:300); b<sub>1</sub>) digalactolipid (92 H<sub>2</sub>) (serum dilution 1:20); b<sub>2</sub>) digalactolipid (92 H<sub>8</sub>) (serum dilution 1:20); c<sub>1</sub>) sulfolipid (29 H<sub>10</sub>) (serum dilution 1:100); d<sub>1</sub>) sulfolipid (15 S<sub>4</sub>) (serum dilution 1:100); b<sub>3</sub>, c<sub>2</sub>, d<sub>2</sub>) control sera with the same dilutions as the respective antisera.

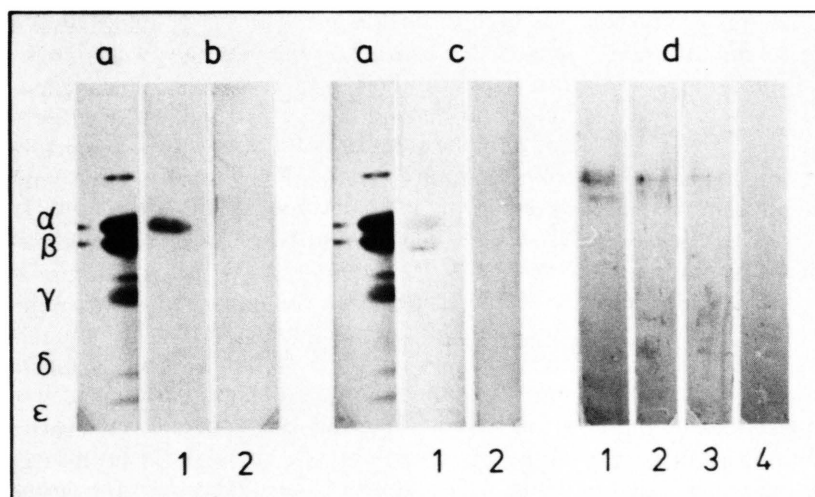


Fig. 5. Reaction of the polypeptides of the CF<sub>1</sub>-complex from spinach with antisera to phosphatidylinositol, phosphatidylcholine and phosphatidylglycerol in the Western Blot procedure. Nitrocellulose membrane with the polypeptides after reaction with antisera to: a) CF<sub>1</sub>-complex (serum dilution 1:300); b<sub>1</sub>) phosphatidylinositol, mixture of the sera 38 H<sub>3</sub>, 39 H<sub>2</sub>, 42 H<sub>4</sub> (serum dilution 1:100); c<sub>1</sub>) phosphatidylcholine (48 H<sub>2</sub>) (serum dilution 1:50); d<sub>1</sub>) phosphatidylglycerol (22 P<sub>2</sub>, 51 D<sub>1</sub>) (serum dilution 1:50); d<sub>2</sub>) phosphatidylglycerol (22 P<sub>2</sub>, 51 D<sub>1</sub>) (serum dilution 1:100); d<sub>3</sub>) phosphatidylglycerol (22 P<sub>2</sub>, 51 D<sub>1</sub>) (serum dilution 1:200); b<sub>2</sub>, c<sub>2</sub>, d<sub>4</sub>) control sera with the same dilution as the respective antisera.

each case to the structure of the CF<sub>1</sub>-complex, the absence of a positive reaction might be due to a too small amount of antigen. But even in the presence of a 3-fold amount of antigen (45  $\mu$ g CF<sub>1</sub> complex instead of 15  $\mu$ g) in the marker experiments, the result was negative with the sulfolipid and a monogalactolipid antiserum.

In order to characterize the antigenic determinants of the lipids and in order to obtain some information on the type of binding of these lipids onto the peptides, the analyzed subunits of the CF<sub>1</sub>-complex were incubated after the transfer to the nitrocellulose membranes with lipases. For the decomposition of the glycolipids we used lipases from *Rhizopus arrhizus* whereas phospholipids were incubated with the phospholipase C from *Chlostridium perfringens*. The further detection reactions are described in Materials and Methods. It was clearly seen that neither the phospholipid antisera nor the glycolipid antisera react with the lipase-treated subunits. Under these condition the antigenic determinants are apparently decomposed. As it is unclear, to what extent lipids are split off the peptides by sodium dodecyl sulfate in the polyacrylamide gel electrophoresis or undergo even a delocalization [27, 28], the immobilized membranes were washed with methanol for 1 h after the transfer of the peptides and before incubation with the lipid antisera. After this pretreatment the glycolipid and phospholipid antisera reacted positively with the  $\alpha$ - and  $\beta$ -subunits.

## Discussion

Marker experiments with monospecific polyclonal glyco- and phospholipid antisera in the Western Blot procedure have shown that the electroneutral galactolipids and the phospholipids phosphatidylcholine and phosphatidylinositol as well as the two electronegative (anionic) lipids sulfoquinovosyldiglyceride and phosphatidylglycerol are bound to the two large subunits  $\alpha$  and  $\beta$  of the CF<sub>1</sub>-complex in spinach chloroplasts. The small subunits  $\delta$  and  $\epsilon$  reacted with none of the antisera tested, even if in undiluted assays the antigen concentration was raised by a factor of at least three. Occasionally the  $\gamma$ -subunit appeared to be marked by one of the monogalactolipid antisera. Thus, with immunological methods it has been demonstrated that the main lipids, which form the bilayer of the thylakoid membrane also occur on the large

subunits of the CF<sub>1</sub>-complex which reaches far into the stroma.

The use of two different antisera in the case of the monogalactolipid and sulfolipid or using a first immunization serum and the serum after one booster immunization in the case of the digalactolipid have shown that either only the  $\alpha$ -subunit reacted or that the  $\alpha$ - and  $\beta$ -subunit were marked with different intensity. As polyclonal antisera with differently reacting antibody populations were used as reagents it can be concluded that the respective lipids are differently bound to the  $\alpha$ - and  $\beta$ -subunits or that the accessibility of the lipid antigenic determinants on both subunits is different. On the other hand it cannot be excluded that the distribution of the lipids on the  $\alpha$ - and  $\beta$ -subunits is different. As the lipid antibodies are not directed towards the fatty acid region, but to the sugar-glycerol-region in the case of the glycolipids and to the glycerol-phosphate-aminoalcohol region in the case of the phospholipids, the positive reaction of the lipid antibodies shows that the lipids are linked *via* the fatty acid residues to the  $\alpha$ - and  $\beta$ -subunit. Between lipids and proteins not only intermolecular interactions exist but the lipids must also be bound co-valently. This is clearly deduced from the fact that after the transfer of the polypeptides, washing of Transmembrane Immobilon P (which was used in this case instead of the nitrocellulose membrane) with a strong polar solvent like methanol, did not abolish the reaction with the glycolipid- and phospholipidantisera. This means that not only the sulfolipid [10] but also the two galactolipids and phosphatidylglycerol are tightly bound. Only after decomposition of the lipids by lipases the antigen-antibody reaction was abolished.

The question concerning the function of the lipids bound to the coupling factor remains unanswered. As the lipids were not detected on the small  $\delta$ - and  $\epsilon$ -subunits it is assumed that these lipids have no function with respect to the binding of the CF<sub>1</sub>-complex to the thylakoid membrane or to the CF<sub>0</sub>-portion of the ATPase. As the two  $\alpha$ - and  $\beta$ -subunits contain the catalytic binding site for the reversible ATPase-reaction and as lipids are only bound to these subunits it appears reasonable to assume, that these lipids stabilize the regulatory properties of the enzyme and play somehow a role in its activation. This idea is supported by

the observation that also the  $\gamma$ -subunit contains a third catalytic binding site [29, 30] and is occasionally reacting with the monogalactolipid antiserum.

Although these immunological experiments do not give any information on the exact amount of lipids bound to these polypeptides, but since amongst others also the two galactolipids are bound, which contain 80–90% highly unsaturated fatty acids as ester component, one could imagine that these lipids are bound as lipid clusters between the hexagonally arranged  $\alpha$ - and  $\beta$ -subunits, thus facilitating conformational changes of the protein complex.

In earlier studies on photosynthetic electron transport we were able to show that the here used antisera inhibited photosynthetic electron transport on the donor side of photosystem II and pho-

tosystem I [31–34]. This inhibition was shown to be due to conformational changes of lipid-protein-complexes, caused by the binding of antibodies. The localization of these inhibition sites in the electron transport chain, as well as the binding of galactolipids, sulfolipid and phospholipids to proteins belonging to the reaction region of photosystem II and photosystem I, as shown by means of the Western Blot procedure supports this view. Subunits are accessible to antibodies also in the native condition. In an ELISA-TEST series intact CF<sub>I</sub>-complexes from spinach chloroplasts did not react with antiserum to monogalactolipid. Therefore it must be assumed that in this isolated condition the monogalactolipids are located in a not accessible condition in the interior of the CF<sub>I</sub>-complex.

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