

Binding of Antibodies onto the Thylakoid Membrane

VII. Localization of Coupling Factor of Photophosphorylation in the Lamellar System of Chloroplasts from *Antirrhinum majus*

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The maximal binding of antibodies against the subunits of the coupling factor of photophosphorylation onto the ultrasonic sediment and to stroma-freed chloroplasts of *Antirrhinum majus* was determined. Different surfaces of the thylakoid membrane are accessible to antibodies in both chloroplast preparations. Stroma-freed chloroplasts bind antibodies only at the outer surface, which is directed towards the stroma in intact chloroplasts. In the ultrasonic sediment, which is the product of ultrasonication and centrifugation, as was demonstrated by electronmicroscopy, the major part of the surface directed towards the inside of the thylakoids is accessible.

Both chloroplasts preparations are able to bind different amounts of antibodies to the five subunits of the coupling factor. While antigenic determinants of all five subunits are present on the surface directed to the outside, the δ -components seems to be lacking on the surface directed towards the inside. The α - and ε -component bind on both surfaces nearly the same amount of antibodies. Antigenic determinants of the β -subunit seem to exist in higher concentrations on the surface towards the outside and those of the γ -subunit on the surface towards the inside.

From the serological investigations it follows that coupling factor molecules span the thylakoid membrane from the outside to the inside. They are not only present in those parts of the thylakoid membrane, exposed in intact chloroplasts to the stroma, but also in stacked thylakoids. The differences between the results of other authors and the present results are discussed.

In the last publication we have reported on the maximal binding of antibodies to the coupling factor of photophosphorylation onto different chloroplast preparations [1]. These studies show among other things that in intact chloroplasts antibodies to coupling factor are not only bound onto the outer surface, which is directed towards the stroma, but also onto the inner thylakoid membrane surface.

In the present publication we have investigated, whether these facts are only valid for single subunits of coupling factor or for the entire coupling factor. The morphological structure of the ultrasonic sediment is also examined by means of electron microscopy.

Methods

Antisera

The antisera to the five subunits of coupling factor were obtained by immunization of rabbits according

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to earlier described procedures [1]. The monospecificity of sera was demonstrated by double diffusion tests in agarose gel [1]. The influence of antisera on photophosphorylation and electron transport in chloroplasts has also been described in earlier publications [1–3].

Quantitative binding of antibodies

The maximal binding of antibodies to stroma-freed chloroplasts of *Antirrhinum majus* and to ultrasonic sediment was carried out according to earlier methods [1, 4]. It was found that saturation values were also obtained by the binding of antibodies against the subunits onto the ultrasonic sediment, if the experiment was carried out in the region of antibody excess. The adsorption experiments were performed during a time period of five months from february to july. *Antirrhinum* plants which were used for chloroplast isolation, had been grown in the greenhouse.



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Electron microscopy studies of the "ultrasonic sediment"

A 10% suspension of stroma-freed chloroplasts in distilled water was sonicated 8 times for 15 sec with ultrasound. The suspension was fractionated by centrifugation for 2 h at $33\,000 \times g$. The sediment was suspended in distilled water and again ultrasonicated for 10 sec in order to suspend the chloroplast preparation homogenously. Thereafter, the fraction was fixed with glutaraldehyde for 2 h (2% in the same buffer). Fixation was carried out at room temperature. After washing and dehydration in a graded series of acetone/water the material was embedded in a low viscosity medium according to Spurr [5]. Serial thin-sections were cut on a Reichert Ultracut microtome, stained with uranyl acetate and lead citrate and examined with a Philips EM 400 electron microscope at 80 kV.

Double diffusion test in agarose gel

The chloroplast preparations, used as antigen, were suspended in 0.06 M $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 8.0 containing 2% Triton $\times 100$ and sonicated twice for 30 sec. The immune precipitation was carried out in 1% agarose gel (agarose from Serva Nr. 11 400, Heidelberg, W.-Germany). The diffusion time was 36 h. The plate was stained with a 2% amido black solution in acetic acid.

Results and Discussion

Characterization of the ultrasonic sediment

Preceding a detailed discussion of the results, attention should be drawn to the fact that only the outer surface of thylakoid membranes of stroma-freed chloroplasts is accessible to antibodies [1, 6]. Ultrasonication of stroma-freed chloroplasts in water followed by a centrifugation results in two fractions: the supernatant, which only contains membrane fragments [3] and the sediment. This ultrasonic sediment mainly consists of thylakoid stacks and separated large thylakoids as the electron-micrographs reveal (Fig. 1). Studies of a large amount of electron microscopic pictures show that thylakoids of the grana stacks are mostly disrupted. Moreover, it can be observed that "membranes" at the outside of grana stacks of the ultrasonic sediment reveal the same thickness as "membranes" inside the

stacks, thus indicating that these membranes represent also partition regions formed by the close contact of the surface of two adjacent membranes as termed by Weier and Benson [7]. Thus it becomes evident that membrane parts originally exposed to the stroma in intact chloroplasts are lacking completely. Instead of these missing membrane parts inner surfaces of thylakoid membranes turn towards antibodies. Consequently, in the ultrasonic sediment there are considerably more inner surfaces available for antibody binding than outer surfaces of thylakoid membranes. This fact is illustrated in the scheme (Fig. 2), in which the inner and outer surface are marked differently. In addition, the ultrasonic sediment contains more or less amorph material (Fig. 1), which obviously consists of decomposed membrane fragments and to some extent of plastoglobuli and of non-chloroplastic contaminations.

Maximal binding of antibodies

As earlier investigations have shown, 1 g of stroma-freed *Antirrhinum* chloroplasts can bind 0.4 g of coupling factor antibodies, whereas the same amount of ultrasonic sediment binds 0.7 g out of these antibodies [1, 8]. Considering that the surface accessible to antibodies is different in both chloroplasts preparations, it is necessary to refer the amount of adsorbed coupling factor antibodies to the maximal antibody binding of both preparations out of an antiserum to stroma-freed chloroplasts, which contains antibodies to most components of the thylakoid membrane [9]. Normalizing this maximal antibody binding to 1 in order to calculate the amount of bound antibodies to coupling factor, the value becomes 0.38 in the case of stroma-freed chloroplasts and 0.49 for the ultrasonic sediment. Thus, coupling factor antibodies must have been adsorbed onto the inner surface of thylakoid membranes, too, because in this preparation the inner face is especially exposed to antibodies. Concerning the localization of the coupling factor molecules it can be suggested that these molecules possibly span the thylakoid membrane. Otherwise, if coupling factor molecules were located only on the surface directed towards the stroma, the ultrasonic sediment would bind considerably lower amounts of antibodies compared to stroma-freed chloroplasts.

Furthermore, earlier studies show that stroma-freed chloroplasts react with antibodies to all five

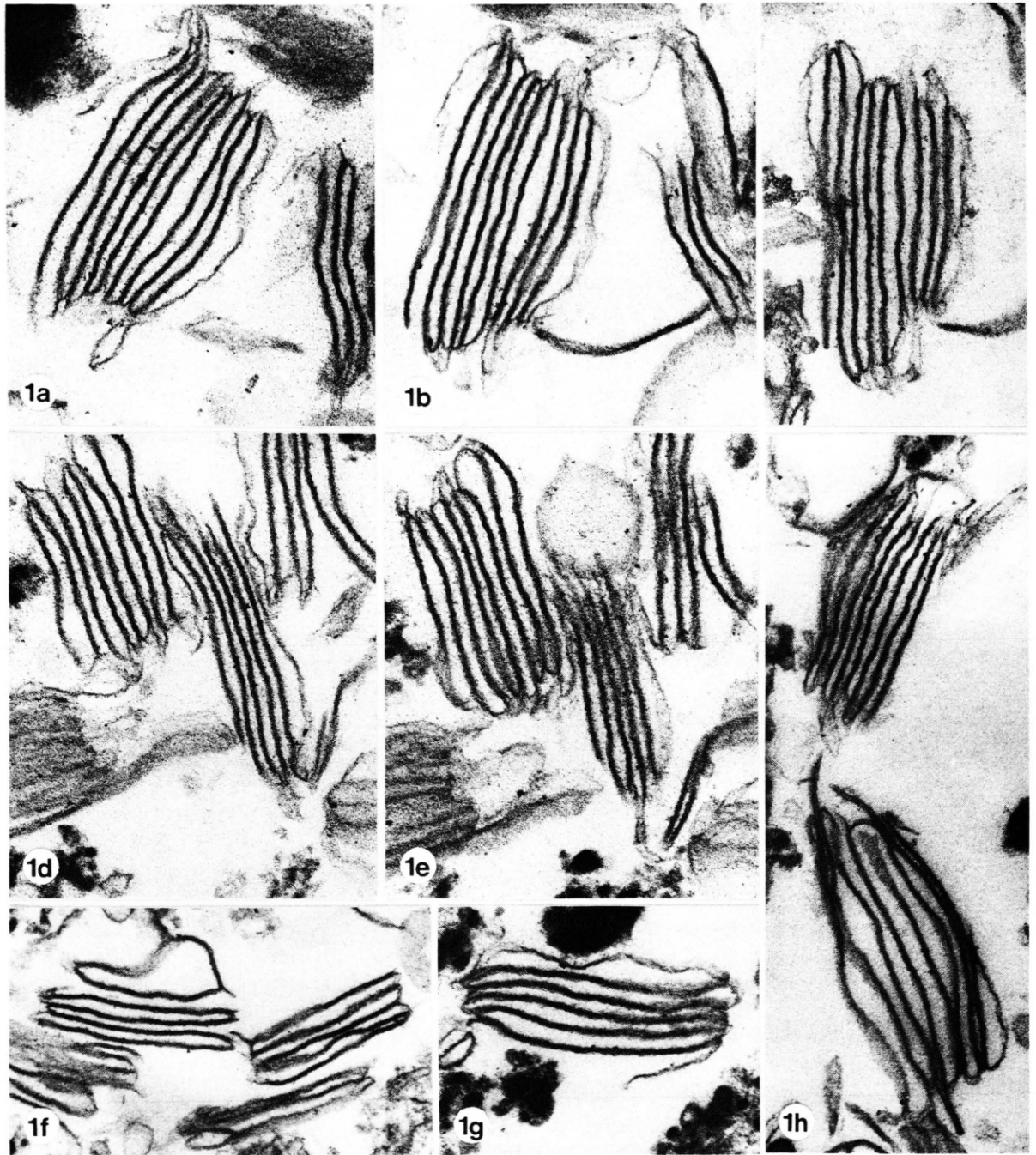


Fig. 1 a–h: Electronmicrographs of thin sections through thylakoid stacks of ultrasonic sediment isolated from stroma-free chloroplasts of *Antirrhinum majus*. Stroma-free chloroplasts were sonicated 8 times 15 sec and subsequently centrifuged for 2 h at $33\,000 \times g$. The obtained sediment – so called ultrasonic sediment – consists of small thylakoid stacks and separated thylakoids. Fig. 1 a–h shows thylakoid stacks from this ultrasonic sediment. After ultrasonication the largest part of the thylakoid membrane, which is directed to the stroma in intact chloroplasts is disrupted. Therefore, in these thylakoid stacks considerable portions of the inner surface of thylakoids are exposed and accessible to antibodies. Magnification: 70000:1.

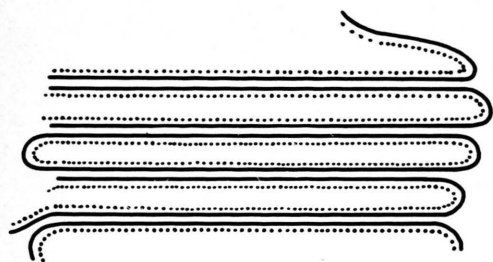


Fig. 2. Schematic graph of a detail from the electron-micrograph. — Thylakoid membrane surface directed towards the outside. --- Thylakoid membrane surface directed towards the inside.

subunits of the coupling factor-molecule [1]. In the present paper this capability is compared with that of the ultrasonic sediment. The monospecificity of antisera was demonstrated formerly [1, 8] as well as their influence on photophosphorylation, electron transport and proton transfer [2, 3].

Results are summarized in Table I. They clearly indicate that antibodies to all five subunits of the coupling factor are bound onto the ultrasonic sediment. In order to compare the results in detail they are referred to the amount of maximal antibody binding of the preparation out of an antiserum to coupling factor. As Table II shows, both, the inner and outer surfaces of the thylakoid membrane bind similar amounts of antibodies to the α - and ϵ -subunit. Whereas stroma-freed chloroplasts adsorb more β - and δ -subunits antibodies than does the ultrasonic sediment, it seems to be the contrary in

Table II. Antibody binding onto stroma-freed chloroplasts and ultrasonic sediment.

Antiserum to	Stroma-freed chloroplasts	Ultrasonic sediment
Coupling factor	1.00	1.00
α -component	0.55	0.44
β -component	0.78	0.49
γ -component	0.65	1.19
δ -component	0.73	0.21
ϵ -component	1.05	1.03

The values are taken from Table I and referred to the antibody amount that chloroplast preparation can maximally bind out of an antiserum to coupling factor.

Table I. Maximal binding of antibodies to stroma-freed chloroplasts, to membrane proteins and to subunits of the coupling factor onto stroma-freed chloroplasts from *Antirrhinum majus* and onto the ultrasonic sediment.

Antiserum to	Stroma-freed chloroplasts	Ultrasonic sediment
Stroma-freed chloroplasts	1.05 ± 0.02	1.44 ± 0.07
Membrane proteins	1.02 ± 0.03	0.74 ± 0.02
coupling factor	0.40 ± 0.04	0.70 ± 0.03
α -component	0.22 ± 0.01	0.31 ± 0.01
β -component	0.31 ± 0.02	0.34 ± 0.02
γ -component	0.26 ± 0.02	0.83 ± 0.06
δ -component	0.29 ± 0.03	0.15 ± 0.01
ϵ -component	0.42 ± 0.04	0.72 ± 0.02

The values refer to 1 g dry weight of the chloroplast preparations. The values for binding of antibodies to stroma-freed chloroplasts and to membrane proteins are taken from earlier publications.

the case of γ -subunit antibodies: they are bound onto the ultrasonic sediment to a greater extent. These data strongly suggest that the polypeptide structure of the coupling factor molecules varies with their accessibility to antibodies either on the outer surface of the thylakoid membrane which is exposed to the stroma or on the inner face directed towards the interior of the thylakoid. Thereby, it is not clear, whether there are antigenic determinants of the δ -subunit on the inner thylakoid face at all, because the ultrasonic sediment also contains outer thylakoid faces. Conspicuously, the ultrasonic sediment adsorbs more antibodies to the γ -subunit than antibodies to the entire coupling factor molecule. But this difference is not quite significant, since the amount of γ -subunit antibody binding varies. Furthermore, it was shown that chloroplasts of *Antirrhinum* plants cultivated at 12 000 lux contain more coupling factor than chloroplasts of plants kept at 20 000 lux. In addition, the amount of coupling factor molecules in chloroplasts changes according to seasonal conditions [10], which may be responsible for the above mentioned differences, too. The present results clearly show that coupling factor molecules penetrate the thylakoid membrane from the outside to the inside face with at least four of the five subunits. Coupling factor molecules are located in stacked as well as in non-stacked thylakoid membranes. These data are in contrast to current conceptions [11–13] suggesting that coupling factor molecules with a diameter of about 90 Å [3] are located only on the outer surface of the thylakoid membrane, which is exposed to the stroma. These ideas are based upon electron microscopic photographs of negative stained preparations, which have been treated with phospho- or silicomolybdate [14]. Since, on the one hand coupling

factor molecules are extracted out of membranes with these reagents [14] and deposited on the thylakoid membranes and the margins of the preparations during evaporation of water and since on the other hand these deposits cannot be observed after staining the sediment with uranylacetate, these data don't appear to be an objection against the presented results. Furthermore, a priori there is no longer any reason for the assumption that the localization of coupling factor is only limited to those parts of the thylakoid membrane exposed towards the stroma, since this idea is mainly based upon sterical considerations. Since the diameter of coupling factor molecules is somewhat larger than that of the thylakoid membranes, the molecules are able to form bulges on membran surfaces for which serological and electron microscopical indications exist [15–17]. As serological investigations clearly reveal, the accessibility of antigenic determinants depends on the condition of the membrane. Thus, antibodies to proteins involved in electron transport [18–21] to pigments [22–29] as well as to anionic lipids [30, 31] and phosphatides [32, 33] are only bound bivalently if the thylakoid membrane is present in a swellable state. Contrary to this, stroma-freed chloroplasts with non-swellable thylakoids adsorb these antibodies in a monovalent reaction.

It is not understandable that Berzborn *et al.* [10] couldn't find coupling factor in the stacked part of thylakoids. Trying to clear up these contradictions it should be noticed that the molecular structure of the preparations could change during the isolation. Such changes were observed by Miller and Staehelin [34]. But there is no doubt that the ultrasonic sediment consists mainly of grana. This is shown by electron-micrographs (Fig. 1). Furthermore the sera, used for quantitative determinations, are monospecific [1]. The serum to the coupling factor reacts, in the double diffusion tests in agarose gel against ultrasonic sediment with only one precipitation band (Fig. 3). To eliminate the unspecific binding of serum components every experiment with quantitative binding was carried out also with control serum, which was taken from the rabbits before immunization. The sediment contains amorph and compact material, which couldn't be seen on electron micrographs. This contamination has only little influence to the results because their surface is much smaller than the surface of the membrane. Concerning localization of the coupling factor molecules in the

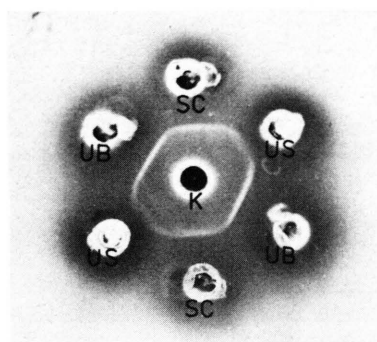


Fig. 3. Immune diffusion in agarose gel in order to demonstrate the presence of coupling factor in the ultrasonic sediment and in the membrane fragments of the ultrasonic supernatant. The chloroplast preparations were treated with 2% Triton $\times 100$. US: ultrasonic sediment; UB: ultrasonic supernatant; SC: stroma-freed chloroplasts; K: anti-serum to coupling factor. The concentration of stroma-freed chloroplasts and ultrasonic sediment was 20 mg dry weight/ml and 40 mg/ml for ultrasonic supernatant.

thylakoid membrane, the now developed ideas are not necessarily in contrast to the results obtained by the freeze etching technique, as the correlation of the morphological structure with the functional units seems not yet to be cleared up in all cases [34].

Summarizing it was shown that the investigations on the maximal binding of antibodies from monospecific antisera to thylakoidmembrane proteins [1, 4, 6, 8, 16, 33] and lipids [1, 4, 6, 9, 23, 33] lead to the following results:

1. The thylakoid membrane surface exposed to the stroma consists preponderantly of proteins whereas the surface directed towards the interior of the thylakoids consists mainly of lipids. Maximally 15% of the outer surface is covered with lipids. The inner surface contains also proteins, the amount of which cannot be estimated by serological investigations.
2. The lipid mixture has a different composition in the outer surface than on the inside face of the membrane. Whereas the surface towards the outside contains preponderantly anionic lipids the surface towards the inside consists preponderantly of neutral lipids.
3. Molecules of the coupling factor span the thylakoid membrane from the outside to the inside of the thylakoid membrane, whereas the portion of

the individual polypeptide molecules on the outside and inside is different. Coupling factor molecules are among the protein molecules present quantitatively the most frequent molecules on the inside of the thylakoids.

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