Inhibition of Electron Transport on the Oxygen-Evolving Side of Photosystem II by an Antiserum to a Polypeptide Isolated from the Thylakoid Membrane

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Dedicated to Prof. Dr. J. Straub on the Occasion of His 65th Birthday

Thylakoid Membrane, Polypeptide, Photosystem II, Antiserum

A polypeptide fraction with the apparent molecular weight 11000 was isolated from stroma-freed chloroplasts from *Antirrhinum majus*. An antiserum to this polypeptide fraction inhibits photosynthetic electron transport in chloroplasts from *Nicotiana tabacum*. The relative degree of inhibition is pH dependent and has its maximum at pH 7.4. The maximal inhibition observed was 93%. The dependence of the inhibition on the amount of antiserum yields a sigmoidal curve which hints at a cooperative effect. A calculation of the Hill interaction coefficient gave the value of 10. The inhibition occurs on the water splitting side of photosystem II between the sites of electron donation of tetramethyl benzidine and diphenylcarbazide. Tetramethyl benzidine donates its electrons before the site where diphenylcarbazide feeds in its electrons. Analysis of the steady state level of the variable fluorescence also indicates that the inhibition site is on the water splitting side of photosystem II. Tris-washed chloroplasts are equally inhibited by the antiserum and the inhibition is also observed in the presence of an inhibitor of photophosphorylation like dicyclohexyl carbodiimide and in the presence of the uncoupler carbonylcyanide m-chlorophenyl hydrazone (CCCP) which means that the inhibitory action is directed towards the electron transport chain. Valinomycin which is supposed to affect the cation permeability of the thylakoid membrane has no influence on the inhibitory action of the antiserum. The same is valid for gramicidin. Methylamine on the other hand can induce a state in the thylakoids in which the antiserum is not effective. If the antibodies are already adsorbed prior to the methylamine addition then the high inhibitory effect by the antiserum remains unchanged upon addition of methylamine.

From the experiments it follows that a component from the vicinity of photosystem II is accessible to antibodies that is, the component is located in the outer surface of the thylakoid membrane. It appears that the inhibitory effect is produced in the course of the light reaction.

Materials and Methods

*The isolation of the polypeptide fraction 11000 from stroma-freed chloroplasts of *Antirrhinum majus* was described previously*. 

Gel electrophoreses were carried out according to Weber and Osborn. In the dodecyl sulfate phosphate gels the acrylamide concentration was 10% whereas in the dodecyl sulfate Tris gels the acrylamide concentration of the separating gel was 12.5%. The gels were stained with Coomassie blue and scanned at λ = 558 nm on a Zeiss PMQ II spectrophotometer fitted with a DISC Ansatz Z K 4.
The isolation of photochemically active stroma-free swellable chloroplasts from Nicotiana tabacum var. John Williams Broadleaf was also described earlier. Light reactions were mainly carried out as reported earlier. A large part of the electron transport reactions was carried out as anthraquinone-2-sulfonate Mehler reaction by measuring the oxygen consumption resulting from the autoxidation of the reduced electron acceptor anthraquinone-2-sulfonate in a Warburg apparatus at 20 °C. The assay composition was mainly as described by Harth et al. The reaction mixture contained: in the main vessel compartment 1.8 ml 0.075 M tricine 0.2 M KCl pH 7.6, 0.1 ml 0.1 M MgCl₂, 0.1 ml 0.05 M K₂HPO₄, 0.1 ml 0.05 M ADP, chloroplasts corresponding to 200 µg chlorophyll, antiserum or control serum as indicated, 0.1 ml 3 × 10⁻³ M anthraquinone-2-sulfonate and 0.1 ml 10⁻³ M NaCN; in the side arm 0.1 ml 0.1 M ascorbate and 0.1 ml 0.005 M tetramethyl benzidine or benzidine and in the center well 0.1 ml 30% KOH. The final volume was 3 ml. The reaction vessels were illuminated from below with 30000 ergs·sec⁻¹·cm⁻² of red light 580 nm < λ < 700 nm, transmitted through a plexiglas filter.

Proton translocation and photophosphorylation if not measured via ³²P-incorporation as described earlier, were measured according to Dilleyn, using a pH-meter model PHM 26 from Radiometer, Copenhagen equipped with a Servogor S Model RE541 recorder.

The preparation of the antiserum to the polypeptide 11000 was described previously as well as the agglutination reactions of this antiserum with chloroplast preparations.

Fluorescence measurements were carried out with a modified Perkin-Elmer Fluorescence Spectrophotometer MPF-3 equipped with a Siemens oscillograph model Oscillar MO 7114.

Results

1. Characterization of the polypeptide fraction 11000

The polypeptide fraction which served for the immunization migrated in the dodecyl sulfate polyacrylamide gel electrophoresis as a single narrow band provided Tris-buffer pH 8.8 was used. Fig. 1 shows an optical scan of the electropherogram after staining of the gel with Coomassie blue. If the electrophoresis was carried out in phosphate buffer at pH 7.1 the polypeptide migrated as a broad diffuse band. Orienting determinations of the amino terminal amino acids revealed that the preparation consists of several polypeptide species which all exhibit the apparent molecular weight of about 11000. The immunological characterization by immune electrophoresis and Ouchterlony double diffusion also hints at more than one component.

2. Determination of optimal experimental conditions

The photoreduction of anthraquinone-2-sulfonate with water as the electron donor in stroma-free swellable chloroplasts from tobacco is considerably enhanced if benzidine/ascorbate or tetramethyl benzidine/ascorbate are added to the reaction mixture. The anthraquinone-2-sulfonate Hill reaction as well as the photoreduction of anthraquinone-2-sulfonate with tetramethyl benzidine/ascorbate as the electron donors are inhibited by the antiserum to the polypeptide 11000. This inhibition has a marked pH dependence which is shown in Fig. 2. The
highest inhibition was at pH 7.4 and the maximal inhibition was 93%. The effect of the antiserum on the Hill reaction lies in a narrower pH range than that of the photooxidation of tetramethyl benzidine. The dependence of the inhibition at this optimal pH of 7.4 on the amount of antiserum added yields a sigmoidal curve shape, which hints at a cooperative effect (Fig. 3). A preliminary calculation of the Hill interaction coefficient yields 10. After the determination of the optimal experimental conditions all reactions described from here onward were carried out with chloroplasts corresponding to 200 μg total chlorophyll at pH 7.4 – 7.5 and in the presence of a saturating amount of antiserum (0.1 ml).

3. Localization of the inhibition site by the antiserum

From a number of experiments the reaction sequence shown in Fig. 4 gives the localization of the inhibition site by the antiserum. The assay was carried out in two Warburg vessels each of which had 3 side arms. One Warburg vessel contained the antiserum and the other the control serum. Two side arms contained each the electron donor to photosystem II namely tetramethyl benzidine and diphenylcarbazide; the third side arm contained 2,6-dichlorophenol indophenol as electron donor to photosystem I. Anthraquinone-2-sulfonate served as an electron acceptor. Before addition of an artificial electron donor to photosystem II water serves as an electron donor. This Hill reaction is clearly inhibited by the antiserum. Addition of tetramethyl benzidine/ascorbate at the time indicated in Fig. 4 does not influence anymore the reaction rate in the sample with antiserum whereas the same sample responds to the addition of diphenylcarbazide. However, the control assay in the presence of the same amount of control serum reacts upon addition of tetramethyl benzidine/ascorbate. From this it follows that the inhibition site is between the sites of electron donation of tetramethyl benzidine/ascorbate and that of diphenylcarbazide. Tetramethyl benzidine thus feeds in the electrons before diphenylcarbazide. The localization of the inhibition site in the region of photosystem II is further supported by the fact that after inhibition of electron transport by the antiserum the typical donor system to photosystem I 2,6-dichlorophenol indophenol/ascorbate yields high rates of anthraquinone-2-sulfonate photoreduction (Fig. 4). In other experiments we were always able to show that the antiserum does not affect photosystem I reactions (Table I, Fig. 13). These results are schematically summarized in Fig. 5.

As a general observation it must be added that despite the fact that agglutination of the chloroplasts occurs immediately upon addition of the antiserum, the inhibition of electron transport becomes only manifest in the course of the illumination period.
This becomes especially obvious in the following fluorescence measurements.

The influence of light on the inhibitory action of the antiserum and the above described localization of the inhibition site is further strengthened by measurements of the steady state level of the variable fluorescence. The assay system contained tetramethyl benzidine/ascorbate as the electron donor and anthraquinone-2-sulfonate as the acceptor. A dark adapted mixture which has received no light prior to fluorescence excitation shows no influence of the antiserum on the steady state level of fluorescence (Fig. 6). If, however, the reaction mixture has been illuminated with red light until the inhibitory effect by the antiserum was established then the fluorescence level was lower in the presence of antiserum than in the presence of control serum (Fig. 7). This shows according to the literature that the inhibition site of the antiserum is on the water splitting side of photosystem I \textsuperscript{13}. If inhibition strikes on the oxygen-evolving side of photosystem II then the fluorescence quencher Q is not or only slowly reduced. In this condition electrons are faster removed by photosystem I than they are supplied by photosystem II. This means that the quencher Q is preponderantly in the oxidized state in which the fluorescence is quenched and consequently low. If the drain of electrons through photosystem I is prevented by the addition of DCMU, fluorescence is immediately high because the quencher Q stays in the reduced state (Fig. 7). Not shown in Fig. 7 is the effect of DCMU on the assay with the antiserum. The addition of DCMU blocks the reoxidation of the reduced quencher which also in the presence of antiserum makes fluorescence rise up again.

As the chloroplasts are agglutinated by the antiserum they scatter stronger than in the presence of control serum. Therefore, an antiserum to a polypeptide 24000 which strongly agglutinates chloroplasts but which does not affect electron transport was tested for fluorescence with and without pre-illumination in the electron transport system TMB/Asc→anthraquinone-2-sulfonate. No effect of the antiserum on the steady state level of variable fluorescence was observed which excludes scattering effects as an error source.

Tris-washed chloroplasts show also in the fluorescence assay that electron transport in the system tetramethyl benzidine/ascorbate→anthraquinone-2-sulfonate is inhibited by the antiserum on the donor side of photosystem II.

4. Influence of uncouplers and inhibitors

The high degree of inhibition at first let us suspect that the antibody action might be somehow
connected with the proton or cation translocation or with some kind of permeability change. The inhibitor of photophosphorylation dicyclohexyl carbodiimide (DCCD) applied in concentrations which entirely suppress the formation of the proton gradient and photophosphorylation (Table I) does not remove the inhibitory effect by the antiserum on the electron transport reaction (Fig. 8). The observed degree of inhibition by the antiserum is still 65%. The reaction rate itself is lower due to the presence of DCCP in the reaction mixture. This leads to the conclusion that primarily the electron transport and not the photophosphorylation is inhibited by the antiserum. The uncoupler carboxyldiene m-chlorophenyl hydrazone (CCCP) and of the photophosphorylation inhibitor N,N'-dicyclohexyl carbodiimide (DCCD) on the inhibition degree of photosynthetic electron transport in tobacco chloroplasts caused by the antiserum in the electron transport system $H_2O \rightarrow$ anthraquinone-2-sulfonate. The assay mixture was $3.3 \times 10^{-6} \text{M}$ in CCCP or $10^{-4} \text{M}$ in DCCD. ○, Assay in the presence of antiserum; ○, in the presence of control serum.

The antibiotic valinomycin also does not influence the inhibitory action of the antiserum on electron transport. The antibiotic is supposed to increase the cation permeability of the membrane. It is seen from Fig. 9 that in the presence of valinomycin the Hill reaction rate of the control decreases in the light until the rate is zero. Upon addition of tetramethyl benzidine the electron transport rate is fully restored. The relative degree of inhibition of the electron transport rate by the antiserum in the system tetramethyl benzidine/ascorbate $\rightarrow$ anthraquinone-2-sulfonate is not changed by the presence of valinomycin (Fig. 9). The antibiotic gramicidin which affects the proton permeability and thereby inhibits photophosphorylation does not alter the influence of the antiserum on electron transport (Fig. 9). The relative degree of inhibition is essentially the same in the presence and absence of gramicidin at pH 7.5. Again, no effect by either antibiotic, namely valinomycin and gramicidin, was observed on the effect of the antiserum on the electron transport rate.

The effect of the uncoupler methylamine is clearly different from that of CCCP. This is demonstrated in Fig. 10. If methylamine is added to the assay after the establishment of inhibition by the antiserum no relieve is observed. If, however, methylamine is added to the chloroplast suspension before serum addition the antiserum has no effect at all on the reaction rate. From this we infer that methylamine changes the morphological structure of the lamellar system and possibly also the state of the thylakoid membrane to a condition in which the antigen is not accessible anymore to antibodies or where binding of antibodies does not induce inhibition (Fig. 10).

Tris-washing of the chloroplasts abolishes the capacity for oxygen evolution. Addition of the artificial electron donor couple tetramethyl benzidine/ascorbate restores electron transport to NADP$^+$ or to an artificial electron acceptor such as...
anthraquinone-2-sulfonate. In this condition the antiserum also exerts its inhibitory effect (Fig. 11). It appears that the relative degree of inhibition is lower than in unwashed chloroplasts. If instead of tetramethyl benzidine the artificial donor diphenylcarbazide is added then the antiserum does not influence the electron transport rate (Fig. 11). This also clearly shows that the inhibition site of the antiserum lies between the site of electron donation of tetramethyl benzidine and that of diphenylcarbazide. Occasionally, it was observed that the antiserum to polypeptide 11000 did not inhibit anymore the electron transport rate in Tris-treated chloroplasts in the presence of the donor couple tetramethyl benzidine/ascorbate. This led us to the idea that Tris-buffer treatment might wash out the polypeptide 11000. This is not the case. By means of our different antisera we have found that amongst other proteins, coupling factor, reductase and plastocyanin were washed out. In contrast to these antisera the antiserum to the polypeptide 11000 gave no precipitation with the supernatant from the Tris-wash. The reason for the loss of inhibition might be that a too complete removal of the above named proteins leads to an altered molecular structure which does not permit access of the antibodies to the antigen or that the active component has lost its functionality. In this latter case antibodies might well become adsorbed. This leads to the formal explanation that the site of inhibition of the antiserum is closely behind the Tris-block. Furthermore, in Tris-washed chloroplasts manganese II ions can serve as electron donor to photosystem II. In the electron transport system $Mn^{2+} \rightarrow$ anthraquinone-2-sulfonate the antiserum inhibits electron transport reaction in the presence of antiserum in the system $H_2O \rightarrow$ anthraquinone-2-sulfonate. The arrows indicate the addition of TMB/Asc and methylamine. ○, Same reaction in the presence of control serum. △, Electron transport reaction in the system TMB/Asc $\rightarrow$ anthraquinone-2-sulfonate in the presence of antiserum. Here methylamine was given to the assay before antiserum addition. △, Same reaction as (△) but in the presence of control serum.

antiserum on the degree of inhibition by the antiserum. ●, Electron transport reaction in the presence of antiserum in the system $H_2O \rightarrow$ anthraquinone-2-sulfonate. The arrows indicate the addition of TMB/Asc and methylamine. ○, Same reaction in the presence of control serum. △, Electron transport reaction in the system TMB/Asc $\rightarrow$ anthraquinone-2-sulfonate in the presence of antiserum. Here methylamine was given to the assay before antiserum addition. △, Same reaction as (△) but in the presence of control serum.

Fig. 10. Characterization of the influence of methylamine on the degree of inhibition by the antiserum. ●, Electron transport reaction in the presence of antiserum in the system $H_2O \rightarrow$ anthraquinone-2-sulfonate. The arrows indicate the addition of TMB/Asc and methylamine. ○, Same reaction in the presence of control serum. △, Electron transport reaction in the system TMB/Asc $\rightarrow$ anthraquinone-2-sulfonate in the presence of antiserum. Here methylamine was given to the assay before antiserum addition. △, Same reaction as (△) but in the presence of control serum.

Fig. 11. Effect of the antiserum on the electron transport in the Tris-buffer washed chloroplasts from tobacco. The electron transport system is TMB/Asc $\rightarrow$ anthraquinone-2-sulfonate or diphenylcarbazide (DPC) $\rightarrow$ anthraquinone-2-sulfonate. ●, In the presence of antiserum, ○, in the presence of control serum. Concentration of DCMU after addition $3.3 \times 10^{-6} \text{M}$ in the TMB experiment and $2 \times 10^{-5}$ in the DPC-assay.

Fig. 12. Electron transport to anthraquinone-2-sulfonate in Tris-washed chloroplasts from tobacco with manganese II as the electron donor. ●, Reaction in the presence of antiserum, ○, reaction in the presence of control serum.
flow, showing that the site of electron donation of manganese is before the inhibition site by the antiserum (Fig. 12).

5. Effect of the antiserum on photophosphorylation reactions

Good photophosphorylation rates are only obtained if the illumination period is short. Under such conditions photophosphorylation is affected to a lower degree than is electron transport under the above described experimental conditions. Phenazine methosulphonate-mediated cyclic photophosphorylation which is a photosystem I reaction is not inhibited by the antiserum (Table I). In line with this observation the rate of proton translocation with PMS as mediator is also not affected by the antiserum (Fig. 13).

In Tris-buffer washed chloroplasts the electron transport system tetramethyl benzidine/ascorbate → anthraquinone-2-sulfonate supports noncyclic photophosphorylation which is DCMU-sensitive and is consequently a photosystem II reaction (Table I). This photophosphorylation reaction is inhibited by the antiserum. The inhibition is a consequence of the coupling of photophosphorylation to electron transport which is clearly seen from the ATP/2e-ratio which is the same in the presence of antiserum and control serum.

Table I. Effect of the antiserum to polypeptide 11000 on photophosphorylation reactions in Tris-buffer washed chloroplasts from wild type tobacco.

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmol[32P]ATP formed (mg Chl)⁻¹·h⁻¹</th>
<th>ATP/2 e</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS+antiserum to polypeptide 11000</td>
<td>205</td>
<td>-</td>
</tr>
<tr>
<td>PMS+control serum</td>
<td>179</td>
<td>-</td>
</tr>
<tr>
<td>Tetramethyl benzidine/ascorbate/anthraquinone-2-sulfonate + antiserum to polypeptide 11000</td>
<td>2.9</td>
<td>0.58</td>
</tr>
<tr>
<td>+ control serum</td>
<td>9.00</td>
<td>0.57</td>
</tr>
<tr>
<td>+ control serum+DCCD</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>+ control serum+DCMU</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

The PMS mediated photophosphorylation assay was illuminated with 120000 lx white light. The other assays were illuminated for 4 min with 30000 ergs·sec⁻¹·cm⁻² of red light 580 nm < λ < 700 nm at 14—15 °C. The electron transport rate for TMB/Asc+control serum → anthraquinone-2-sulfonate was 15.7 μmol O₂ uptake·(mg Chlorophyll)⁻¹·h⁻¹ and 5.0 μmol O₂ uptake·(mg Chlorophyll)⁻¹·h⁻¹ in the presence of antiserum. DCCD, dicyclohexyl carbodiimide was 10⁻⁴ M in the assay; DCMU 10⁻⁵ M in the PMS assay and where indicated.

Discussion

In the present paper we have described the effects of an antiserum to a polypeptide with the apparent molecular weight 11000. This antiserum inhibits photosynthetic electron transport between the sites of electron donation of tetramethyl benzidine and diphenylcarbazide. According to the literature, these donors are supposed to feed in their electrons on the oxygen evolving side of photosystem II. From all hitherto investigated antisera to purified polypeptide fractions this is the only one with such properties, whereas all the others inhibit either photosystem I reactions or inhibit between the two photosystems. Consequently, the polypeptide participates in reactions which occur on the water splitting side of photosystem II. This does not mean, however, that polypeptide 11000 is necessarily an electron transport component. The inhibition could also be due to a conformational change within the polypeptide 11000 itself or due to a conformational change of a protein molecule from which polypeptide 11000 is a part. Moreover, the inhibition can be due to an alteration of the molecular structure of the membrane, which would be the change of positions of proteins in relation to each other.

The fact that a component which plays a role in the vicinity of photosystem II and in the water splitting reaction is located in the outer surface of the thylakoid membrane is at first glance opposed to concepts advanced in the literature. According to this view photosystem II is located on the inner surface of the thylakoid membrane. However, we should like to emphasize at this point that our ob-
The capacity for oxygen evolution was somehow connected with the presence of stacked thylakoids and that antibodies did not enter into partitions. At this point a satisfactory explanation for the high effectiveness of the antiserum cannot be given. One possible interpretation would be that under our experimental conditions the polypeptide 11000, originally located in the partitions, becomes accessible to antibodies due to a rearrangement. This rearrangement could be induced or favored by light.

Another, not easily explainable effect, is the influence of light on the onset of inhibition. This influence of light is best seen in the fluorescence assay and was originally found in the ferricyanide Hill reaction. A minimum reaction time in the light of two minutes was necessary until the effect just began to show up. A mere preillumination in the presence of antiserum but in the absence of the electron transport components of the assay system and subsequent completion of the assay gave no or only a low inhibition.

It looks as if the complete assay system establishes in the light a membrane condition, possibly a redox state, which represents the condition in which inhibition becomes possible. Here, the observation by Giaquinta et al. would fit into ours. These authors observed that diazonium benzene [35S]sulfonate was incorporated into the lamellar system when electron flux through photosystem II and plastoquinone occurred. Also, in this case a rearrangement of the membrane or in the membrane might be induced by light.

Finally, it should be noted that, as shown earlier and again in this paper, antiserum to the proteins of the thylakoid membrane are at best only slightly species specific.

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