Localization and Functional Characterization of Three Thylakoid Membrane Polypeptides of the Molecular Weight 66000

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Three polypeptide fractions with the apparent molecular weight 66 000 were isolated from stroma-freed *Antirrhinum* chloroplasts which were solubilized with dodecyl sulfate. Antisera to these fractions affect electron transport in distinctly different ways. For the characterization of the three antisera photochemical reactions of chloroplast preparations with artificial electron donors and acceptors as well the analysis of fluorescence rise curves were used. Antiserum 66 000 PSI-96 inhibits electron transport apparently on the acceptor side of photosystem I, provided the antibodies are adsorbed onto the outer surface of the thylakoid membrane. Antiserum 66 000 PSI-88 probably acts directly on the reaction centre I or on its immediate vicinity, if the antibodies are adsorbed at the inner surface of the thylakoid membrane. Antiserum 66 000 PSII-42 inhibits electron transport in the region of photosystem II. The antigen towards which the antiserum is directed appears to belong to the reaction centre II, as also in the condition of high inhibition degrees, the fluorescence intensity remains unchanged. The antigenic determinants are located at the outer surface of the thylakoid membrane.

Dodecyl sulfate polyacrylamide gel electrophoresis of thylakoid membrane proteins, carried out according to Weber and Osborn, yields near the starting point three relatively sharp bands [1]. The apparent molecular weights of these bands correspond to 66 000, 62 000 and 59 000 respectively [2]. The latter two bands are components of the coupling factor of photophosphorylation [3, 4]. Despite the fact that the band corresponding to 66 000 molecular weight appears to be narrow, analysis of the photometer curve revealed that it consisted of several components. In the following we report on the preparative separation of the fraction with the apparent molecular weight 66 000 into three components. Furthermore, we attempted to localize the components in the thylakoid membrane and we characterized their function in photosynthesis by their antisera. We proceeded essentially according to the earlier described method [5–8]. As the membrane proteins are not soluble in water they were solubilized with sodium dodecyl sulfate and fractionated by gel chromatography. After the removal of the dodecyl sulfate the polypeptides are denatured, but contain apparently still native antigen determinants. This is shown by the fact that the antisera to these fractions react with functioning stroma-free swellable chloroplasts.

In the following we try to characterize three polypeptide fractions which exhibit the same molecular weight. Therefore, the earlier nomenclature is not sufficient anymore. For the easier distinction we add to the apparent molecular weights the name of the photosystems in the region of which the antigen is effective and for clarity sake the number of the antiserum. This appears also advantageous, as antisera which are directed towards the same antigen occassionally differ somewhat with respect to their action. Among the sera which are described in the following, the numbers 66 000 PSI-96 and 66 000 PSI-88 inhibit in the region of photosystem I, whereas the third, 66 000 PSII-42, exerts its action in the region of photosystem II.

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Abbreviations: PMS, phenazine methosulfate; DCPiP, 2,6-dichlorophenol indophenol; A-2-sulf, anthraquinone-2-sulfonate; DCMU, N,N′-3,4-dichlorophenyl dimethylurea; DPC, diphenyl carbazide; TMB, tetramethyl benzidine.

Materials and Methods

Isolation of the polypeptides: Stroma-freed chloroplasts of *Antirrhinum majus* corresponding to 4.5 g dry weight were dissolved in 450 ml 0.01 M sodium phosphate buffer (pH 7.2) containing 2.2% sodium dodecyl sulfate and 1% mercaptoethanol. The solution was stirred for 20 hours at room temperature and subsequently centrifuged (60 min at 30000 × *g* at 22 °C) in order to remove undissolved com-

ponents. Not following these indications may lead to different results.

The separation of the polypeptides was achieved by gel filtration through eight in series connected columns (diameter 21.5 cm, height 80 – 90 cm) with Sepharose 6B (Pharmacia). The elution buffer was 0.01 M sodium phosphate buffer (pH 7.2) containing 0.25% sodium dodecyl sulfate and 0.1% mercaptoethanol. Fractions of 250 ml were collected. The running speed was 1 to 2 cm per hour. The fractionation was carried out with a setting of WFN, Köln. The quality of the separation was verified by polyacrylamide gel electrophoresis. Fractions with the apparent molecular weight 66 000 were pooled and concentrated (Amicon, H1DP10 hollow fiber). The further processing followed different routes. For the isolation of the fractions 66 000 PSI the concentrate was fractionated by adsorption onto hydroxylapatite (Bio-Gel HT, Bio-Rad). Before applying the preparation to hydroxylapatite the preparation was transferred by dialysis to 0.1 M sodium phosphate buffer pH 6.4, containing 0.1% dodecyl sulfate and 0.1% mercaptoethanol. Elution was done with buffers of increasing ionic strength [9]. Fractions whose conductivity was 29 – 30 mS/cm contained the antigen 66 000 PSI-96. If in the starting material components of the coupling factor were present, these were eluted at lower ionic strength. The combined fractions yield in the gel electrophoresis besides a portion which is aggregated, a single sharp band. In a separate run fractions with lower conductivity (27 – 28 mS/cm) which hardly contained aggregated parts were pooled. They contained the antigen 66 000 PSI-88. For the isolation of the antigen 66 000 PSII-42 a concentrated preparation, which contained polypeptides with the apparent molecular weight 66 000 was transferred by dialysis into 0.1 M Tris-HCl buffer (pH 9.2) containing 0.25% dodecyl sulfate and 0.1% mercaptoethanol. Upon gel filtration through CL-6B Sepharose (Pharmacia) the formerly uniform appearing preparation was separated into fractions of differing molecular weights. The fractions with the apparent molecular weight 66 000 were pooled. An additional purification was achieved on hydroxylapatite. Fractions which were eluted at high ionic strength were pooled. The listed fractions were dialysed against 0.01 M sodium phosphate buffer (pH 7.2) containing 0.1% dodecyl sulfate and 0.1% mercaptoethanol and subsequently concentrated by ultrafiltration (Amicon, PM10 membrane), until the protein content was 0.3 – 0.5%. Dodecyl sulfate was removed according to ref. 10. For immunization the preparations were transferred by diafiltration (Amicon, PM10 membrane) into 0.06 M potassium-sodium phosphate buffer (pH 7.8).

It proofed to be necessary to keep bacteria away from all solutions. For this purpose the used buffers were sterilized either by heating or filtration (Sartorius Membranfilter, pore width 0.45 μm). Sterilization of the separation plants was achieved by applying occasionally diethylpyrocarbonate to the columns.

Immunization of the rabbits was carried out as described by Radunz [11].

The isolation of photochemically active stroma-free swellable chloroplasts from *Nicotiana tabacum* "John William's Broadleaf" was described by Homann and Schmid and Schmid *et al.* [12, 13].

Rates of photosynthetic electron transport reactions were determined and *photophosphorylation reactions* were carried out as described previously [8, 12–14].

Fluorescence measurements were carried out as described earlier [8]. Excitation wavelength 435 nm, excitation slit 40 nm; emission wavelength 685 nm, emission slit 40 nm.

Results

The principle of the antigen preparation was the following: The polypeptide fraction 66 000 was obtained by gel filtration. The fractionation of this preparation was achieved by chromatography on hydroxylapatite. By this step impurities, such as components of the coupling factor with the apparent molecular weights 62 000 and 59 000 were removed. The fraction 66 000 PSI-88 was eluted before fraction 66 000 PSI-96. Because of overlapping of the components the yield is small. The fraction 66 000 PSII-42 is obtained by repeated gel chromatography of the starting preparation, with the difference that in this case the pH of the elution medium is 9.2. From the originally uniform appearing preparation, components of lower molecular weights are separated. The not decomposed major portion, which exhibits a molecular weight corresponding approximately to the original one, was used for immunization after chromatography on hydroxylapatite. It should be noted that the polypeptide fractions 66 000 PSI do not change their typical properties if treated with alcaline buffer. In alcaline medium they are not transformed into polypeptides of type 66 000 PSII. Because of the insolubility of the polypeptides all operations were carried out in the presence of dodecyl sulfate. In order to check the uniformity up to now the dodecyl

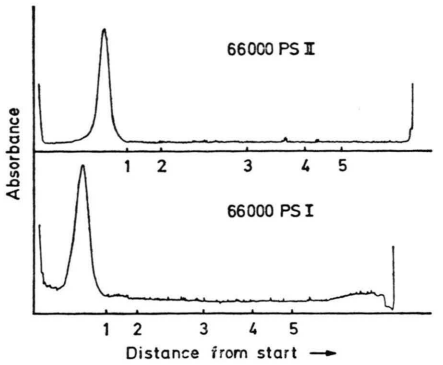


Fig. 1. Optical scans of polyacrylamide electropherograms of polypeptide fractions of the type 66 000 PSII-42 (upper curve) and of the type 66 000 PSI-96 (lower curve). The numbers 1–5 refer to the positions of the molecular weight markers used: 1 bovine serum albumin 67 000; 2 ovalbumin 45 000; 3 chymotrypsinogen 25 000; 4 myoglobin 17 800; 5 lysozyme 14 300.

sulfate polyacrylamide gel electrophoresis was used. The obtained apparent molecular weight depends on the conditions used. It was repeatedly observed that the apparent molecular weight of the purified fractions appears to be higher than that of the starting preparations. For the nomenclature of the preparations this circumstance is not respected. As an example for the obtained degree of purity we show in Fig. 1 the optical scan of the stained gels. Because of aggregation of the polypeptides after removal of dodecyl sulfate the serological purity criteria such as the Ouchterlony double diffusion test and the immune electrophoresis are only of limited value. As is seen from the following results, the sera 66 000 PSI-96 and 66 000 PSII-42 are apparently monospecific with respect to their action on electron transport, whereas this point is not so clear with serum 66 000 PSI-88.

Antiserum 66 000 PSI-96. This antiserum inhibits the photoreduction of anthraquinone-2-sulfonate in the presence of DCMU with dichlorophenol indophenol as the donor. The inhibition is also observed if the donor concentration is high namely 0.8 to 1.2 mM in the assay. In this case electron transport is not dependent on plastocyanin [15] or on cytochrome f [16]. Consequently, the inhibition site is situated behind these two electron carriers *. The degree of inhibition depends on the assay conditions. As seen from Table I the addition of KCl,

* The term before and after is used in the sense of the direction of electron flux from water to $NADP^+$.

Table I. Dependence of the inhibitory action of the antiserum 66 000 PSI-96 on the anthraquinone-2-sulfonate reduction with DCPiP as the electron donor in tobacco chloroplasts on the composition of the assay system.

Additions		Oxygen uptake [μmol · (mg chlorophyll)$^{-1}$ · h^{-1}]	% Inhibition
none	antiserum	569	0
	control serum	569	
ADP, P_i, Mg^{++}	antiserum	507	24
	control serum	665	
KCl	antiserum	262	58
	control serum	627	
KCl, ADP, P_i, Mg^{++}	antiserum	273	52
	control serum	563	
Gramicidin	antiserum	323	22
	control serum	415	
KCl, Gramicidin	antiserum	240	46
	control serum	446	

Reaction in 0.05 M Tricine pH 7.2; KCl when used was 0.1 M in the assay; ADP and inorganic phosphate 1.66×10^{-3} M and Mg^{2+} 3.3×10^{-3} M in the assay; gramicidin 0.3 μg/ml assay where indicated. The reaction was run in the presence of 10^{-6} M DCMU at 20 °C.

ADP, P_i, and Mg^{2+} increases the effectiveness of the antiserum. Under these conditions in the presence of saturating amounts of antiserum the maximal inhibition is 80 per cent (Fig. 2). Occasionally, inhibition degrees of up to 96 per cent were observed. The pH-optimum lies at approximately pH 7.2 (Fig. 3). If it was correct that the high concentration of $DCPiPH_2$ are directly photooxidized by P_{700} [15], then the inhibition would be located on the acceptor side of photosystem I. The influence of the antiserum on photophosphorylation reactions shows that PMS-mediated cyclic photophosphorylation under standard assay conditions (2 min of illumination) is stimulated by the antiserum whereas the non-cyclic photophosphorylation in the system $H_2O \rightarrow$ A-2-sulf is inhibited to the same degree as electron transport (Table II). The stimulation of PMS-mediated cyclic photophosphorylation becomes smaller soon after the onset of illumination, is zero after approximately 5 minutes and then leads to an inhibition (Table III). For the evaluation of the phenomenon it must be borne in mind that the reaction rate also in the presence and absence of control serum becomes quickly smaller. Therefore, one usually uses a reaction time of only 2–3 minutes and considers only initial rates. However, the fact remains that in the assay with antiserum the reaction rate decreases faster than in the control.

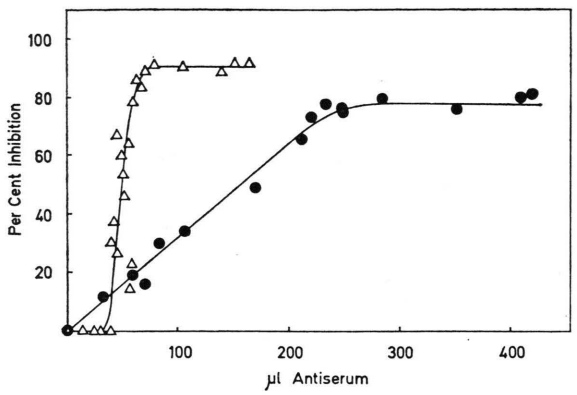


Fig. 2. Dependence of the degree of inhibition on the amount of antiserum added. ● 66 000 PSI-96, electron transport system DCPiP/asc → A-2-sulf; △ 66 000 PSII-42, electron transport system TMB/asc → A-2-sulf; the amount of antiserum is calculated for chloroplasts containing 25 µg chlorophyll per assay.

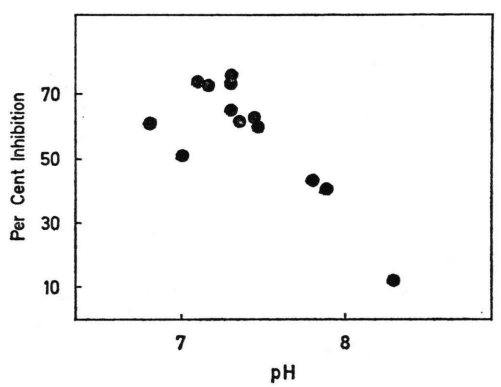


Fig. 3. Dependence of the degree of inhibition by the antiserum 66 000 PSI-96 on the pH of the assay medium.

It might be that stimulation as well as the inhibition is due to secondary effects of the antiserum. If, as we assume, linear electron transport is blocked immediately behind the primary electron acceptor X, a temporary stimulation can be expected, because more electrons will flow through the cycle. If the decrease of the reaction rate of PMS-mediated cyclic photophosphorylation is generally dependent on the amount of electrons flowing through the cycle, then a faster decrease of the rate in the assay with antiserum is to be expected. As also different explanations are possible this hypothesis must be experimentally verified. However, we feel that the polypeptide 66 000 PSI-96 is to be localized on the acceptor side of photosystem I or possibly is a component of a complex acceptor molecule X. In this context it should be noted that if the antiserum contained antibodies to the component of the coupling factor with the molecular weight 62 000, PMS-mediated cyclic photophosphorylation under the conditions described in Table II would have been inhibited. Also, antibodies to coupling factor could not be detected in the Ouchterlony double diffusion test. For completeness sake it should be added that the DCPiP Hill reaction is not affected by the antiserum.

Fluorescence intensity is increased by the antiserum (Fig. 4) but the fluorescence rise time appears to be longer in the presence of antiserum. Hence, in the presence of functioning photosystem II the oxidation of Q^{17} by photosystem I is inhibited. The slowed down fluorescence rise suggests that

Table II. Effect of the antisera on photophosphorylation reactions in wild type tobacco chloroplasts.

Antiserum	[µmol ATP formed · (mg chlorophyll)$^{-1}$ · h^{-1}]		
	Non cyclic $H_2O \rightarrow K_3[Fe(CN)_6]$	Non cyclic $H_2O \rightarrow$ A-2-sulf	Cyclic PMS-mediated
66 000 PSI-96	41.2	15.4	581
Control serum	42.3	51.2	431
% Inhibition	2.6	70	0 (35% stimulation)
Control serum + 10^{-6} M DCMU	0	0	431
66 000 PSI-88	43	47	460
Control serum	43	52	522
% Inhibition	0	10	12
Control serum + 10^{-6} M DCMU	0	0	522
66 000 PSII-42	30	37.0	466
Control serum	44	50.2	519
% Inhibition	32	26	10
Control serum + 10^{-6} M DCMU	0	6	519

The reaction was carried out at 20 °C in 120 000 lx white light. The values are averages of 4 independent determinations.

Table III. Dependence of the effectiveness of the antiserum 66 000 PSI-96 on the reaction time in PMS-mediated cyclic photophosphorylation in wild type tobacco chloroplasts.

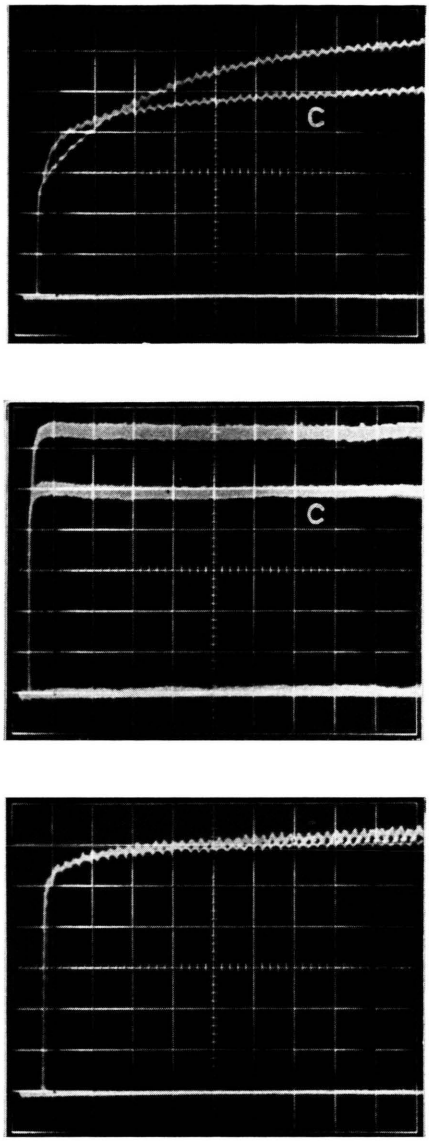
Illumination time [min]		[μmol ATP formed · (mg chlorophyll)$^{-1}$ · h^{-1}]	Effect of the antiserum
2	antiserum	581	35% stimulation
	control serum	431	
3	antiserum	483	17% stimulation
	control serum	413	
6	antiserum	273	4.3% inhibition
	control serum	285	
15	antiserum	41.7	74% inhibition
	control serum	158.6	

The reaction was run in 120 000 lx white light at 20 °C in the presence of 3.3×10^{-5} M PMS and 10^{-6} M DCMU in the assay.

photosystem I is at first still functioning. Consequently, the inhibition does not affect photosystem I but may be located on its acceptor side behind the primary acceptor X. Upon addition of DCMU the fluorescence of the control is lifted to the level of the assay with antiserum. DCMU does not increase the fluorescence level measurably if electron transport by the antiserum was already inhibited by more than 90 per cent. For references on Kautsky effect see [18, 19].

For the characterization of the inhibitory effect of the antiserum it should be noted again that the effect of the antiserum is not directed towards photophosphorylation. This is shown by the fact that the inhibition by the antiserum is also seen in the presence of gramicidin (Table I).

Antiserum 66 000 PSI-88. In contrast to the antiserum 66 000 PSI-96 this antiserum does not affect electron transport in chloroplast preparations under appropriate conditions. With green wild type tobacco chloroplasts, however, occasionally a more or less strong inhibition of the photoreduction of anthraquinone-2-sulfonate with high concentrations of DCPiPH_2 as the electron donor is observed (Fig. 5). With chloroplasts of the tobacco mutant Su/su var. aurea no appreciable inhibition of the above named reaction was found (Fig. 6). However, after ultrasonication the antiserum inhibits the same electron transport reaction in the aurea mutant up to 70 per cent (Fig. 6). The DCPiP Hill reaction or the photooxidation of diphenyl carbazide with DCPiP as the electron acceptor is not affected by



a)

b)

c)

Fig. 4. Influence of the antiserum 66 000 PSI-96 on the kinetics of the fluorescence rise. Wild type tobacco chloroplasts suspended in 0.05 M Tricine pH 7.2, 0.1 M KCl; electron donor ascorbate, electron acceptor anthraquinone-2-sulfonate. The assay with control serum is labelled with C. a) Time scale 0.1 sec/unit; b) time scale 5 sec/unit; c) as a) but with addition of DCMU.

the antiserum. The antiserum inhibits PMS-mediated cyclic photophosphorylation in unsonicated wild type tobacco chloroplasts on the average by 12% (Table II). In chloroplasts from the aurea mutant no inhibition was observed. This could imply that the inhibitory effect of photophosphorylation in

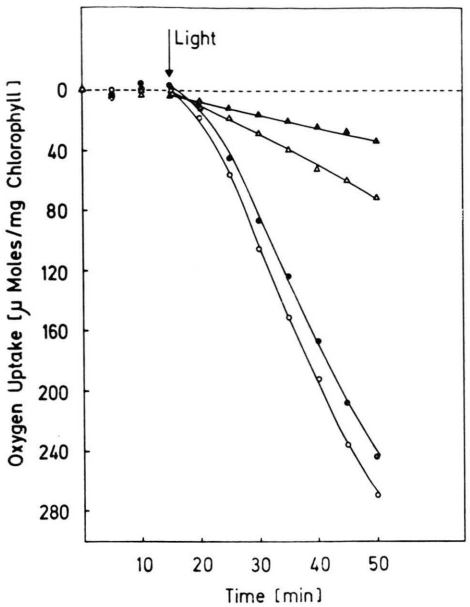


Fig. 5. Anthraquinone-2-sulfonate photoreduction with DCPiP/ascorbate as the electron donor in wild type tobacco chloroplasts. ○ reaction in the presence of control serum without ultrasonication; ● reaction in the presence of antiserum 66 000 PSI-88 without ultrasonication; △ reaction in the presence of control serum with chloroplasts after short ultrasonication; ▲ reaction in the presence of antiserum 66 000 PSI-88 with chloroplasts after short ultrasonication.

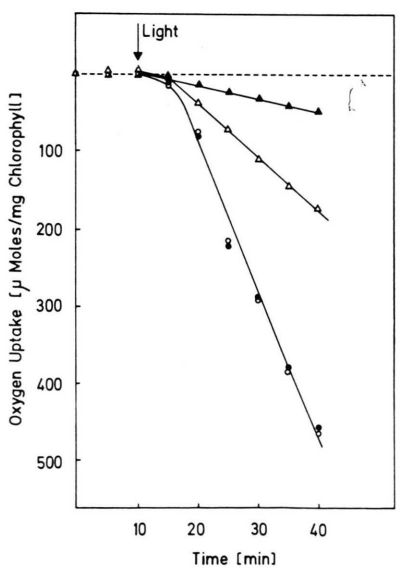


Fig. 6. Anthraquinone-2-sulfonate photoreduction with DCPiP/ascorbate as the electron donor in chloroplasts of the tobacco aurea mutant Su/su var. aurea. Symbols as in Fig. 5.

wild type tobacco chloroplasts is due to antibodies to coupling factor. This, however, seems not to be the case as the antiserum does not react with a coupling factor preparation in the Ouchterlony double diffusion test. Also from the fact that none of the tested photophosphorylation reactions in the aurea mutant is inhibited by the antiserum, it follows that no inhibitory action of the antiserum to coupling factor is present.

From the observed inhibition of electron transport in sonicated chloroplasts and from the fact that PMS-mediated cyclic photophosphorylation is slightly inhibited in unsonicated wild type chloroplasts it follows that the inhibition site must be located in the region of the reaction centre I. As cyclic photophosphorylation under the conditions used [20] runs via P_{700} and the primary electron acceptor X and as coupling factor is not involved in the inhibitory action, the suspicion arises that polypeptide 66 000 PSI-88 is a component of the reaction centre I itself. Moreover, the antiserum 66 000 PSI-88 acts in the electron transport system before the antiserum 66 000 PSI-96.

Similar effects were observed with the earlier described antiserum to Fraction 1 [21]. Fraction 1 was obtained by fractionation of stroma-freed chloroplasts, which were solubilized by means of sodium deoxycholate. The antiserum does not affect the photoreduction of anthraquinone-2-sulfonate with high concentrations of DCPiP using unsonicated chloroplasts, but inhibits after ultrasonication completely. Therefore, little doubt exists, that the antiserum to this fraction is directed towards the same component as the antiserum to polypeptide 66 000 PSI-88. According to our view, the higher degree of inhibition is due to the fact that Fraction 1 contains more native antigenic determinants than polypeptide 66 000 PSI-88. The identity of these two antisera is further substantiated by the observation that only the antiserum 66 000 PSI-88 inhibits the photoreduction of anthraquinone-2-sulfonate in this fraction whereas antiserum 66 000 PSI-96 does not (Fig. 7). Moreover, the antiserum 66 000 PSI-88 and the antiserum to Fraction 1 exert the same effect on fluorescence. Both antisera cause a lowering of the steady state level of the fluorescence rise curve (Fig. 8a). This could be interpreted as a diminuation of the ratio Q_{red}/Q_{ox} which occurs when the inhibition is on the donor side of photosystem II as observed in the case of the antiserum

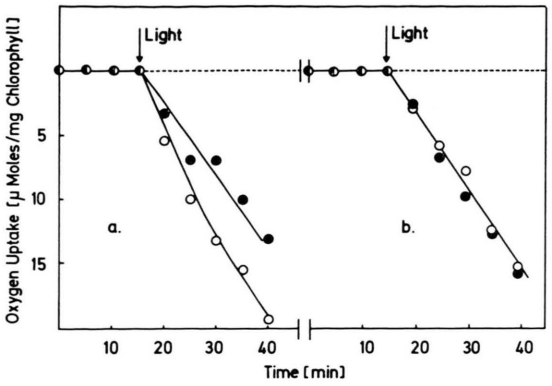


Fig. 7. Effect of the antisera 66 000 PSI-88 and 66 000 PSI-96 on the anthraquinone-2-sulfonate reduction with Fraction 1 from deoxycholate solubilized chloroplasts. Electron donors DCPiP/ascorbate. a) ○ reaction in the presence of control serum; ● reaction in the presence of antiserum 66 000 PSI-88; b) ○ reaction in the presence of control serum; ● reaction in the presence of antiserum 66 000 PSI-96.

to polypeptide 11 000 [8]. DCMU addition does not bring the steady state level of the fluorescence rise curves in the presence of antiserum and control serum together (Fig. 8b). However, the fluorescence level, lowered in both samples by the preillumination, is brought up by DCMU. Hence, the lowering of the fluorescence intensity has in this case nothing to do with Q, but is probably due to some structural alteration of the thylakoid membrane induced by the antiserum. For references see ref. 22. The situation is different with ultrasonicated chloroplasts. Here, the antiserum rises the fluorescence level provided the chloroplast preparation had not been exposed to too much light prior to fluorescence excitation (Fig. 8c). DCMU lifts the fluorescence level of the control to that of the antiserum sample (Fig. 8 d). This hints at a slow-down of electron transport in the region of photosystem I if the antibodies can be adsorbed at the inner surface of the thylakoid membrane or if antigen molecules become exposed otherwise. Additionally, it should be noted that the antiserum 66 000 PSI-88 agglutinates stroma-freed chloroplasts. As the only one among the three antisera it precipitates chloroplast fragments obtained by sonication and subsequent centrifugation (ultrasonic supernatant [23, 24]). A final conclusion would be that if the antiserum 66 000 PSI-88 is monospecific, the polypeptide 66 000 PSI-88 spans the thylakoid membrane.

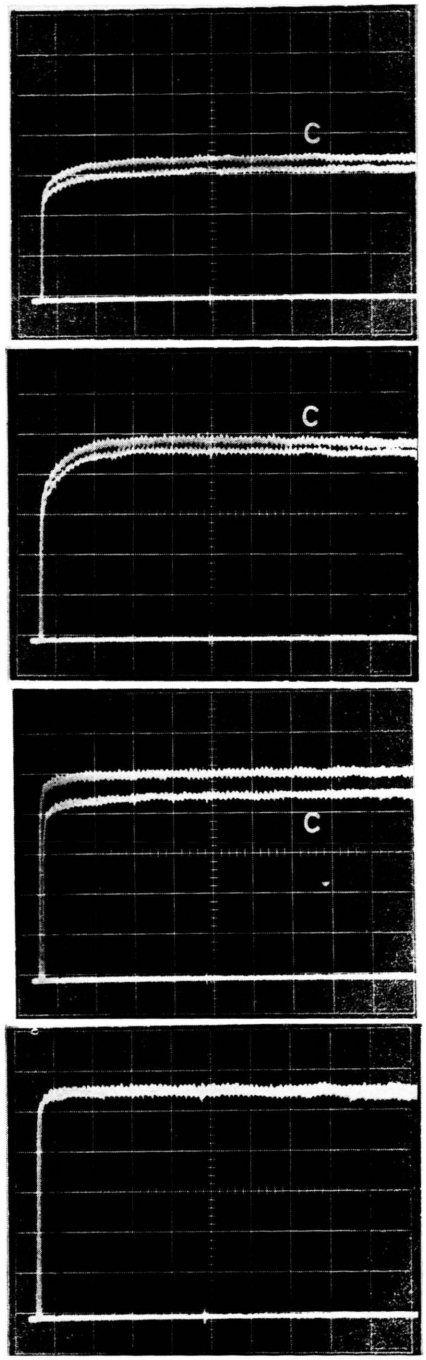


Fig. 8. Influence of the antiserum 66 000 PSI-88 on the kinetics of the fluorescence rise. Chloroplasts of the tobacco aurea mutant suspended in 0.05 M Tricine pH 7.2 and 0.1 M KCl; electron donor ascorbate, electron acceptor anthraquinone-2-sulfonate. The assay with control serum is labelled with C. Time scale in pictures 0.1 sec/unit. a) and b) without ultrasonication; c) and d) after short ultrasonication; a) and c) in the absence of DCMU; b) and d) in the presence of DCMU.

Whether the antisera to stroma-freed chloroplasts used by Berzborn *et al.*[5] and that used by Regitz *et al.*[25] contained antibodies to the antigens 66 000 PSI-96 and PSI-88, cannot be decided at this state of investigation.

Antiserum 66 000 PSII-42. This antiserum inhibits just as the antiserum to the polypeptide fraction 11 000 the anthraquinone-2-sulfonate Hill reaction and the photooxidation of tetramethyl benzidine with the same acceptor[8]. Different to the antiserum to polypeptide fraction 11 000 the antiserum also inhibits the photooxidation of diphenyl carbazide in Tris-washed chloroplasts (Fig. 9). This infers that the site of inhibition lies behind the site of electron donation of diphenyl carbazide. The photooxidation of diphenyl carbazide in chloroplasts not treated with Tris-buffer is also inhibited by the antiserum (Fig. 10). However, here the inhibition is observed after a time lag of 4 – 6 minutes. As shown in Fig. 11 diphenyl carbazide addition causes in the assay of the anthraquinone-2-sulfonate Hill reaction or tetramethyl benzidine photooxidation already inhibited by the antiserum, a temporary increase in the reaction rate. The pH-optimum of the inhibition lies at approximately pH 7.8 for the electron transport reaction between tetramethyl benzidine and anthraquinone-2-sulfonate in chloroplasts not treated with Tris-buffer (Fig. 12). The dependence of the degree of inhibition on the amount of antiserum added shows a sigmoidal curve shape (Fig. 2) as in the case with the antiserum to polypeptide fraction 11 000. The maximal degree of inhibition is more than 90 per cent. Hence, the antiserum inhibits in the region of photosystem II. No effect is observed on photosystem I reactions such as the photoreduction of anthraquinone-2-sulfonate with DCPiP/ascorbate as the donor in the presence of DCMU (Table IV).

A further characterization is possible by investigation of the influence of the antiserum on the

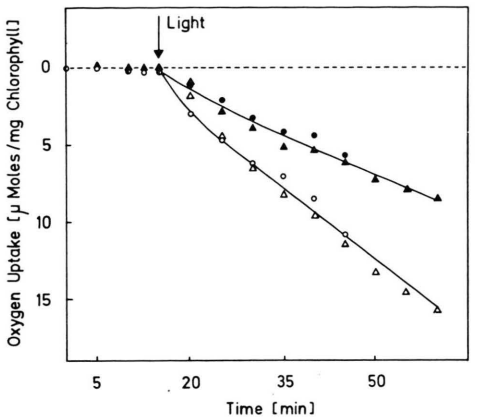


Fig. 9. Effect of the antiserum 66 000 PSII-42 on Tris-washed wild type tobacco chloroplasts; △ photooxidation of tetramethyl benzidine with anthraquinone-2-sulfonate as the electron acceptor in the presence of control serum; ▲ same reaction as △ in the presence of antiserum; ○ photooxidation of diphenyl carbazide with anthraquinone-2-sulfonate as the electron acceptor in the presence of control serum; ● same reaction as ○ in the presence of antiserum.

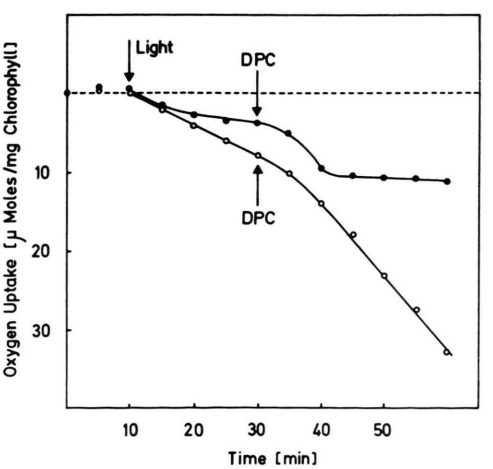


Fig. 10. Influence of diphenyl carbazide (DPC) addition on the reaction kinetics of the anthraquinone-2-sulfonate reduction in the presence of antiserum 66 000 PSII-42. ● Antiserum; ○ control serum. DPC indicates the time of addition.

Table IV. Comparison of the degree of inhibition of various electron transport reactions in wild type tobacco chloroplasts by the antisera.

Reaction	% Inhibition		
	66 000 PSI-96	66 000 PSI-88	66 000 PSII-42
$H_2O \rightarrow$ A-2-sulf	70 ± 10	0 – 4	≧90
TMB/asc → A-2-sulf	70 ± 10	0 – 3	≧90
DPC → A-2-sulf	75 ± 10	0 – 8	50 – 60 *
$H_2O \rightarrow$ DCPiP	0 – 2	0 – 2	
DCPiP/asc → A-2-sulf + 10^{-6} M DCMU	80 ± 10	0 – 30 **	0 – 3
DCPiP/asc → A-2-sulf + 10^{-6} M DCMU short ultrasonication	80 ± 10	50 ± 20	0 – 3

* Tris-washed chloroplasts; ** depending on the state of the chloroplast preparation. With chloroplasts of the aurea mutant either no or only a slight inhibition is observed.

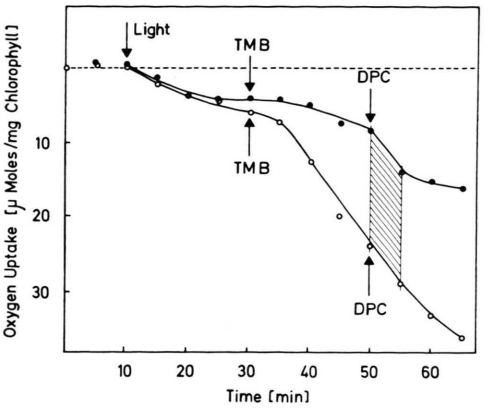


Fig. 11. Influence of subsequent additions of tetramethyl benzidine (TMB) and diphenyl carbazide (DPC) on the reaction kinetics of the anthraquinone-2-sulfonate Hill reaction in wild type tobacco chloroplasts. ○ Control serum; ● antiserum 66 000 PSII-42. The dashed parts mark the time span in which the inhibition is relieved.

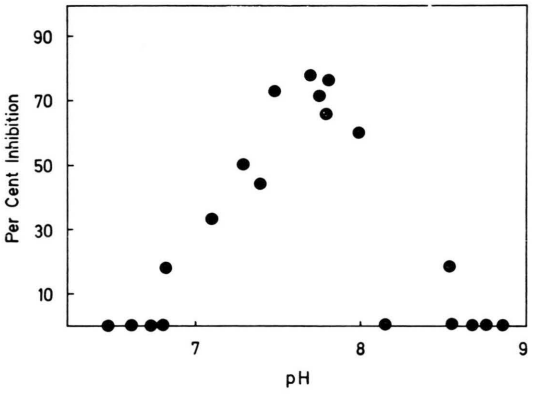


Fig. 12. Dependence of the degree of inhibition of the antiserum 66 000 PSII-42 on the pH of the assay. Reaction: Photoreduction of anthraquinone-2-sulfonate with tetramethyl benzidine/ascorbate as electron donor in wild type tobacco chloroplasts.

Kautsky effect. The antiserum causes either no effect on fluorescence or it slightly increases fluorescence temporarily (Fig. 13). Repeated illuminations do not change this observation. If the variable portion of fluorescence depends on the ratio Q_{red}/Q_{ox}, this result infers that this ratio is unchanged also under conditions in which electron flow is inhibited by more than 90%. Consequently, the adsorption of antibodies influences the oxidation as well as the reduction of the quencher to the same extent. If the reaction of the antiserum is monospecific, unchanged fluorescence together with blocked electron transport can be explained if the antigen is a component

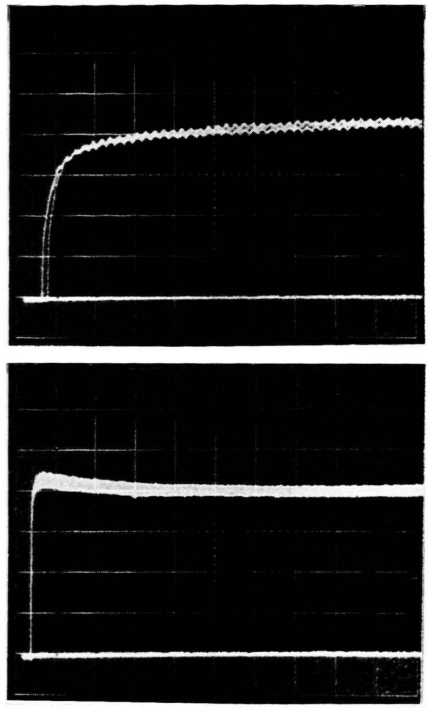


Fig. 13. Influence of the antiserum 66 000 PSII-42 on the kinetics of the fluorescence rise in wild type tobacco chloroplasts. Chloroplasts suspended in 0.05 M Tricine, 0.1M KCl pH 7.2; electron donor ascorbate; electron acceptor anthraquinone-2-sulfonate. a) Time scale 0.1 sec/unit; b) time scale 5 sec/unit. No difference between antiserum and control serum is observed.

of reaction centre II and if intermolecular interactions exist between the natural electron donor and the natural acceptor. It is also thinkable that interactions between Q and the antigen occur which regulate the oxidation and reduction of Q to the same extent. Whether this view is correct remains to be seen in future investigations. It should be noted that after addition of DCMU the fluorescence rise curves stay at the same level.

Discussion

Some results of the above described investigations are compiled in Table IV. It is clearly seen that the antisera, although directed towards polypeptide fractions of the same apparent molecular weight are distinctly different with respect to their effect on electron transport. The three antisera can also be distinguished by their influence on the fluorescence rise curve of chloroplasts. As can be seen, the sera 66 000 PSI-96 and 66 000 PSII-42 are mono-

specific, at least as far as the investigated reactions are concerned. Furthermore, the antigens 66 000 PSI-96 and 66 000 PSII-42 are located at the outer surface of the thylakoid membrane and are, therefore, fully accessible to antibodies. In order to exert an effect on electron transport in the case of antisera of the type 66 000 PSI-88 the thylakoids must be torn up by ultrasonication or the antigen molecule must be made accessible by trypsin digestion. Apparently only the antigen-antibody reaction at the inner surface of the thylakoid membrane leads to an inhibition of electron transport. Binding of antibodies onto the outer surface, however, remains without effect on electron transport, but causes fluorescence changes, which apparently are not exclusively due to the redox state of the quencher Q. The antisera can also be distinguished by the curves which are obtained if the degree of inhibition of the reaction rate is plotted versus the amount of antiserum added. Especially interesting are such cases in which the curve shape is sigmoidal, because a cooperative effect must be assumed in which several antigens belong to a functional unit and cooperate together. From all this it appears certain, that the three polypeptides play a role at different sites of the electron transport system, which does not mean, however, that the polypeptides are electron carriers, because the antibodies can also affect the carrier by conformational changes induced by the antibody binding provided intermolecular interactions exist between the protein and the carrier. The proteins would exert a regulating function then as an underlining for the carriers.

Less certain appears the exact functional localization of the effects in the electron transport system. A complicating factor in this context is, that the extent of the effect on the reaction rate as well as on the Kautsky effect depend in a more or less pronounced form on the condition or state of the chloroplast preparation. This condition is influenced by the isolation medium and the time which is required for the chloroplast isolation. A role plays apparently also the season in which the plants have been grown. Decesive can be the pH of the reaction medium. The maximum of inhibition does by no means coincide with the pH-optimum of the reaction rate. Furthermore, important for the inhibition are also potassium and magnesium ions as well as the presence of phosphate and adenosindiphosphate. Moreover, the listed factors have a different bearing on the three antisera. These circumstances although advantageous for the distinction of the sera and antigens, complicate the exact functional localization. With the above described investigations, differences in the condition of the chloroplast preparation have above all hampered the investigations on the effect of the antiserum 66 000 PSI-88.

Despite some remaining uncertainties in the details, our method to characterize the insoluble thylakoid membrane proteins via their antisera has proven to be a valuable tool.

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