

Mobility of Chloroplast Coupling Factor 1 (CF₁) at the Thylakoid Surface as Revealed by Freeze-Etching after Antibody Labelling

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Freeze-fracturing and 60 sec deep-etching of isolated chloroplast thylakoid systems exposed large areas of the outer surface (matrix side) of the thylakoids. If the thylakoid systems were first treated with antisera against chloroplast coupling factor 1 (CF₁), the 14 nm particles at the outer surface appeared aggregated. Between clusters these particles were absent. Since there is no change in the number of particles/area after treatment with antibodies, it is concluded that the 14 nm particles are mobile within the surface of the thylakoid. The antisera contained only antibodies against CF₁; therefore the 14 nm particles at the outer surface are identified to be CF₁. The implication of a mobile ATP-synthetase (CF₁) for the mechanisms of photophosphorylation is discussed.

Introduction

The methods, results and interpretations of investigations of chloroplast thylakoid membranes by freeze-fracturing have been extensively reviewed^{1–3}. According to the now generally accepted interpretation of the events which lead to the final micrograph, four different views of one membrane can be distinguished. Freeze-fracturing of chloroplasts yields two fracture faces of the thylakoid membrane with distinctly different morphological appearance⁴. With the “double replica” technique, it has been shown⁵ that these two faces represent the complementary aspects of the fracture plane. If the fracture occurs along an internal hydrophobic region⁶, the face which shows densely packed small particles corresponds to a view of the outer half of the membrane from inside. This face is called the “C” face by Park³ and the “outer fracture face” (OFF) by Mühlethaler⁷. The fracture face containing large particles represents the complementary inner half of the membrane as seen from the outside. It is the “B” face or “inner fracture face” (IFF). The difference in density of particles both on the OFF and the IFF seems to be due to the stacking of thylakoids in the grana region^{8, 9}.

To reveal the real outer or inner surfaces, the fractured membranes are exposed to the vacuum in order to remove a thin layer of ice by sublimation^{6, 7, 10}. This technique of freeze-drying (freeze-etching) the surfaces to a very small extent is optimal only if a fixed specimen is frozen in distilled water to avoid the formation of an eutectic. The outer surface (OS), *i. e.* the surface towards the matrix of the chloroplast, or “A” face, reveals two populations of particles with an average size of 10 nm and 14 nm respectively. The inner surface (IS) or “D” face, which faces towards the interior of the thylakoid, has only one type of complex particles which often show a central depression and a substructure of three to five units¹.

The identification of all these particles and their correlation with photosynthetic activities of the chloroplast membranes is urgently required. Histochemical approaches are ambiguous, if possible at all¹¹; and most of the probes which have been used for chemical labelling¹² are not visible in the electron microscope. On the other hand it can be questioned whether the static picture derived from electron microscopy is a true picture at all, or whether the membrane might be in constant flow and change; the distribution of the described particles across and along the thylakoid membrane might not be constant. We believe that labelling with antibodies against characterized components of the thylakoid

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will be a powerful tool to investigate both these questions.

This first publication reports the effects on particles in the thylakoid membrane of an antiserum against chloroplast coupling factor 1 (CF₁). Identification of the 14 nm particles at the OS as CF₁ is confirmed, and their mobility within the plane of the membrane is deduced.

Materials and Methods

Plants of corn salad (*Valerianella locusta*, L.), used in February, had been grown in the field in Switzerland and were purchased in the local market. Plants of *Spinacea oleracea*, used in August, had been grown in the field in Bochum (West Germany).

Chloroplast thylakoid systems were isolated according to a modified procedure of McCarty and Racker¹³, by blending with a medium containing 0.4 M sucrose, 20 mM tris-HCl pH 8 and 10 mM NaCl. After differential centrifugation, the chloroplasts were osmotically shocked twice by suspending in 10 mM NaCl. They were then collected by centrifugation and resuspended in 0.2 M sucrose, 20 mM tris-HCl pH 8 and 10 mM NaCl.

Chlorophyll was extracted with 80% acetone, and its concentration determined according to Kirk¹⁴.

Chloroplast coupling factor 1 (CF₁) was isolated from spinach chloroplasts as described¹⁵ and purified to homogeneity^{15, 16}.

Immunization of rabbits was carried out as follows: About 1 mg of pure CF₁ protein in 1 ml of buffer was mixed with 1.5 ml Freund Adjuvant (Difco), compl., and sonicated for 5 sec to yield a paste. This was injected in small portions into the foot pads of one foot and intradermally into the skin of the back. After 4 weeks, 1 mg CF₁ was injected intravenously in 0.5 ml of physiological buffer to boost the antibody response. Seven, nine and eleven days after the booster injection, blood was taken from the ear vein and the procedure repeated one month later. The sera were lyophilized without dialysis. An antiserum against ferredoxin-NADP reductase was produced as described¹⁷. Control sera had been taken from the rabbits before immunization.

Ringtests, double diffusion and immunoelectrophoretic analyses were carried out according to standard procedures¹⁸, and agglutination of washed thylakoid systems as described¹⁹.

Incubation of thylakoid systems with sera. 0.5 ml of the suspension of isolated thylakoid systems, containing 100 µg chlorophyll, was incubated at 20 °C for 15 min with 0.1 ml reconstituted serum (40

mg/ml in distilled H₂O). Two different control sera, one antiserum against ferredoxin-NADP reductase and two different antisera against CF₁ were used. A third control was incubated with 0.1 ml of a medium containing 0.1 M NaCl and 20 mM tris-HCl pH 8. The samples were spun down (0 °C, 5 min, 10,000×g), washed twice with 0.1 M NaCl and 20 mM tris-HCl buffer, and once with 0.1 M NaCl.

Fixation. The pellet was homogenized in 5 ml fixation medium containing 1.5% glutaraldehyde (Union Carbide, Genève) + 1.5% acrolin (Polysciences Inc., Warrington Pa.) in 25 mM Na-cacodylate buffer (Fluka, Buchs) pH 7.3. Subsequently it was incubated for 10 min at 24 °C and 2 hours at 4 °C. Thylakoids of *Valerianella* were fixed at 0 °C (2 hours).

Freeze-fracturing was performed as described by Moor^{20, 21}, but with the following modifications: Prior to freezing, the fixed samples were washed three times in distilled water to avoid the formation of an eutectic during freezing. The samples were broken in a vacuum of 2×10^{-6} Torr and at a temperature of -100 °C. The cold microtome knife was used as a shield and cold trap whilst ice was sublimed from the specimen surface for 60 sec. For replication with platinum and carbon, electron beam guns were used²². All the micrographs have been photographically reversed, so that evaporated platinum appears white and the shadows of structures appear black.

Particle counting was done on pictures with a final magnification of 107,000 times. All particles within an area corresponding to 0.3 µm² were counted, in squares corresponding to 0.01 µm² and twelve micrographs from each treatment were scored. Micrographs were not selected for this purpose, but chosen for their technical quality.

Results

1. Characterization of the antisera against CF₁

The two antisera used here (our ref. 19–7 and 55–11) were very probably monospecific. In immunoelectrophoretic analysis with *crude* CF₁ preparations they yielded only one arc, provided the analysis was carried out under conditions which minimize dissociation of CF₁ into subunits, which are discernable antigens¹⁶. No crossreaction could be detected with ribulosediphosphate carboxylase, ferredoxin, ferredoxin-NADP reductase, plastocyanin or cytochrome f from spinach. As indicated by the agglutination titer serum 55–11 contained 3 times as much antibodies as serum 19–7.

2. Agglutination of chloroplast thylakoid systems

Isolated thylakoid systems are agglutinated by antisera against CF₁, as observed by others^{23, 24} and by us. This reaction exhibits a zone phenomenon¹⁹. Since we did not expect to be able to expose outer thylakoid surfaces within these large three-dimensional clusters, a ratio of antiserum to particles and an overall dilution was chosen to minimize this macroscopic effect. For the same reason, the thylakoid systems were homogenized as much as possible after each wash following the incubation with the sera. It is remarkable, but not surprising in the light of earlier observations¹⁹, that the antiserum against CF₁ from spinach crossreacted with the CF₁ at the surface of thylakoid systems from *Valerianella* or *Pisum sativum*. However, it was clearly seen by phase contrast microscopy that class I chloroplasts isolated from peas as described²⁵, were not agglutinated by antisera against CF₁; *i. e.* no crossreacting antigenic determinant is present at the outer surface of the envelope.

3. Electron microscopy

Control sample treated with
0.1 M NaCl

The thylakoid systems of the chloroplasts were dispersed throughout the specimen and, due to the treatment, they were seen to be slightly swollen. Due to the extensive etching few of the exposed membrane areas showed the characteristic particles of the IFF or OFF; the others were often large (up to 3 μ m), and we can be sure that most of them corresponded to outer surfaces of thylakoids since there were round disks of grana size with exactly the same typical structure*. As shown in Fig. 1**, the 14 nm particles and the smaller particles (10 nm) are both randomly dispersed over the surface. The mean distance between the particles is several times their diameter. Occasionally, small aggregates were seen in the spinach preparation, which were absent in the thylakoids from *Valerianella*. The density of large and small particles at the OS is given in Table I.

* Only a few surfaces were entirely different and could be residual envelopes. To the best of our knowledge, this type of chloroplast surface has not been described in the literature.

** Figs 1–4 see Tables on page 696 a–b.

Table I. Particles at the outer surface of washed thylakoids of spinach. Number of particles/ μ m² on micrographs from control thylakoid systems (salt treated) and thylakoid systems treated with antiserum 19–7 against CF₁. The mean values and their standard deviations are given.

	Larger Particles	Smaller Particles	Ratio s/l
Control	1310 \pm 77	1562 \pm 50	1.19
Antiserum	1415 \pm 64	1662 \pm 63	1.18

Sample treated with control serum or antiserum against ferredoxin NADP reductase

When isolated thylakoid systems were treated with serum from unimmunized rabbits, the micrographs after freeze-etching (Fig. 2) showed no difference compared with the control. This is noteworthy because it means that no detectable protein contamination, from the incubation with the rather large amount of protein, is left at the OS; *i. e.* the washing procedure before fixations was sufficient to remove any unspecifically adsorbed protein. Particle counting was not done with this preparation. The samples after treatment with antiserum against ferredoxin-NADP reductase had the same appearance (not shown).

Samples treated with antiserum against CF₁

Thylakoid systems of chloroplasts isolated from either *Valerianella* or spinach and treated with an antiserum against CF₁, show two obvious differences when compared with the controls. Often several of the thylakoid systems were clustered. Within these clusters, IS, IFF and OFF were seen as in the control. Around the periphery of these large clusters, exposed outer surfaces could be seen; but they did not look typical. The 14 nm particles at this surface seemed aggregated and no longer randomly distributed. In contrast, the smaller particles were still randomly dispersed (Figs 3 and 4). The aggregates contained a variable number of particles. After incubation with serum 19–7 they were usually seen as individual entities and the aggregates were rather flat and did not show piling up of material. After incubation with serum 55–11 often all spaces between the particles were filled by material. Additional small structures related to antibody molecules were not resolved. Such molecules would be expected to be Y-shaped, each arm 3 to 5 nm^{26–28}. Individual bridges between the

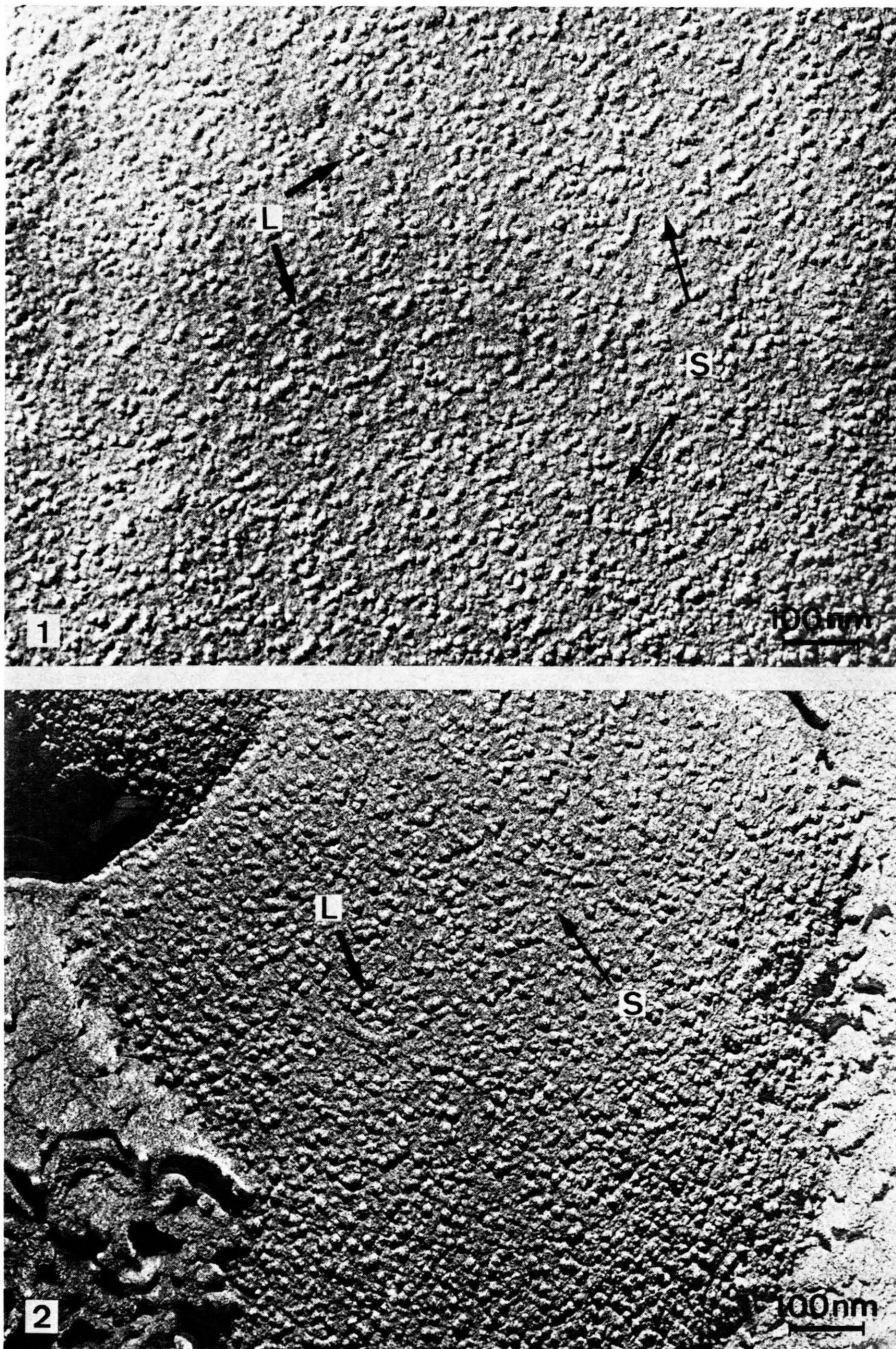


Fig. 1. Thylakoid outer surface of spinach chloroplast showing large (L) and small (S) particles revealed by deep-etching. Control sample with 0.1 M NaCl.

Fig. 2. Similar sample treated with a control serum lacking antibodies against CF₁. Both micrographs are magnified 100.000 : 1.



Fig. 3. Thylakoid outer surface of spinach chloroplast deep-etched after treatment with antiserum 19-7 containing antibodies against CF_1 .

Fig. 4. As Fig. 3, but after treatment with an antiserum against CF_1 , from a different animal (55-11). Both micrographs are magnified 100.000 : 1.

particles were not resolved. The surface between the twodimensional aggregates was essentially devoid of the 14 nm particles, though the smaller ones remained.

The number of particles on the micrographs was counted in order to give quantitative significance to their density (Table I). It is obvious from the Table that no significant differences can be deduced from the numbers. The amount of both larger and smaller particles counted in an area of $0.3 \mu\text{m}^2$ at the OS were the same whether or not the isolated thylakoid systems were treated with antibodies against CF_1 . Enumerating the particles/area within the aggregates would have required the determination of each aggregate separately and did not seem worthwhile.

No changes at the IS could be detected in the preparations. Changes at the IFF or OFF were not observed, but cannot be excluded with certainty.

Discussion and Conclusions

1. It seems possible that, after addition of the antisera, some of the large particles are removed from the thylakoid surface into the supernatant thus yielding the empty areas, and that others stay in place and bind antibodies thus building up the observed patches with higher particle density as compared with the controls. But the following arguments speak against this possibility:

The phenomenon of macroscopic agglutination shows that at least some of the antigen, (*i.e.* the large particles, see below), is rather tightly bound to the membrane; the agglutinated thylakoid systems cannot be shaken apart easily and dispersed evenly. It seems unreasonable to assume that monospecific antibodies would pull some of the particles out off the thylakoid whereas others could withstand such removal. There is no evidence for differences in the binding of the 14 nm particles. Besides this the overall number of large particles/area was not decreased (Table I).

The particle counting was done on micrographs from samples treated with serum 19–7. It could be clearly seen in most of these micrographs that the aggregates contain only 14 nm particles (Fig. 3). Connecting antibodies were never resolved; Even in the samples treated with antibodies against ferredoxin-NADP reductase no additional small particles could be detected. Neither the overall number

of particles/area nor the proportion of small particles was significantly increased (Table I). Thus *individual* antibodies seem not to be visible. Therefore it is very probable that those 14 nm particles which are missing in the empty areas contributed to the higher particle density in the aggregates. It cannot be ruled out with certainty that these particles, due to antibody action, were removed into the solution and then reattached to the thylakoid surface; but most of the aggregates after treatment with serum 19–7 were flat and not piled up as would be expected if reattachment had occurred.

We conclude, therefore, that the aggregates are formed by lateral movement of the particles at the membrane surface. Since the mean distance of the particles in the control was greater than the maximum which can be bridged by an antibody, we propose that the antibody first reacts by only one of its binding sites with a particle, that this complex moves laterally within the surface of the thylakoid until it meets another particle, and that the antibody then reacts with its second binding site. From quantitative precipitation experiments it can be calculated that up to 10 antibody molecules react with one CF_1 molecule in solution (Berzborn, unpublished experiments). Thus, the observed aggregates can be easily explained by a repetition of the proposed process.

We suggest that the reaction with the antibodies does not by itself induce the capacity of lateral movement at the thylakoid surface, but that this capacity is a property of the 14 nm particles *in vivo*. This is in agreement with the work of Frye and Eddin²⁹ who observed, after labelling with fluorescent antibody, lateral mobility of membrane antigens at the surface of fused human and mouse cells. The authors did not consider their finding to be an artifact of the reaction with the antibodies, but to reflect a physiological property of membrane surface proteins in general.

2. Aggregation of particles in the membrane of red blood cells (ghosts) has been observed previously, after rather drastic treatments such as with urea³⁰, pronase³¹ or trypsin³², or after pH changes in ghosts³³ or mitochondria³⁴ or repeated washings of ghosts in distilled water³⁵. Such treatments seem to cause unspecific aggregation, whilst in our experiments the aggregation was due to a specific antibody. Comparable aggregation of the immunoglobulin molecules at the surface of lymphocytes

by antibodies³⁶ has been observed, and of the red blood cell surface antigens after treatment with concanavalin A³⁷. The reaction of antibodies with spectrin at the inner surface of ghosts caused movement of sites at the outer surface³⁸.

Based on observations of the mobility of some peripheral or integral membrane components, dynamic membrane concepts have been previously proposed^{39, 40}. We consider that our results on a surface antigen of the thylakoid membrane are best interpreted in accordance with such concept.

Recently, mobility of integral particles in the OFF and IFF of the thylakoid membrane was reported^{41, 42}. However, in these cases, which are in general agreement with our results and conclusions concerning the surface particle, neither the biochemical function of the particles at the fracture faces is known, nor the exact mechanism which causes their aggregation, which is related to stacking⁴² and (or) to changes in the physical state of the lipids⁴³.

3. The large particles at the OS could have been aggregated, because they were connected by antibodies directly, or the antibodies could have connected mobile base pieces carrying the 14 nm particles. We suggest that the particles themselves are the reacting antigens at the thylakoid surface for the following reason:

The patches with little substructure are probably due to a higher number of antibodies attached to each antigen within the aggregate, because they are predominant, if higher amounts of antibodies are used for incubation, *e.g.* the samples treated with serum 55–11 (Fig. 4). In contrast to individual antibodies, *several* antibodies connecting the antigens or even bound with only one binding site, due to antibody excess, seem to be visible in our deep etching preparations as an amorphous cementing material. Since the 14 nm particles were covered by the antibodies, these have reacted with the particles themselves and not underneath.

Therefore, and because monospecific antisera against CF₁ were used, we propose that the reacting mobile 14 nm particle at the outer surface of the thylakoid is the coupling factor 1 of photophosphorylation. Thus the identification of this particle would be achieved.

From experiments using EDTA washing of isolated thylakoid systems combined with freeze etching, it is known that EDTA removes a large

particle from the outer surface of the thylakoid^{4, 44}. It has been suggested by estimation of ATPase activity in the extract and by the results of reconstitution experiments combined with negative staining^{45–47} that CF₁ is identical with the removed large particle. In the earlier papers, only some of the EDTA removable particles could be proposed to be CF₁ and, indeed, there is evidence from immunological^{19, 48} and electron microscopic^{4, 45} work, that there are two types of large particles attached to the thylakoid surface and that one of them is the ribulose-diphosphate carboxylase. Since in our micrographs, all larger particles at the outer surface seem to be aggregated by the antibodies, and since there is no antibody present which would react with the carboxylase, it follows that our treatment removed this protein entirely, and that there is no other large protein protruding from the membrane.

On the other hand, our particle counts are not in agreement with the number given by Mourakami⁴⁹. It is possible that, in his case, the carboxylase was not removed entirely. Also, dependent on physiological conditions or osmotic effects the overall number of enzymes/area might change.

4. If CF₁ is freely mobile within the plane of the thylakoid membrane, this would effect the design and interpretation of experiments on chloroplast structure and function. If there is any binding protein in the membrane for CF₁, this should be mobile. Studies on the agglutination of thylakoid systems by antibodies prepared against separated subunits of CF₁^{50, 51} showed an anisotropy of attachment of CF₁ to the thylakoid. Though there is some discrepancy in the results, CF₁ seems to be prevented from tumbling, whereas capable to perform lateral motions, as concluded in this paper.

A mobile CF₁ also would have to be considered in investigations on the distribution of CF₁ in the different regions of the thylakoid system^{52–55} and on the development of this membrane structure.

A coupling factor which is not fixed at a certain site in the membrane would have considerable implications for the mechanism of photophosphorylation. Since antibodies against CF₁ inhibit photophosphorylation^{23, 56}, it can now be argued that this is because the antibodies prohibit CF₁ to move randomly close to an electron transport domain: *i.e.* transport of the high energy intermediate, produced

by light driven electron transport, to ATP-synthetase molecules (CF_1) over some distance would not be tolerated. We are looking for antisera with specificities which would allow this problem to be solved.

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