# Polypeptides of the Thylakoid Membrane and Their Functional Characterization

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From stroma-freed chloroplasts of *Antirrhinum majus* polypeptides with the apparent molecular weights 44 000, 26 000 and 20 000 were isolated.

The antiserum to a polypeptide with the molecular weight 44 000 inhibits the photoreduction of anthraquinone-2-sulfonate with dichlorophenol indophenol/ascorbate when the concentration of the electron donor dichlorophenol indophenol is low. The antiserum enhances the rate of phenazine methosulfate-mediated cyclic photophosphorylation. The variable fluorescence yield is increased by the antiserum. It is assumed that this polypeptide plays a role in electron transport between the two photosystems. From two polypeptides with the apparent molecular weight 26 000 one seems to belong to the reaction center of photosystem II as it inhibits the photooxidation of tetramethyl benzidine and diphenyl carbazide with suitable electron acceptors and inhibits electron transport between water and silicomolybdate. Variable fluorescence is not or not too strong decreased by the antiserum. The other polypeptide of the apparent molecular weight 26 000 inhibits the photoreduction of anthraquinone-2-sulfonate with high concentrations of dichlorophenol indophenol as the electron donor. Phenazine methosulfate-mediated cyclic photophosphorylation is also inhibited by the antiserum. Therefore, we should like to associate it with the reaction center of photosystem I. The antiserum to the polypeptide with the apparent molecular weight 20 000 inhibits the photoreduction of anthraquinone-2-sulfonate with low and high concentrations of the electron donor dichlorophenol indophenol. It enhances phenazine methosulfate-mediated cyclic photophosphorylation. The polypeptide, therefore, should be functionally involved on the acceptor side of photosystem I.

The results obtained up-to-now on the function and localization of the polypeptides in the thylakoid membrane are summarized.

In several publications we have characterized a series of polypeptides of the thylakoid membrane by means of their antisera [1–13]. In the present publication we report on a polypeptide with the apparent molecular weight 44 000, two polypeptides with the apparent molecular weight 26 000 and a polypeptide with the apparent molecular weight 20 000. The polypeptides were isolated as usual from stroma-freed chloroplasts of *Antirrhinum majus*. Thus, the first screening of the polypeptides involved in electron transport reactions of the thylakoid membrane is concluded.

## Materials and Methods

*Stroma-freed* chloroplasts of *Antirrhinum majus* and stromafree swellable tobacco chloroplasts used for the functional tests were prepared as described earlier [14, 15].

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*Abbreviations:* DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; DCMU, N,N'-3,4-dichlorophenyl dimethylurea; Q, quencher of photosystem II.

*Preparation of the polypeptides:* The antigens 44 000 PSI-93 and 20 000 PSI-5 were prepared from a sucrose palmitate-stearate extract by gel filtration on Sepharose CL-6B (Pharmacia) after addition of sodium dodecyl sulfate (0.25%) to the extract [13]. The antigen 26 000 PSII-76 was isolated by repeated gel chromatography on Sepharose 6B and Sephadex G-150 as described earlier [6, 16]. The antigen 26 000 PSI-58 was freed from dodecyl sulfate after prefractionation of the dodecyl sulfate containing solution on Sepharose. For this purpose the solution, containing 3% Triton X-100 and 6 M urea, was freed by ion exchange chromatography from dodecyl sulfate and subsequently fractionated by chromatography on DEAE-Sepharose CL-6B (Pharmacia). Prior to this procedure the preparation was transfered to 0.025 M Tris-HCl buffer, pH 8.0, containing 0.1% mercaptoethanol, 1% Triton X-100, containing in addition 1% *n*-butanol. Elution was done with continuously increasing Tris-buffer concentrations of constant pH. The other components of the elution buffer were the same as described above. Fractions with a conductivity of 12–16 mS/cm were dialyzed after addition of 0.25% dodecyl sulfate against 0.01 M sodium

phosphate buffer pH 7.2, containing 0.25% dodecyl sulfate, 0.1% mercaptoethanol and 0.02% sodium azide. Subsequently, the preparation, containing 17 mg protein, was concentrated to 20 ml and subjected to gel filtration on Sephacryl S-200 Superfine (Pharmacia) (separation length 3.60 m, diameter 5 cm). Fractions, which in the dodecyl sulfate polyacrylamide gel electrophoresis exhibited an apparent molecular weight 26 000, were used for immunization after the removal of dodecyl sulfate.

*Immunization and serological tests* were carried out as described earlier [5, 17].

*Tests for electron transport reactions and photophosphorylation reactions* were all carried out as described earlier [4, 5, 18].

*Silicomolybdate* was prepared freshly before use according to the literature [19, 20].

*Fluorescence measurements* were also described previously [12].

## Results and Discussion

*On a polypeptide with the apparent molecular weight 44 000.*

Recently, we have reported on the effect of antisera which were directed to four polypeptides with apparent molecular weights between 40 000 and 50 000 on photosynthetic electron transport [12]. In the meantime two additional polypeptides were isolated from a sucrose palmitate-stearate extract whose molecular weights also fall into this region. One of the antisera had the same effect as that against 45 000 PSI-4 which apparently inhibits electron transport on the acceptor side of photosystem I [12].

The second polypeptide exhibited an apparent molecular weight 44 000. The antiserum to this polypeptide inhibits the chloroplast-mediated photoreduction of anthraquinone-2-sulfonate by DCPIP/ascorbate. Hence, as the serum is effective in the region of photosystem I, we designate it 44 000 PSI-93, with 93 being the serum number. The inhibition occurs only at low concentrations of the electron donor. According to Fujita and Murano this means that the inhibition site is on the donor side of photosystem I [21]. Fitting into this conclusion, the antiserum enhances PMS-mediated cyclic photophosphorylation (Table I). Non-cyclic photophosphorylation is inhibited to approximately the same extent as electron transport. On the other hand, photophosphorylation with ferricyanide as the electron acceptor and water as the donor is not affected by the antiserum (Table I). Other photosystem II reactions are also unaffected. The effect of the antiserum on electron transport is only measurable after an illumination period of several minutes. This is also seen with the fluorescence induction curve. An increase of the Q-dependent fluorescence yield shows up only after a preillumination of several minutes, if the chloroplasts were well

Table I. Effect of the antisera 44 000 PSI-93, 20 000 PSI-5, 26 000 PSII-58, and 26 000 PSII-76 on photophosphorylation reactions in tobacco chloroplasts.

| Antiserum | [$\mu$mol ATP formed · (mg Chlorophyll)$^{-1}$ · h$^{-1}$] | | |
|---|---|---|---|
| | PMS | $H_2O \rightarrow K_3Fe(CN)_6$ | $H_2O \rightarrow$ Anthraquinone-2-sulfonate |
| | Cyclic | Non-cyclic | Non-cyclic |
| 44 000 PSI-93 | 545 ± 30 | 75 ± 6 | 16 ± 1 |
| Control serum | 482 ± 30 | 75 ± 5 | 55 ± 6 |
| 20 000 PSI-5 | 680 ± 42 | 75 ± 5 | 26 ± 2 |
| Control serum | 486 ± 30 | 75 ± 5 | 55 ± 3 |
| 26 000 PSI-58 | 0 | 75 ± 5 | 17 ± 1 |
| Control serum | 482 ± 29 | 78 ± 6 | 56 ± 3 |
| 26 000 PSII-76 | 407 ± 28 | 23 ± 2 | 17 ± 1 |
| Control serum | 484 ± 31 | 76 ± 4 | 56 ± 4 |

The values are averages of at least 5 individual determinations. The mean error of the average value is indicated.

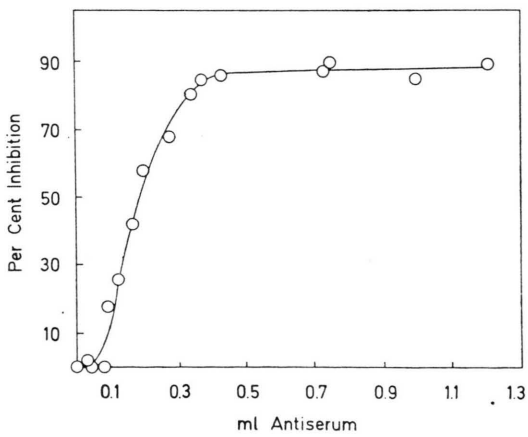


Fig. 1. Dependence of the degree of inhibition of electron transport caused by the antiserum 44 000 PSI-93 on the amount of antiserum added. Electron transport reaction DCPIP/ascorbate → anthraquinone-2-sulfonate. DCPIP-concentration 0.15 mM.

adapted to the dark. The dosis-effect curve shows a lag phase, that is small amounts of serum have no effect, whereas after exceeding a threshold value the inhibition reaches its maximal extent fast (Fig. 1). We do not dare to decide whether the curve increases after the lag phase in a hyperbolic or sigmoidal manner. The question is not unimportant as it would give an indication whether only one or several antigens play a role in every electron transport chain.

*Polypeptides with molecular weights between 20 000 and 30 000*

In the region of the apparent molecular weights between 20 000 and 30 000 electropherograms show an accumulation of partially overlapping bands. The gels show after staining at least six individual bands. If these gels are scanned before staining with 680 nm light and after staining with Coomassie Blue with 558 nm light, two protein bands with high red absorption are recognized. Their apparent molecular weights are approximately 27 000 and 23 000 and they are probably components of the lightharvesting-complex ([22], for further references see ref. 23). Burke *et al.* have prepared antisera to this complex [24]. The antisera exerted no effect on electron transport but had an effect on the excitation energy distribution between photosystem II and I. This influence on excitation energy distribution proved to be dependent on the type and concentrations of the ions present in the assay [24]. We have often immunized rabbits with polypeptide fractions of this molecular weight region. The obtained antisera were with a few exceptions without any effect on electron transport, although antigen-antibody reactions with chloroplasts occurred [6, 25]. In the following we report on these exceptions.

An antiserum to the polypeptide with the apparent molecular weight 20 000, in the following designated as 20 000 PSI-5 inhibits the photoreduction of anthraquinone-2-sulfonate with DCPIP/ascorbate at high and low concentrations of the electron donor. The dosis-effect curve shows a sigmoidal shape and reaches its saturation value at approximately 80% (Fig. 2). The effect of the serum is only seen after an illumination period or is increased in the course of the illumination. Also, the fluorescence induction shows a Q-dependent increase of the fluorescence yield after preillumination. From low temperature fluorescence measurements it appears that antiserum 20 000 PSI-5 lowers the energy spill-over with dark adapted chloroplasts. Preillumination abolishes this effect. At room temperature the serum lowers the fluorescence yield only slightly if DCMU is present. Photosystem II reactions are not influenced. A more precise localization of the serum effect is possible as the serum enhances cyclic photophosphorylation by 40% whereas noncyclic photophosphorylation with ferricyanide as the acceptor is not inhibited (Table I). Therefore, we should like to assume that the serum exerts its effect on the acceptor side of photosystem I.

Finally, two polypeptides with the apparent molecular weights 26 000 were isolated. The antiserum to the first component (26 000 PSI-58) inhibits the photoreduction of anthraquinone-2-sulfonate by high and low concentrations of the electron donor DCPIP. The amount of serum added plotted against inhibition of electron transport yields a sigmoidal

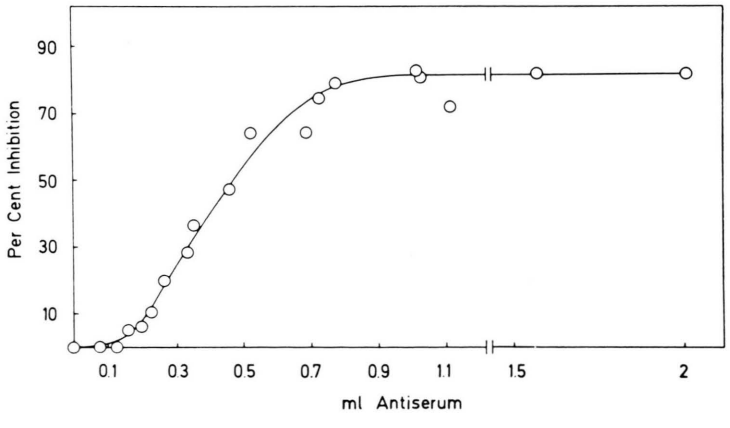


Fig. 2. Dependence of the degree of inhibition of electron transport caused by the antiserum 20 000 PSI-5 on the amount of antiserum added. Electron transport reaction as in Fig. 1. DCPIP-concentration 0.9 mM.

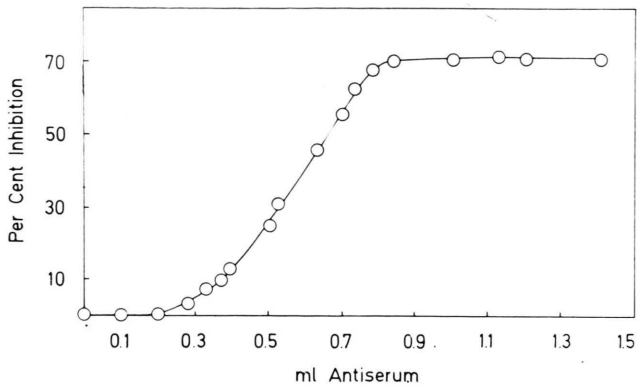


Fig. 3. Dependence of the degree of inhibition of electron transport caused by the antiserum 26 000 PSI-58 on the amount of antiserum added. Electron transport reaction and DCPIP-concentration as in Fig. 2

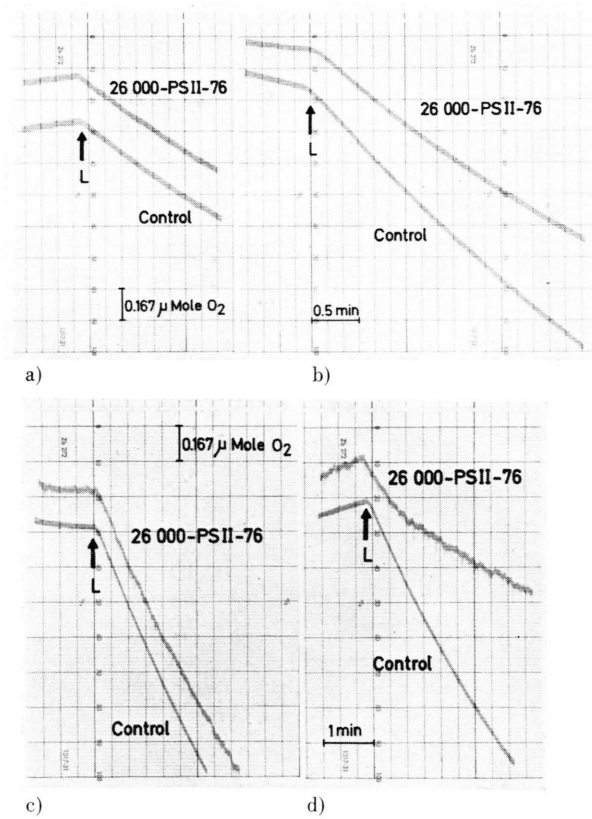


Fig. 4. Photoreduction of silicomolybdate with water as the electron donor in the presence of the antiserum 26 000 PSII-76. Original registrations of oxygen evolution measured with the oxygen electrode. The arrow indicates the onset of oxygen evolution upon illumination. a) and b) without addition of ADP, $P_i$ and $Mg^{2+}$ to the assay; c) and d) scan in the presence of ADP, $P_i$ and $Mg^{2+}$ in the assay. The greater effectiveness of the antiserum in b) and d) is caused by a longer illumination of the complete reaction mixture before registration.

curve (Fig. 3). PMS-mediated cyclic photophosphorylation is completely inhibited by the antiserum. Photosystem II reactions are not inhibited (Table I). Antiserum 26 000 PSI-58 has no effect on the distribution of excitation energy between the photosystems if the chloroplasts are dark adapted. After preillumination, however, the antiserum enhances the energy spill-over. The same is valid for the Q-dependent fluorescence yield. Hence, we feel that

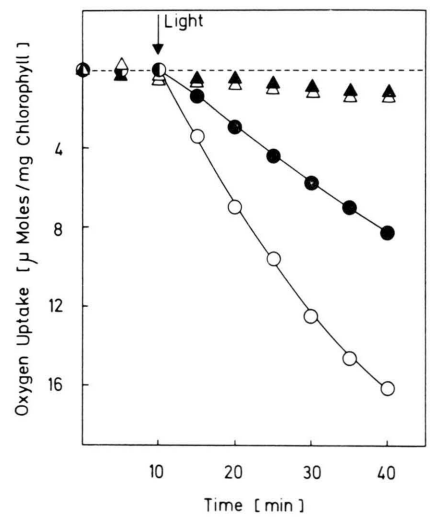


Fig. 5. Effect of the antiserum 26 000 PSII-76 on the photooxidation of diphenyl carbazide in Tris-washed tobacco chloroplasts with anthraquinone-2-sulfonate as the electron acceptor; (●) reaction in the presence of antiserum, (○) reaction in the presence of control serum; diphenyl carbazide concentration 1.7 mM in the assay. The triangels give the rate of the remaining Hill reaction after Tris-treatment; (▲) in the presence of antiserum, (△) in the presence of control serum.

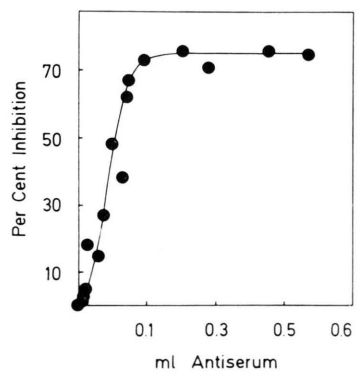


Fig. 6. Dependence of the degree of inhibition of electron transport caused by the antiserum 26 000 PSII-76 on the amount of antiserum added. Electron transport reaction tetramethyl benzidine/ascorbate → anthraquinone-2-sulfonate.

the antigen 26 000 PSI-58 belongs to the reaction center of photosystem I.

The second serum to a polypeptide fraction with the molecular weight 26 000 is not entirely uniform with respect to its effectiveness. It inhibits to a small extent photosystem I reactions and obviously contains a small amount of antibodies to the antigen 26 000 PSI-58. Its main effect, however, lies on the photosystem II side. The photoreduction of silicomolybdate with water as the electron donor is inhibited by 60% (Fig. 4 d). In order to obtain a high degree of inhibition this assay had to contain ADP, Pi and $Mg^{++}$ and had to be preilluminated (Fig. 4 a, b, c). The photooxidation of diphenyl carbazide is also inhibited (Fig. 5). As is expected, the same is true for the photooxidation of tetramethyl benzidine. The dosis-effect curve for the reaction tetramethyl benzidine to anthraquinone-2-sulfonate shows rather a hyperbolic than a sigmoidal shape (Fig. 6). The effect of the antiserum resembles in many respects that of the serum 66 000 PSII-42, described earlier [11]. The serum

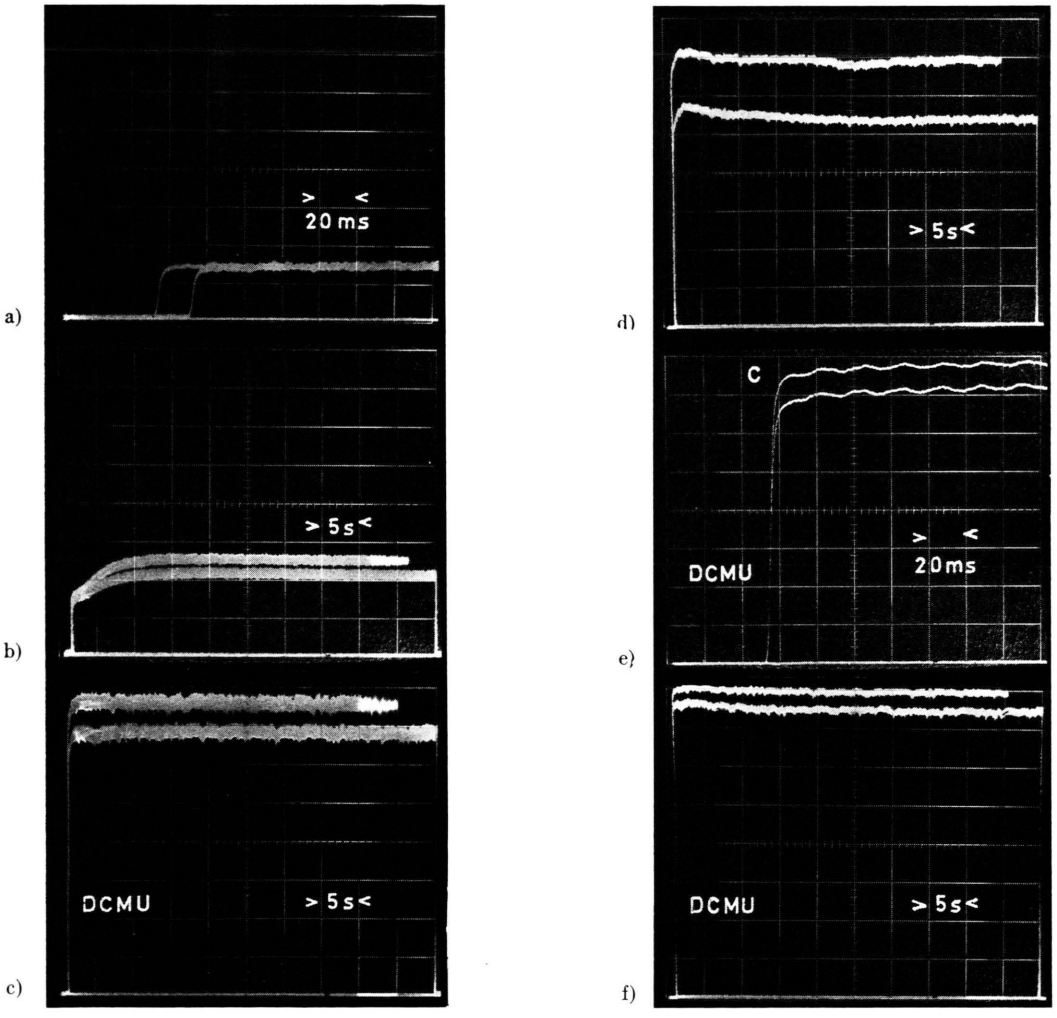


Fig. 7. Effect of the antiserum 26 000 PSII-76 on the fluorescence rise.
In the dark adapted sample initial fluorescence ($F_0$) is not influenced by the antiserum (a), whereas the variable fluorescence ($F_{var}$) is lowered by the antiserum (b). This lowering of the fluorescence yield, however, is apparently not Q-dependent, as DCMU increases the difference (c). This goes for the fluorescence excitation with low light intensity ($\approx$ 0.1 Watt/m$^2$). Excitation with strong light (42 Watt/m$^2$) increases the difference between the assay with antiserum and control serum (d). In the presence of DCMU the antiserum lowers $F_0$ and $F_{var}$ under these light conditions (e, f). The shorter scan or the one denoted with c is the fluorescence rise curve in the presence of control serum. The scale on the ordinate corresponds to 200 mV in a), b) and c), and to 5V in d), e) and f).

lowers the fluorescence yield of dark adapted chloroplasts in weak and strong exciting light (Fig. 7 b, d). In weak light DCMU does not abolish but rather enlarges this difference although it increases the fluorescence yield in the assay with antiserum as well as in the one with control serum (Fig. 7 c). Excitation with strong light shows that in the presence of DCMU also the level of the initial fluorescence ($F_0$) is lowered (Fig. 7 e) which is not the case in the absence of DCMU (Fig. 7 a). Also in strong exciting light the difference in the assay with control and antiserum does not disappear entirely in the presence of DCMU (Fig. 7 f). From this it appears that Q-dependent variable fluorescence is less affected by the antiserum than would be expected from the inhibition of electron transport. The situation becomes less clear after a preillumination period as in this case the lowering of the fluorescence level is superimposed on a small rise of the fluorescence yield, which is due to the contamination by 26 000 PSI-58 (Fig. 8 a – f). If one disregards the effect of the contamination with 26 000 PSI-58

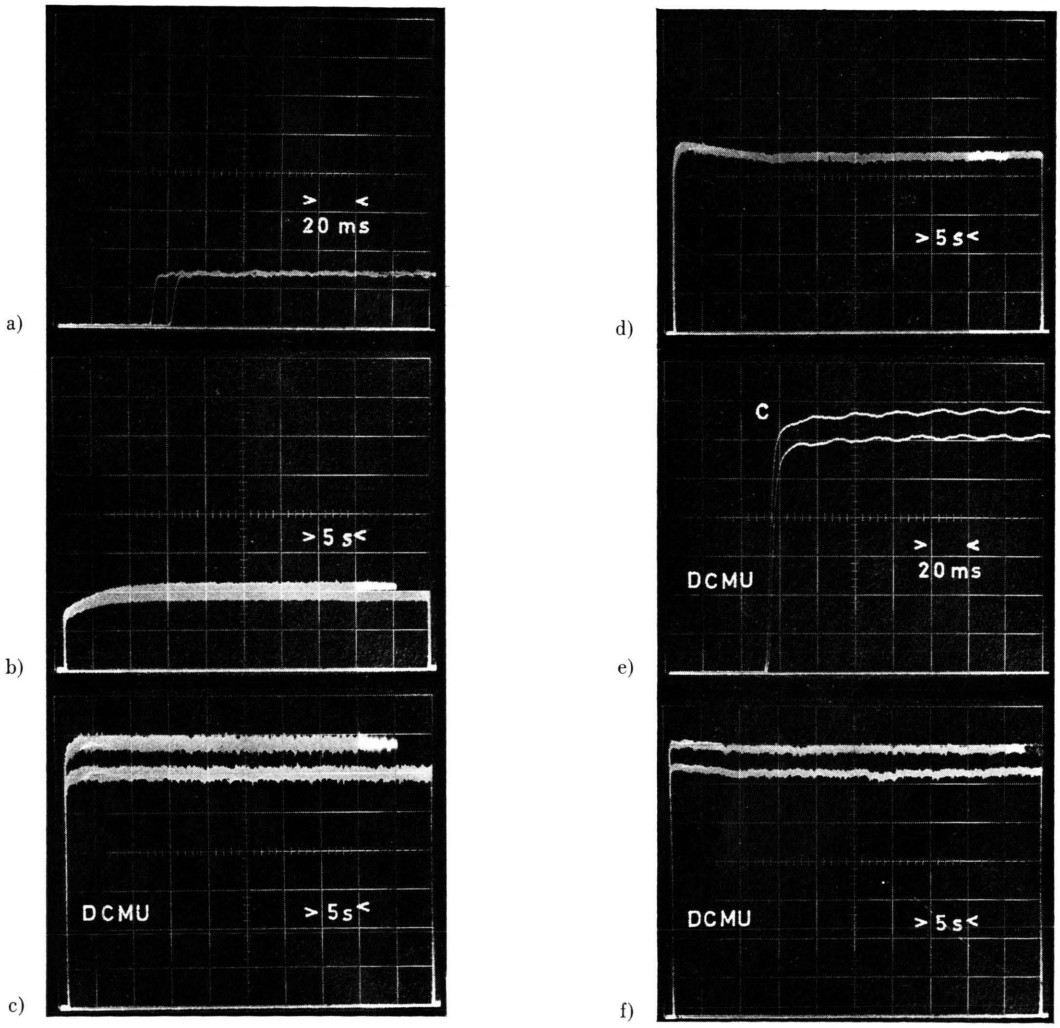


Fig. 8. Effect of the antiserum 26 000 PSII-76 on the fluorescence rise in preilluminated chloroplasts. Also after preillumination the antiserum does not affect $F_0$ (a), lowers the variable fluorescence (b) and DCMU increases the difference between the sample with antiserum and control serum (c). This is valid for the fluorescence excitation with low light intensity. In strong light the antiserum does not exhibit an effect on fluorescence (d). Nevertheless, in the presence of DCMU $F_0$ is lowered by the antiserum (e). This effect is also seen with low registration speed (f). The shorter scan or the one denoted with c represents the fluorescence rise in the presence of control serum. The scale on the ordinate corresponds to 200 mV in a), b), and c), and to 5V in d), e) and f).

the antiserum effect on fluorescence is similar to that of 66 000 PSII-42 [11]. The antiserum enhances the energy spill-over from photosystem II to photosystem I with dark adapted and preilluminated chloroplasts. It should be noted that preillumination increases as with other antisera the effect. Therefore, the antigen 26 000 PSII-76 could well belong to the reaction center of photosystem II.

*Survey of the functional localization of the polypeptides described up-to-now*

We have reasons to believe that we have by now investigated the major part of the polypeptides which play a role in electron transport in the thylakoid membrane. In the following we give a short survey of our results.

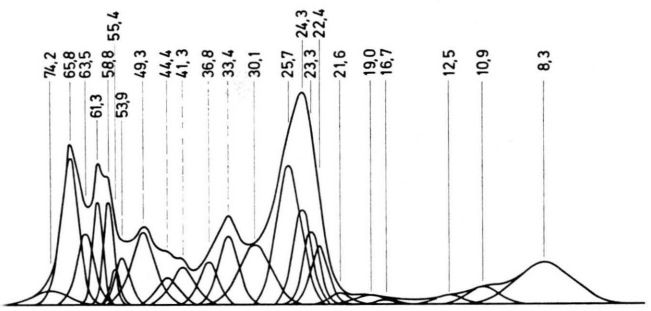


Fig. 9. Photometer curve of a Coomassie Blue stained dodecyl sulfate polyacrylamide gel electropherogram of the thylakoid membrane polypeptides (acrylamide concentration 10%, buffer pH 7.2, wavelength 558 nm) resolved with a computer. The numbers over the peaks are to be multiplied by 1000 and represent the apparent molecular weights of the polypeptides.

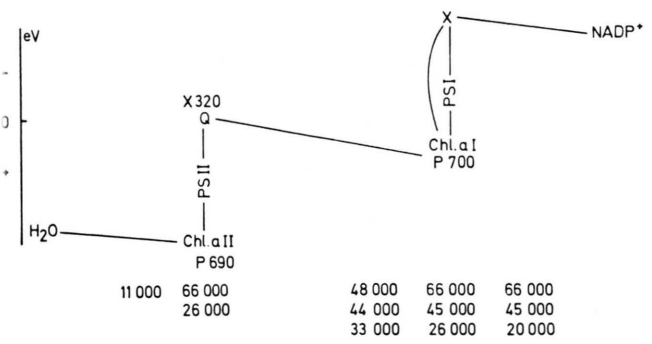


Fig. 10. Scheme which illustrates the sites of functional involvement of the polypeptides listed. The numbers are the apparent molecular weights.

An antiserum to a polypeptide with the apparent molecular weight 11 000 (11 000 PSII-15) inhibits electron transport on the donor side of photosystem II [8, 9]. This serum inhibits the reaction rate of the photooxidation of tetramethyl benzidine but not the rate of the diphenyl carbazide photooxidation. The antiserum diminishes the initial signal amplitude of the 690 nm and the 320 nm absorption change in repetitive flash spectroscopy [9]. It should be noted that the real molecular weight of this polypeptide is 6300 [26].

Two antisera directed to polypeptides with the apparent molecular weights 66 000 (66 000 PSII-42) and 26 000 (26 000 PSII-76) affect the reaction center of photosystem II or its immediate vicinity. Both sera inhibit the photooxidation of tetramethyl benzidine and diphenyl carbazide [11].

Antisera to polypeptides with the apparent molecular weights 33 000 (33 000 PSI-60), 44 000 (44 000 PSI-93) and 48 000 (48 000 PSI-3) inhibit electron transport between the two photosystems. These antisera inhibit the photoreduction of anthraquinone-2-sulfonate by DCPIP/ascorbate at low concentrations but not at high concentrations of the electron donor [6, 12].

Antisera to polypeptides with the apparent molecular weights 66 000 (66 000 PSI-88) and 45 000 (45 000 PSI-1) inhibit in the immediate vicinity or the reaction center of photosystem I itself. These antisera inhibit the photoreduction of anthraquinone-2-sulfonate by DCPIP/ascorbate at high and low concentrations of the electron donor in the presence of DCMU. Moreover, these sera inhibit PMS-mediated cyclic photophosphorylation [11, 12].

The polypeptides with the apparent molecular weights 66 000 (66 000 PSI-96), 45 000 (45 000 PSI-4) and 20 000 (20 000 PSI-5) play a role on the acceptor side of photosystem I. The antisera inhibit the photoreduction of anthraquinone-2-sulfonate by DCPIP/ascorbate at high and low concentrations of the donor [11, 12]. The antisera to the polypeptides with the apparent molecular weights of 20 000 and 45 000 do not affect PMS-mediated cyclic photophosphorylation [12]. The serum to the polypeptide with the apparent molecular weight 66 000 at first enhances this reaction, which, however, in the further course of the reaction leads to an inhibition [11].

As far as we know, none of these polypeptides is identical to one of the known electron carriers. This is shown by comparison with antisera to plastocyanin [5], cytochrome f [10], ferredoxin $NADP^+$-reductase [1, 4] and to ferredoxin [3]. In the course of these investigations we also obtained antisera which were directed to the subunits of the coupling factor of photophosphorylation [13].

Concerning the localization in the electron transport chain we rely on the literature [27, 28]. The view on these reactions is sometimes controversal which leads to some uncertainties in our localization experiments.

With the exception of polypeptide 66 000 PSI-88 the antigenic determinants, whose binding to antibody molecules causes on inhibition of electron transport are located in the outer surface of the thylakoid membrane [11]. According to results of Nelson *et al.* we think it possible, that polypeptide 66 000 PSI-88 is bound to $P_{700}$ [29]. The antiserum to this polypeptide inhibits electron transport only after the disrupture of the thylakoids by ultrasonication. Therefore, the antigen 66 000 PSI-88 is located inside the thylakoid membrane or spans the membrane [11].

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