Localization and Function of Cytochrome f in the Thylakoid Membrane
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Dedicated to Professor Rolf Hosemann at the Occasion of His 65th Birthday
Thylakoid Membrane, Cytochrome f, Antiserum

A monospecific antiserum to cytochrome f agglutinates stroma-free swellable chloroplasts from tobacco and Antirrhinum. Consequently, antigenic determinants towards which the antiserum is directed are located in the outer surface of the thylakoid membrane. The antiserum inhibits linear photosynthetic electron transport. Just as described earlier for the antiserum to polypeptide 11000 this inhibition develops in the course of the light reaction. Ultrasonication in the presence of antiserum abolishes the light requirement and the maximal inhibition of the electron transport reaction is immediately observed. Electron transport in chloroplasts from a tobacco mutant which exhibits only photosystem I-reactions is also inhibited by the antiserum. No time lag in the light for the onset of inhibition is observed with these chloroplasts. As chloroplasts of this mutant have only single unfolded thylakoids it appears that light might preponderantly open up partitions. If the light effect is interpreted in this way, cytochrome f should be located in the partition regions but nevertheless in the outer surface of the thylakoid membrane. However, a rearrangement of molecules in the membrane by which the accessibility of cytochrome f is changed cannot be excluded. The inhibition of linear electron transport by the antiserum is approximately 50 per cent and can only be increased to 75% upon the addition of antibodies to plastocyanin. The inhibition by the antiserum to cytochrome f as well as the combined inhibition by the antiserum to cytochrome f and plastocyanin can be by-passed by DCPIP. It appears that cytochrome f and plastocyanin cannot be connected in series in the electron transport chain but are both closely associated in the thylakoid membrane.

Preparation of cytochrome f from the tobacco aurea mutant N. tabacum var. Su/su²

As all purifications of cytochrome f described in the literature are hampered by the chlorophyll extraction, chloroplasts from the tobacco aurea mutant...
Su/su² which contain less than 1/10 of the chlorophyll of the wild type chloroplasts were used as starting material. For our cytochrome f preparation the “Total Chloroplast Particle Fraction” described by Singh and Wassermann¹² was prepared from 14 kg of tobacco aurea leaves. This “Total Chloroplast Particle Fraction” was extracted with 90% ethanol as described by Garewal, Singh, and Wassermann¹³. The pellet from the extraction was suspended in 2% Triton X-100 (Serva, Germany), 4 M urea 0.05 M Tris HCl pH 8 and sonicated in small portions for 5 min each with the necessary cooling intervals. After centrifugation the supernatant was already distinctly reddish in color. The supernatant was applied onto a DEAE (Whatman DE 22) cellulose column as described by Garewal et al.¹⁵. The column was equilibrated with 2 M urea 2% Triton, 0.05 M Tris and 0.005 M dithiothreitol (Boehringer, Germany). The supernatant in 4 M urea 2% Triton 0.05 M Tris and 0.005 M dithiothreitol was loaded onto the column and eluted with the same mixture. The elution was monitored at 280 nm and yielded a distinct 4 step elution diagram: A sharp stepwise increase of the optical density, then a peak of a light green fraction, and a reddish brown turbid fraction, and at last in comparison to the other peaks a very low peak containing the cytochromes. The cytochrome containing fractions were collected and brough by dialysis to 50% saturation of (NH₄)₂SO₄. During this procedure two distinct layers were formed within the dialysis bag. A brown-reddish precipitate in the bottom layer which contained purified cytochromes and a foamy reddish-brown top layer which contained amongst others a red carotenoid protein. The top layer was removed and discarded. The bottom layer was spun for 15 min at 30000 x g and the sediment dissolved in the minimum amount of 2% Triton, 4 M urea, 0.05 M Tris pH 8 containing 0.005 M dithiothreitol. This yellow-reddish solution was applied to a DEAE column (DE 22 Whatman 70 – 80 cm high; 2.65 cm diameter). The column was equilibrated as before with a solution containing 4 M urea, 2% Triton, 0.05 M Tris and 0.005 M dithiothreitol. Elution was done with the same solvent. The principal elution pattern had changed in comparison to the first column. Only 1 wide but low protein peak emerged, yielding yellow solutions. The elution was stopped when the yellow color of the fractions had become very weak. On top of the column retention of some red material was visible. For the cytochrome f preparation only the tail of the protein peak without the peak fractions was collected and dialysed against a solution of 2% Triton, 0.05 M Tris, 0.005 M dithiothreitol. Upon removal of the urea the cytochrome f precipitated and was collected by centrifugation. The precipitate was dissolved in a minimum of the solution used for the column elution. This procedure resolubilized the major part of the sediment. The remaining undissolved part was again separated from the solution by centrifugation and the sediment was discarded. The solution was dialyzed against the urea-free medium and the resulting precipitate redissolved in the urea containing medium. For the immunization of the rabbits a fraction which appeared uniform in the dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2) was dialyzed against 3 changes of 5 l 0.005 M phosphate and then diluted with 0.005 M phosphate to give a final Triton concentration of less than 0.5%. This Triton-containing solution was injected into the rabbits. For the dodecyl sulfate polyacrylamide gel electrophoresis the sediment resulting after the removal of the urea by dialysis was washed once with water and dissolved in 2% dodecylsulfate containing 0.1% mercaptoethanol and incubated at room temperature for 24 hours. The gels were stained with Coomassie Blue and the optical density scanned with a Zeiss PMQ2 Spectrophotometer. The measurement was carried out at 558 nm.

The preparation of the antiserum was carried out with the above preparation by injecting approximately 1 mg protein into rabbits according to the method and the time schedule described for the antisera to plastocyanin¹⁰ and to ferredoxin-NADP⁺-reductase¹⁴.

The absorption spectrum of cytochrome f was taken with a Cary Model 118 Spectrophotometer.

Results

Characterization of the cytochrome f used for immunization

The cytochrome f preparation yielded in the dodecyl sulfate polyacrylamide gel electrophoresis after treatment with mercaptoethanol one fast running single band which corresponds to an apparent molecular weight 13600 provided the protein has been incubated a sufficiently long time with the mercaptoethanol-containing dodecyl sulfate solution (Fig. 2). Otherwise, before staining, two red bands are observed which correspond to the apparent molecular weights 26900 and 13600 (Fig. 2). The spectrum of cytochrome f dissolved in 4 M urea, 0.05 M Tris 2% Triton in the presence of 0.005 dithiothreitol is shown in Fig. 3 and exhibits all the characteristics of cytochrome f described in the literature¹⁵. Prolonged incubation of cytochrome f with a dodecyl
Fig. 2. Optical scan of the dodecyl sulfate polyacrylamide gel electropherogram of the isolated cytochrome f. Two bands are seen if the preparation is not incubated long enough with mercaptoethanol-containing sodium dodecyl sulfate (upper curve).

sulfate solution containing mercaptoethanol leads to an altered spectrum in which all absorption bands are shifted to shorter wavelength.

Fig. 3. Spectrum of the pure cytochrome f used for the injection into the rabbits.

Serological reactions of the antiserum to cytochrome f with different chloroplasts preparations

As seen in Table I, various chloroplast preparations react differently with the antiserum to cytochrome f. As in the case of the antisera to plastocyanin and ferredoxin NADP+-reductase agglutination is observed with stroma-free swellable chloroplasts from tobacco and Antirrhinum. Consequently, antigenic determinants towards which the antiserum is directed are located in the outer surface of the thylakoid membrane. On the other hand, stroma-freed chloroplasts from tobacco and Antirrhinum specifically adsorb the antibodies but do not agglutinate. The specific adsorption is demonstrated by a positive Coombs test and a mixed antigen agglutination with cytochrome f according to Uhlenbruck. Ultrasonication leads to agglutination of the preparation (Table I). The table also shows the amount of antigen which is necessary to exhaust one ml of antiserum.

Serological tests for monospecificity of the antiserum to cytochrome f

The antiserum obtained by the injection of the cytochrome f preparation is monospecific. Only one single sharp immuno precipitation band is observed in the Ouchterlony double diffusion test if the antiserum is run against the complex polypeptide mixture obtained by dissolution of tobacco chloroplasts in Triton X-100 (Fig. 1 a). The antiserum is not species specific because chloroplasts from

<table>
<thead>
<tr>
<th>Chloroplast type</th>
<th>Reaction</th>
<th>Amount of chloroplasts in mg dry weight by which 1 ml of antiserum is exhausted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroma-freed chloroplasts from Antirrhinum</td>
<td>specific adsorption (no agglutination)</td>
<td>40</td>
</tr>
<tr>
<td>Stroma-free swellable Antirrhinum chloroplasts</td>
<td>agglutination</td>
<td>38</td>
</tr>
<tr>
<td>Ultrasonic sediment from stroma-freed Antirrhinum chloroplasts</td>
<td>agglutination</td>
<td>15</td>
</tr>
<tr>
<td>Stroma-freed chloroplasts from wild type tobacco</td>
<td>specific adsorption (no agglutination)</td>
<td>45</td>
</tr>
<tr>
<td>Stroma-free swellable chloroplasts from wild type tobacco</td>
<td>agglutination</td>
<td>43</td>
</tr>
<tr>
<td>Stroma-free swellable chloroplasts from the tobacco mutant NC95</td>
<td>agglutination</td>
<td>—</td>
</tr>
<tr>
<td>Ultrasonic sediment from stroma-freed wild type tobacco chloroplasts</td>
<td>agglutination</td>
<td>25</td>
</tr>
</tbody>
</table>

Chloroplasts were prepared according to refs 8 and 9.

Table I. Serological reactions of the antiserum to cytochrome f with chloroplast preparations from Antirrhinum majus and N. tabacum.
Fig. 1 a. Serological test to show the monospecificity of the antiserum to cytochrome f. The center well contained the antiserum. (1) Chloroplasts from parsley; (2) chloroplasts from tobacco; (3) french pressed Chlorella; (4) tobacco chloroplasts; (5) chloroplasts from Antirrhinum majus; (6) tobacco chloroplasts. The chloroplast preparations according to Homann and Schmid⁹, were dissolved in 1% Triton.

Fig. 1 b. The center well contained chloroplasts from tobacco, dissolved in 1% Triton. (1) Antiserum to cytochrome f; (2) control serum; (3) antiserum to plastocyanin; (4) antiserum to ferredoxin NAD⁺-reductase; (5) antiserum to cytochrome f and (6) antiserum to the lamellar system of Antirrhinum majus.
parsley, *Antirrhinum* and *Chlorella* cells dissolved in Triton gave an identical immuno precipitation band (Fig. 1 a). This shows that the cytochromes f from *Chlorella* and tobacco are serologically identical. The cytochrome f molecules of these species might, however, differ in other respects. The serological difference of the antiserum to cytochrome f with the antisera to ferredoxin-NADP*-reductase and plastocyanin is verified in the Oudetlerony double diffusion test when these antisera are tested against tobacco chloroplasts dissolved in Triton X-100 (Fig. 1 b).

*Effect of the antiserum to cytochrome f on photosynthetic electron transport*

The chloroplasts used for the following tests are stroma-free swellable chloroplasts from either wild type tobacco or from the yellow leaf patches of the variegated tobacco mutant *N. tabacum* var. NC95 10. The antiserum to cytochrome f inhibits photosynthetic electron transport in chloroplasts from wild type tobacco (Fig. 4). It appears that the inhibition develops in the course of the light reaction (Fig. 4) just as described earlier for the inhibitory action of the antiserum to polypeptide 11000 11. A minimum of 5 min is necessary for the onset of inhibition. This could mean that in the light antigenic determinants become accessible in such a way that the inhibition of electron transport becomes possible. This inhibition is observed in the electron transport system tetramethyl benzidine/ascorbate $\rightarrow$ anthraquinone-2-sulfonate. However, the inhibition in this electron transport system is relieved upon addition of 2,6-dichlorophenol indophenol/ascorbate (Fig. 4). Obviously, DCPiP can by-pass the inhibition. From this observation and that of Fujita and Murano who observed that high concentrations of DCPiP by-passed plastocyanin 18 it appears that cytochrome f acts similarly and near the site of plastocyanin. A brief sonication of the stroma-free swellable chloroplasts in the presence of antiserum to cytochrome f abolishes the induction period. The inhibition is immediately high and barely increases with time (Fig. 5). The maximal inhibition of electron transport which is observed under these conditions is around 50 per cent. Only if to these sonicated chloroplasts antiserum to plastocyanin is added, can the inhibition be brought up to 75% (Fig. 6). It should be noted that the amount of antiserum to plastocyanin added was so low that no effect of this antiserum by itself on electron transport was observed 10. The 75 per cent inhibi-
Inhibition of electron transport in tobacco chloroplasts which were sonicated for 5 sec in the presence of antisem to cytochrome f. After sonication in addition antiserum to plastocyanin was added. Electron transport system as in Figs 4 and 5. (O) Assay in the presence of control serum; (®) assay in the presence of antiserum.

It is remarkable that the inhibitory effect caused by the antibodies becomes only apparent in the light. This fact was already observed with the antiserum to polypeptide 11000 which affects electron transport on the watersplitting side of photosystem II. Hence, the question arises what does light do to the lamellar system. If one uses stroma-free swellable chloroplasts prepared from the yellow leaf patches of the variegated tobacco mutant NC95 then it appears that no ultrasonication is necessary to obtain the immediate inhibitory effect by the antiserum (Fig. 8). Undisrupted thylakoids give immediately an inhibition which is not further increased by light. As earlier investigations have shown that chloroplasts from the tobacco mutant NC95 have single separated and unfolded thylakoids it appears that a brief ultrasonication might open up partition regions. Consequently, cytochrome f, or at least protein which belongs to the cytochrome f molecule is located in the outer surface of the thylakoid membrane, but inaccessibly to antibodies because it is located in the partitions. This holds only if the mutation has not altered the molecular structure of the thylakoid membrane. It should be mentioned that chloroplasts of the tobacco mutant NC95 contain cytochrome f which is serologically identical to that of the wild type tobacco.

* While this paper was submitted to this journal Kunert, Böhme and Böger reported on reactions of plastocyanin and cytochrome 553 in Scenedesmus. These authors conclude from their studies that both plastocyanin and cytochrome f can donate their electrons also directly to P700. Our results can be interpreted in the same way.
Table II. Effect of the antiserum to cytochrome f on photophosphorylation reactions in chloroplasts from wild type tobacco.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Ultrasonication [sec]</th>
<th>Photophosphorylation [μmol ATP formed × mg Chl⁻¹ h⁻¹]</th>
<th>Inhibition [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS + antiserum</td>
<td>0</td>
<td>433</td>
<td>7</td>
</tr>
<tr>
<td>PMS + control serum</td>
<td>0</td>
<td>467</td>
<td></td>
</tr>
<tr>
<td>PMS + antiserum</td>
<td>5</td>
<td>134</td>
<td>23</td>
</tr>
<tr>
<td>PMS + control serum</td>
<td>5</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>PMS + antiserum</td>
<td>15</td>
<td>49</td>
<td>17</td>
</tr>
<tr>
<td>PMS + control serum</td>
<td>15</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Benzidine/ascorbate → MV + antiserum</td>
<td>5</td>
<td>5.2</td>
<td>47</td>
</tr>
<tr>
<td>Benzidine/ascorbate → MV + control serum</td>
<td>5</td>
<td>9.8</td>
<td></td>
</tr>
</tbody>
</table>

Illumination for 2 min with 120 000 lx white light at 20 °C. PMS-concentration 3.3 × 10⁻⁵ M.

The antiserum to cytochrome f inhibits photophosphorylation reactions only if the chloroplasts are sonicated in the presence of antiserum (Table II). Without ultrasonication the degree of inhibition is very low. This is true for chloroplasts from wild type tobacco and for those of the tobacco mutant NC95 (Table III). Chloroplasts from the tobacco mutant NC95 exhibit only photosystem I-dependent PMS-mediated cyclic photophosphorylation. However, for the understanding it should be noted that already a short ultrasonication affects the phosphorylation rate in tobacco chloroplasts (Tables II and III). Despite this fact PMS mediated cyclic photophosphorylation is distinctly inhibited by the antiserum. The latter results confirm the data of Racker et al. if one excludes reaction artifacts induced by ultrasonication. Photophosphorylation in the electron transport system benzidine/ascorbate → methylviologen which is comparable to that described in Fig. 5 shows approximately 50 per cent inhibition (Table II). For the discussion of this

(Fig. 9). Hence, the primary effect of light appears to be the opening of the partition regions. In addition, a rearrangement of molecules in the membrane induced by light is not excluded. In this context it should be noted that Böhme investigated the reduction kinetics of cytochrome f in digitonin treated thylakoid fragments from spinach chloroplasts. From the effect of light on the course of the reduction kinetics he concluded that the accessibility of cytochrome f in the thylakoid membrane is changed in the light. He proposed a conformational or structural change in the membrane induced by light.

Table III. Effect of the antiserum to cytochrome f on PMS-mediated cyclic photophosphorylation in chloroplasts from the yellow leaf patches of the variegated tobacco mutant NC95.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Ultrasonication [sec]</th>
<th>Photophosphorylation [μmol ATP formed × mg Chl⁻¹ h⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS</td>
<td>0</td>
<td>303</td>
</tr>
<tr>
<td>PMS + antiserum</td>
<td>0</td>
<td>360</td>
</tr>
<tr>
<td>PMS + control serum</td>
<td>0</td>
<td>235</td>
</tr>
<tr>
<td>PMS + antiserum</td>
<td>5</td>
<td>37</td>
</tr>
<tr>
<td>PMS + control serum</td>
<td>5</td>
<td>76</td>
</tr>
</tbody>
</table>

Illumination for 2 min 120 000 lx white light at 20 °C. PMS-concentration 3.3 × 10⁻⁵ M.
Fig. 9. Serological test to show the presence of cytochrome f in the lamellar system of chloroplasts of the variegated tobacco mutant NC95. The centerwell contained antiserum to cytochrome f. (1) Cytochrome f preparation; (2) chloroplasts from wild type tobacco; (3) chloroplasts from the yellow leaf patches of the variegated tobacco mutant NC95; (4) cytochrome f preparation; (5) chloroplasts from wild type tobacco; (6) chloroplasts from the yellow leaf patches of the variegated tobacco mutant NC95; the chloroplasts were dissolved in 1% Triton.
result it must be borne in mind that the photophosphorylation reactions are short time experiments. The 2 min reaction time are not sufficient to establish the light effect of the inhibitory action of the antiserum.

The dependence of the relative degree of inhibition on the amount of antiserum added yields a sigmoidal curve shape which hints at a co-operative effect.

Discussion

From our experiments it follows that cytochrome f which is involved in linear electron transport is accessible to antibodies and, therefore, located in the outer surface of the thylakoid membrane. As the antiserum is monospecific the observation of an agglutination and an inhibition of electron transport have the same importance for the localization of cytochrome f in the thylakoid membrane. The degree of inhibition of 50% by the antiserum to cytochrome f which can only be increased by the addition of antiserum to plastocyanin shows that plastocyanin and cytochrome f cannot be connected or are at least not exclusively connected in series in the electron transport chain. This observation fits into the results by Haehnel who found that part of the cytochrome f is situated in a side path of the linear electron transport chain. It would also fit into the results by Kunert et al. who observed in Scenedesmus that cytochrome f and plastocyanin can feed in their electrons separately but directly into P700. Furthermore, from the fact that the inhibitory action of the antiserum to cytochrome f and the combined inhibition by the antiserum to cytochrome f and plastocyanin can be both by-passed by dichlorophenol indophenol it follows that both components must be closely associated in the thylakoid membrane.

On the other hand, the fact that PMS-mediated cyclic photophosphorylation is only inhibited if chloroplasts are sonicated in the presence of antiserum, leads to the conclusion that the cytochrome f which is involved in cyclic photophosphorylation is only accessible to antibodies during ultrasonication. This is turn could mean that this cytochrome f is located inside the membrane which agrees with the data of Racker et al. However, ultrasonication might induce reaction artifacts and according to Rumberg and Witt high concentrations of PMS react directly with P700. If cytochrome f was located at the inner surface of the thylakoid membrane the antiserum should also be effective if added after ultrasonication as experimentally shown by Koenig et al. Consequently, it appears that cytochrome f molecules have separated active sites for linear and cyclic electron transport. The inhibition site for linear electron transport is accessible from the outside whereas the inhibition site for cyclic electron transport is located inside the membrane and therefore, cannot react with the antibodies. In this case all cytochrome f molecules would be located in the outer surface of the thylakoid membrane. In this context it must be borne in mind that cytochrome f in the thylakoid membrane consists of several polypeptide chains of the apparent molecular weight of 13600. This explains why the values for the molecular weights reported in the literature differ so much from each other and are much higher than ours. In the presence of dodecyl sulfate which separates the polypeptide chains from each other also Davenport reports on a molecular weight which is very similar to ours. Also, the interpretation of an observed cooperative effect, requires that the cytochrome f molecule is composed of several polypeptide chains.

The authors thank Miss T. Akmandor, Miss E. Schölzel and Mrs. C. Essmann for technical assistance.
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18 Y. Fujita and F. Murano, Plant and Cell Physiol. 8, 269 [1967].