Antisera to the Coupling Factor of Photophosphorylation and Its Subunits

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Stroma-freed chloroplasts were extracted with sucrose palmitate-stearate containing buffer. After the addition of dodecyl sulfate and mercaptoethanol to the extract a series of polypeptides was isolated from the mixture by gel filtration. These polypeptides were later used for immunization. Antisera to four polypeptides reacted in the Ouchterlony double diffusion test with authentic coupling factor yielding a precipitation band. According to the observed apparent molecular weights the polypeptides are the α, β, δ and ε subunits of the coupling factor. An antiserum to the γ subunit has been obtained already previously.

All antisera inhibit photophosphorylation reactions and electron transport considerably. Addition of gramicidin inhibits photophosphorylation completely whereas gramicidin restores electron transport in the assays with the antisera to the α, β, γ and δ subunit. In the case of the antiserum to the ε subunit gramicidin does not regenerate electron transport. As in the presence of the serum to the ε subunit pH changes in the suspension medium are not observed, this serum seems to open a proton channel. Also, upon addition of dicyclohexyl carbodiimide (DCCD) pH changes in the suspension medium in the assay with antiserum do not reoccur. According to these unexpected results the identity of the antigen with the ε subunit of the coupling factor is not certain.

ATP-ase reactions are only inhibited by the antisera to the α and γ subunit and what is thought to be the ε subunit. The antiserum to the α subunit uncouples electron transport as the only one when used in sufficient concentrations. The dosis-effect curves of the inhibition of the electron transport exhibits a maximum. The dosis-effect curves for the other components rise after a lag phase in an approximately hyperbolic manner. The inhibitory action on electron transport is exerted by all antisera in the region of the reaction center I or in its immediate vicinity. This is thought to be due to the fact that a protein of the reation center I is inhibited in its function by the increasing proton concentration inside the thylakoid. The inhibition of electron transport by the antiserum to the ε subunit is considered to be a direct serum effect.

Besides the increase in fluorescence yield, due to the inhibition of electron transport in the region of photosystem I, decreases of the fluorescence yield are observed in the presence of DCMU, which do not depend on the redox state of Q but rather on the condition of the thylakoid membrane. Moreover, the antisera affect in a differing manner the energy spill-over of excitation from photosystem II to photosystem I.

In several publications we have shown, that it is possible to isolate polypeptides by gel filtration from stroma-freed chloroplasts which were solubilized by sodium dodecyl sulfate. These polypeptides are functionally characterized and localized in the thylakoid membrane via their antisera [1–6]. Due to the large number of polypeptides in the thylakoid membrane, the isolation of the pure polypeptides by gel filtration alone is difficult and requires considerable technical facilities. In addition, in special cases adsorption chromatography on hydroxylapatite [5] or ion exchange in 50% ethanol were applied [6]. The separation of the polypeptides after the solubilization with sodium dodecyl sulfate by gel chromatography is faciliated, if preceded by a fractionation of the chloroplast preparations in the presence of neutral detergents. However, neutral detergents frequently cause formation of aggregates, which do not dissolve again with dodecyl sulfate. We observed, that after a prefractionation with sucrose palmitate-stearate 7 no aggregation occurs.

In the following proteins are characterized which were solubilized with sucrose palmitate-stearate. After the dissociation of these proteins with sodium dodecyl sulfate and the isolation of their polypeptides the obtained polypeptides were used for the immunization of rabbits. The investigation showed that some of the sera inhibited all photophosporylation reactions tested to a high extent. At first we did not intend to publish these results separately as the coupling factor and its subunits is the subject

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Abbreviations: DAD, diaminodurene; DCCD, dicyclohexyl carbodiimide; DCMU, N,N′-3,4-dichlorophenyl dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; Q, quencher of photosystem II.

of many publications ([7, 8], for references see [9, 10]). An own publication, however, seemed justified to us, when we realized that some of our antisera exhibited hitherto undescribed properties or properties which differed from the already described ones.

Materials and Methods

Isolation of the polypeptides: Approximately 4 g of stroma-freed chloroplasts of *Antirrhinum majus*, suspended in 80 – 100 ml water, were supplemented with the equal volume of a solution of the following composition: 0.02 M sodium phosphate buffer pH 7.2, 2.5 ml mercaptoethanol and 250 mg sucrose palmitate-stearate 7 (Serva) per 1000 ml. Subsequently, 3 volumes of 0.01 M sodium phosphate buffer were added, which was saturated with sucrose palmitate-stearate 7 and contained 0.25% mercaptoethanol. The sucrose palmitate-stearate containing buffer solutions were sterilized in an autoclave with the 0.25% mercaptoethanol added after cooling. The suspension of chloroplasts in this solution was stirred for 90 minutes at room temperature and subsequently centrifuged for 1 h at 32 000 × *g* and 20 °C. The sediment was washed twice with the 0.01 M buffer. The combined supernatants were supplemented with such an amount of sodium dodecyl sulfate, as to give a final concentration of 0.25%. The collected extracts of three preparations, containing approximately 300 mg protein, were concentrated to 100 ml (Amicon, PM 10 membrane) and subjected to a gel filtration on Sepharose CL-6B (Pharmacia) (Separation length 700 cm, diameter 10 cm). The elution buffer was 0.05 M Tris-HCl buffer pH 7.5, containing 0.25% sodium dodecyl sulfate, 0.2% mercaptoethanol and 0.5% sodium chloride. Polypeptide fractions, which appeared uniform in the gel electrophoresis were, as described earlier, freed from dodecyl sulfate and then used for immunization [1, 5]. Prior to the removal of the detergent by anion exchange the sodium chloride containing Tris buffer was replaced by 0.01 M sodium phosphate buffer pH 7.2 free of chloride.

Isolation of the coupling factor: Coupling factor of photophosphorylation from tobacco was essentially prepared as described by Lien and Racker [11] a difference being the extraction from the chloroplasts which had to be carried out with 2 mM EDTA instead of 0.75 mM.

Serological methods: With the polypeptides and authentic coupling factor rabbits were immunized as described earlier [12]. The other serological tests were carried out also as described previously [13].

Electron transport reactions and photophosphorylation reactions were carried out as described earlier [13 – 15] with stroma-free swellable tobacco chloroplasts prepared according to Homann and Schmid [16]. Light triggered ATP-ase activity was determined according to Carmeli and Avron [17]. pH changes in the suspension medium were measured according to Dilley [18].

Fluorescence measurements were carried out also as described earlier [6].

Results and Discussion

Effect of the antisera on photochemical reactions

Sucrose palmitate-stearate containing buffer extracts from stroma-freed chloroplasts 3% of their dry weight. In the extract we detected by means of antisera ferredoxin-NADP-reductase, carboxydismutase and coupling factor of photophosphorylation. After addition of sodium dodecyl sulfate and mercaptoethanol several polypeptides were isolated from this extract. By immunization of rabbits antisera to the polypeptides were obtained. Among these some reacted with an authentic coupling factor preparation. By their apparent molecular weights the antigens were identified as the α, β, δ and ε subunit of the coupling factor. Due to the unexpected properties of the polypeptide, which, according to its molecular weight, we think to be the ε subunit, it is uncertain whether this component is identical with the ε subunit of other authors [9, 10]. The antiserum to the γ subunit has been obtained earlier [6].

The antisera to coupling factor and its five subunits agglutinate stroma-freed chloroplasts. Applied in the Ouchterlony double diffusion test against coupling factor all antisera react with a precipitation band. The five antisera inhibit all types of photophosphorylation reactions (Table I). According to Nelson *et al.* only the sera to the α and β subunit agglutinate and only the sera to the α and γ subunit affect photophosphorylation [19]. Our differing result may be explained by the fact that not all precipitating or agglutinating sera to an individual polypeptide also affect its function. We have repeat-

Antiserum to	[μmol ATP formed · (mg Chlorophyll)$^{-1}$ · h^{-1}]		
	$H_2O \rightarrow K_3Fe(CN)_6$	$H_2O \rightarrow$ anthraquinone-2-sulfonate	PMS
	Non-cyclic	Non-cyclic	Cyclic
α subunit	10 ± 1 *	6 ± 1 *	63 ± 3 *
Control serum	75 ± 5	55 ± 6	485 ± 30
β subunit	20 ± 1	10 ± 1	150 ± 14
Control serum	75 ± 5	55 ± 4	497 ± 30
γ subunit	23 ± 1	17 ± 1	40 ± 1
Control serum	76 ± 3	55 ± 6	486 ± 29
δ subunit	15 ± 1	11 ± 1	121 ± 13
Control serum	75 ± 4	59 ± 6	483 ± 30
ε subunit	5 ± 1	3 ± 1	19 ± 1
Control serum	73 ± 4	57 ± 4	485 ± 30

Table I. Effect of the antisera to the coupling factor subunits on photophosphorylation reactions in tobacco chloroplasts.

The values are averages of at least 5 determinations. The mean error of the average value is indicated.
* This degree of inhibition is observed with 0.6 ml antiserum with which in Figure 2 electron transport appears restored.

edly observed that one antiserum did agglutinate chloroplasts whereas the other also affected the function [6].

The five antisera inhibit the photoreduction of anthraquinone-2-sulfonate with dichlorophenol indophenol (DCPIP)/ascorbate, even if the concentration of the electron donor is high (0.9 mM). According to Fujita and Murano high concentrations of DCPIP bypass plastocyanin as the electron donor to photosystem I [20]. Consequently, the inhibition occurs at the reaction center of photosystem I or in its immediate vicinity. If diaminodurene (DAD) is used as the electron donor instead of DCPIP generally the same degree of inhibition is observed [21]. As the antisera agglutinate stroma-freed chloroplasts and inhibit photophosphorylation and electron transport antigenic determinants of these polypeptides are accessible to antibodies. Therefore, models of the coupling factor do not describe its structure properly, if the polypeptide chains do not reach all the free surface of the membrane-bound coupling factor molecule. The inhibition of electron transport by the antisera to the α, β, γ and δ subunit is probably due to the increase of the proton concentration or the electrochemical membrane potential ([22], for further references see [23, 24]). The acidification inside the thylakoid is stronger if ATP-synthesis is inhibited.

That the decrease of pH is the cause for the inhibition of electron transport is shown by the fact that gramicidin relieves the inhibition induced by the antisera. This effect on electron transport in the region of reaction center I might be due to the fact that an acid sensitive protein of reaction center I is situated at the inner surface of the thylakoid membrane. Our polypeptide 66 000 PSI-88 might be the candidate [5]. An antiserum to a chloroplast fraction, which we obtained after dissolution of stroma-freed chloroplasts with deoxycholate equally inhibited electron transport only at the inner surface of the thylakoid membrane [25]. In both cases the antiserum seems to be directed towards the same polypeptide.

The inhibition of electron transport by the antiserum to the ε subunit is not relieved by gramicidin. In the presence of the antisera to the α, β, γ or δ subunit the pH-value in the suspension medium increases upon illumination. In the presence of the antiserum to the ε subunit, on the other hand, no alcalisation of the suspension medium occurs (Fig. 1). The ΔpH curve does not differ from the one in the presence of gramicidin. Consequently, the adsorption of an antibody molecule onto the ε subunit of the coupling factor might cause an open-

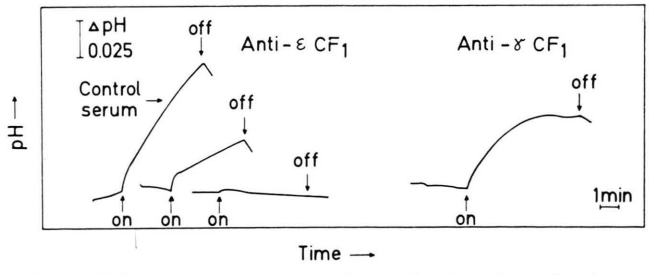

Fig. 1. Effect of the antisera to the ε subunit and γ subunit of coupling factor (CF_1) on the light-induced pH-change in the suspension medium of tobacco chloroplasts. In the case of the antiserum to the ε subunit the effect of two concentrations of antiserum is shown in the second and third curve. The curves are registered under phosphorylating conditions in the presence of PMS.

ing of a proton channel. As the antiserum reacts with coupling factor it appears improbable that the antiserum action is directed towards the proton pump. No indications were observed which would question the monospecificity of the antiserum. Chloroplasts solubilized with 1% Triton X-100 yielded with the antiserum only one single immunoprecipitation band. If the above considerations are correct the inhibition of photophosphorylation caused by the antiserum to the ε subunit is not due to a direct inhibition of the ATP-synthesis but rather due to the fact that no proton gradient is built up. Consequently, the inhibition of electron transport by this antiserum is directly caused by antibody adsorption. This rises the question whether the ε subunit itself is also an electron transport component or whether it is coupled to an electron transport component by intermolecular interactions. At any rate it should be borne in mind that gramicidin would not abolish the inhibition of electron transport if the antiserum contained in addition antibodies to an electron transport component. Until the monospecificity of the antiserum is established beyond doubt we do not wish to draw further conclusions. As the antisera to the α, β, γ and δ subunit all inhibit photophosphorylation we cannot have yet a precise idea on their special function from the presented data. Also the dosis dependence of the serum action gives no further indication for the β and δ subunit. A pecularity is shown by the dosis-effect curve of the α subunit. This curve at first rises linearly with increasing amounts of added antiserum, reaches a maximum and then decreases again (Fig. 2). Also, at the high antiserum concentrations photophosphorylation remains inhibited (Table I). With some reservations the course of the curve can be interpreted that binding of one antibody molecule per functional unit is sufficient to inhibit photophosphorylation and electron transport. The adsorption of a second antibody molecule then causes uncoupling (Fig. 2), a condition in which electron transport is not inhibited anymore. The curves for the β and δ subunit are given in Figs 3 and 4. They show a lag phase, but then increase in a more or less hyperbolic manner. In the dosis-effect curve of the serum to the ε subunit the lag phase is missing (Fig. 5). A sigmoidal curve shape is seen with a curve of the γ subunit [6]. In the literature differing reports are found concerning the subunits to which ATP-ase activity is linked [9, 10,

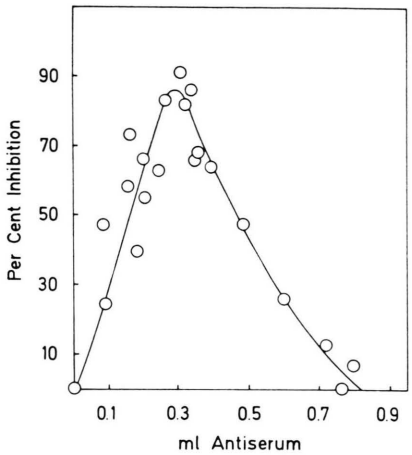

Fig. 2. Dependence of the degree of inhibition of electron transport caused by the antiserum to the α subunit on the amount of antiserum added in tobacco chloroplasts. Electron transport reaction DCPIP/ascorbate → anthraquinone-2-sulfonate. DCPIP-concentration 0.9 mM.

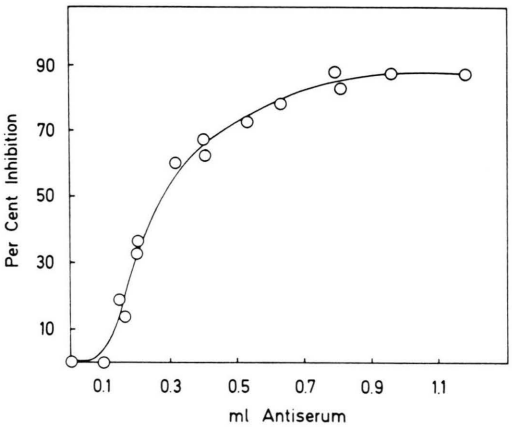

Fig. 3. Dependence of the degree of inhibition of electron transport caused by the antiserum to the β subunit on the amount of antiserum added. Electron transport reaction and donor concentration as in Fig. 2.

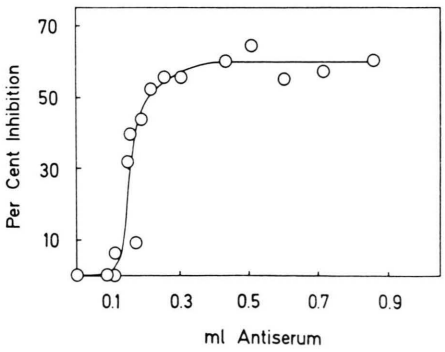

Fig. 4. Dependence of the degree of inhibition of electron transport caused by the antiserum to the δ subunit on the amount of antiserum added. Electron transport reaction and donor concentration as in Fig. 2.

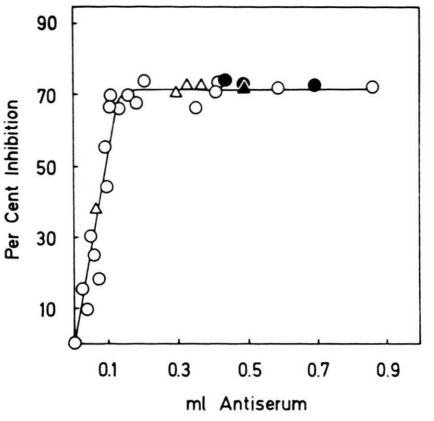

Fig. 5. Dependence of the degree of inhibition of electron transport, caused by the antiserum to the ε component, on the amount of antiserum added. (○) Electron transport reaction DCPIP/ascorbate → anthraquinone-2-sulfonate, DCPIP concentration 0.9 mM in the assay; (△) reaction as (○) but in the presence of 0.7 μg gramicidin per ml assay; (●) Electron transport reaction DAD/ascorbate → anthraquinone-2-sulfonate, DAD-concentration 0.2 mM; (▲) reaction as (●) but in the presence of 0.7 μg gramicidin per ml assay.

26]. Our antiserum to the α subunit inhibits the magnesium dependent light-triggered ATP-ase in chloroplasts (Table II). From two sera to the γ subunit one serum inhibits the ATP-ase (Table II)

Table II. Effect of the antisera to the coupling factor (CF_1) subunits on the light-triggered ATP-ase in tobacco chloroplasts

Antiserum to	[μmol[γ-P^{32}]-ATP hydrolyzed · (mg Chlorophyll)$^{-1}\cdot h^{-1}$]	% Inhibition
α subunit	21±2	38±4
Control serum	34±3	
β subunit	35±4	3±0.5
Control serum	36±4	
γ subunit 0.1 ml	31±3	
0.2 ml	27±3	
0.3 ml	19±2	
0.4 ml	19±2	47±5
Control serum	36±4	
δ subunit	43±5	*
Control serum	36±4	
ε subunit	5±0.5	85±9
Control serum	35±4	
Coupling factor (CF_1)	13±2	63±8
Control serum	35±4	

* No significant effect of the antiserum, possibly a slight stimulation. The mean error of the average value is indicated.

whereas the other stimulates [2]. The two sera are obviously directed towards different antigenic determinants of the γ subunit. Antibody binding in one case induces a conformational change which activates the ATP-ase whereas antibody binding in the other case causes inhibition. If an antibody activates an enzyme, this is due to a conformational change in the enzyme molecule. If, however, it inhibits an enzyme, this action can be due to the blocking of the active center or equally to a conformational change. In addition, it should be noted, that we had obtained earlier an antiserum to the α subunit which also stimulated the ATP-ase [1, 2]. The monospecificity of the earlier serum, however, was uncertain [1]. In addition, to the listed antisera also the serum to the ε subunit inhibits the ATP-ase activity. The degree of inhibition is higher than with the other antisera (Table II). The antisera to the β and δ subunit were without effect (Table II). However, conclusions concerning the uninvolvement of a component can be drawn with certainty only, if a larger number of antisera to the same component has been investigated.

Effects of the antisera on the chlorophyll a fluorescence of chloroplasts

The sera to coupling factor and its five subunits cause an increase of fluorescence yield. This is expected, if the inhibition of electron transport occurs in the region of photosystem I. In the presence of DCMU the difference between the fluorescence yields of the assay with antiserum and the assay with control serum not only disappears but the control fluoresces stronger than the assay with antiserum. From this it appears that the increase in fluorescence yield depends on the redox state of the quencher Q of photosystem II. To this fluorescence increase a decrease not depending on Q is superimposed. This decrease of the fluorescence yield not depending on Q is probably due to an alteration of the structure of the thylakoid membrane [27, 28] which either causes an increased transfer of excitation energy from photosystem II to photosystem I or a radiationless de-excitation. Hence, all coupling factor antisera influence the chlorophyll a fluorescence in a twofold manner, namely by a change of the electron transport speed and an alteration of the molecular structure of the thylakoid membrane.

The individual antisera mainly differ in the time, which is necessary to obtain the change of the flu-

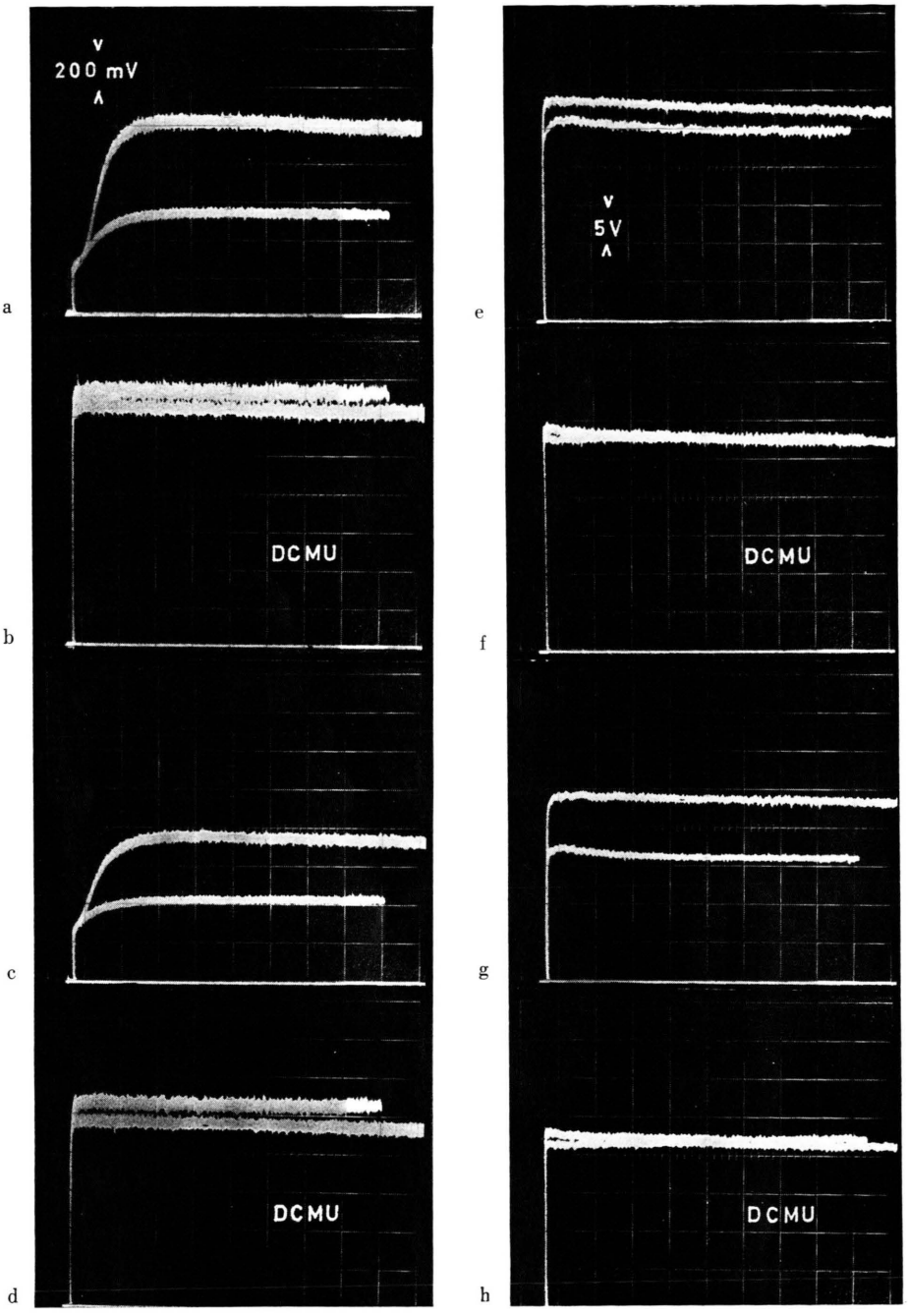

Fig. 6. Effect of the antiserum to the ε subunit on the fluorescence rise. Every picture shows the fluorescence rise curve of the assay with antiserum and that with control serum. The shorter scan represents the control. The time scale is 5 sec per division.
a) dark adapted; fluorescence excitation with weak exciting light (0.08 W/m^2); e) as a) but with strong exciting light (40 W/m^2); b) and f) the same assay as a) and e) but in the presence of 10^{-6} M DCMU; c) and g) correspond to the assays a) and e) but preilluminated for 3 min with red light with 190 W/m^2 prior to fluorescence excitation; d) and h) correspond to c) and g) but in the presence of DCMU.

orescence yield after switching on the exciting light. With chloroplasts, well adapted to the dark, the sera to coupling factor and its γ subunit cause either no or only a very slight increase of the fluorescence yield [6]. The increase only shows up after a preillumination of 3 minutes with 190 W/m^2 of red light. The other antisera do not show this preillumination effect if the exciting light is weak (Fig. 6 a). After a preillumination one rather observes a somewhat smaller increase of the fluorescence yield caused by the antiserum than with dark adapted assays (Fig. 6 c). If, however, excitation is done with strong light, the antisera cause with dark adapted chloroplast preparations either no or only

a weak increase in fluorescence yield (Fig. 6 e). Only after preillumination the excitation with strong light leads to a fluorescence increase (Fig. 6 g). The presented fluorescence rise curves are the original registrations obtained with the serum to the ε subunit. The described fluorescence yield changes are due to a change in the variable portion (F_{var}). Initial fluorescence (F_0) remains constant. In the presence of gramicidin only with the antiserum to the ε subunit no change in the Q-dependent fluorescence is found. This is to be expected, as addition of gramicidin does not restore electron transport. With the other antisera addition of gramicidin causes the disappearance of the difference between the assays with antiserum and control serum. In the presence of DCMU the antisera to the α, γ, δ and ε subunit cause in comparison to the control a more or less clear decrease of the fluorescence yield (Fig. 6 b, d, f, h). In the excitation with weak light the initial fluorescence F_0 is not affected whereas in the excitation with strong light also F_0 is decreased by the antisera. With the preillumination it matters apparently whether the assay contained already during the preillumination the DCMU or whether it was added later. With the antisera to the β subunit we observed in some cases in the presence of DCMU an increase of the fluorescence yield especially if no preillumination had taken place. The two latter points need clarification. Special investigation is still needed to solve the question why with the individual sera the fluorescence yield change is observed immediately after switching on the exciting light, whereas with others a more or less long preillumination is required, before the sera show an inhibitory effect.

Our results indicate that the coupling factor may occur in the membrane in three states, which manifest themselves by differing reactivities of the antisera. One state is realized in dark adapted chloroplasts and is at first barely changed in weak exciting light. The second state is reached within a short time in strong exciting light whereas the third state requires irradiation of several minutes. It is a characteristic of the third state that this state is maintained also after a prolonged dark period. Thus, it obviously depends among other things on the state of the thylakoid membrane and the conformation of its molecules whether an active serum affects the function or not. Such alterations of states, depending on light, may exist in the living cell. Thus, with chloroplast preparations, which were isolated in the afternoon, frequently no preillumination was necessary in order to see the increase in fluorescence yield caused by the antiserum, whereas chloroplasts isolated from leaves harvested in the morning, showed the effect only after preillumination.

Table III. Effect of the antisera on the low temperature fluorescence emission in tobacco chloroplasts at 77 °K.

Serum	*F* 735 / *F* 685	
	Dark [%]	Preilluminated [%]
Anti-α CF_1	6±1	22±1
	16±2	26±2
Anti-β CF_1	−13±1	0±1
	− 2±2	4±1
Anti-γ CF_1	− 3±1	23±2
	− 4±1	18±3
Anti-δ CF_1	− 9±1	9±1
	− 9±2	9±2
Anti-ε CF_1	− 5±1	29±1
	2±2	25±2

Every value is the average of five recordings. The mean error of the average value is indicated.

The effect which the individual antisera exert on the intersystem energy migration, that is measured at 77 °K as the ratio of the fluorescence emission at 735 and 685 nm, is also differing (Table III). Here too, an influence of preillumination is seen. It should be noted that after a preillumination of 3 minutes the preparations were kept in absolute darkness for 15 minutes prior to freezing. As the assays contained ascorbate as the electron donor and anthraquinone-2-sulfonate as the electron acceptor, the dark adapted and preilluminated samples should be in the same redox state. The considerable differences found in parallel determinations with dark adapted chloroplasts are due to the fact that also after a darkening of 15 minutes the thylakoid membrane of the different preparations is not in the same condition. After preillumination the parallel determinations fit reasonably well together. The antisera to the α, γ and ε subunit enhance after preillumination the energy spill-over from photosystem II to photosystem I considerably. The effect of the antiserum to the δ subunit is smaller and that to the β subunit very low.

It is presumed that the altered distribution of excitation energy over the photosystems is due to changes of distances and orientation of individual chlorophyll molecules or of structural units with several chlorophyll molecules. It is noteworthy, that the adsorption of antibodies to coupling factor molecules should cause such distance and orientation changes. Already Mohanty *et al.* had found that coupling factor plays a role in structural changes of the thylakoid membrane which in turn cause fluorescence changes [28]. The new finding is, however, that the kind and extent of the fluorescence changes seem to depend on the subunit onto which the antibody is bound. On the other hand alterations of the membrane structure, as caused by illumination, influence the conformation of the coupling factor ([29], for references see [23, 30]). Such interactions are only possible if the coupling factor molecules are not only loosely bound to the membrane surface but are rather integrated within the membrane.

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