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 in the light 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 antisera 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.

PMS-mediated cyclic photophosphorylation in chloroplasts from wild type tobacco and the tobacco mutant NC95 is only inhibited if the chloroplasts are sonicated in the presence of antiserum. If one disregards, that ultrasonication might cause reaction artifacts, it is thinkable that the cytochrome f, involved in the PMS-mediated cyclic photophosphorylation reaction, might be located inside the membrane.

The basic studies by Duysens [1] and Witt [2] have shown that cytochrome f is oxidized by photosystem I. However, after the discovery of plastocyanin [3] the discussion arose whether cytochrome f or plastocyanin reduces the photooxidized P_{700} [4, 5]. In addition, incited by Mitchell's chemiosmotic theory of photophosphorylation, the concept of the sidedness of the thylakoid membrane has led to the question where cytochrome f should be located [6, 7]. Due to some data by Hauska, McCarthy, and Racker [6] and by Racker *et al.* [7] cytochrome f was placed on the inside face of the thylakoid membrane. In context with our investigations on the effects of antisera to components of the thylakoid membrane we report in the following on a monospecific antiserum to cytochrome f. With respect to the localization of the cytochrome f in the membrane part of our results differs from the current concept.

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Abbreviations: DCMU, N-N′-3,4-dichlorophenyl dimethyl urea; DCPiP, 2,6-dichlorophenolindophenol; TMB, tetramethyl benzidine; PMS, phenazine methosulfate; MV, methylviologen.

Materials and Methods

Chloroplast preparations: Stroma-freed chloroplasts from wild type tobacco *N. tabacum* var. John William's Broadleaf and *Antirrhinum majus* were prepared according to Kreutz and Menke [8]. Stroma-free swellable chloroplasts from wild type tobacco, the yellow leaf patches of the variegated tobacco mutant from *N. tabacum* NC95 and from *Antirrhinum majus* were obtained according to Homann and Schmid [9]. *Light reactions and photophosphorylation reactions* were carried out in the assay systems described previously [10, 11].

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/su[2] 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 Wassermann[12] 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 Wassermann[13]. 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.*[13]. 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 $(NH_4)_2SO_4$. 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 $30000 \times g$ 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 plastocyanin[10] and to ferredoxin-$NADP^+$-reductase[14].

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 yields 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 literature[15]. Prolonged incubation of cytochrome f with a dodecyl

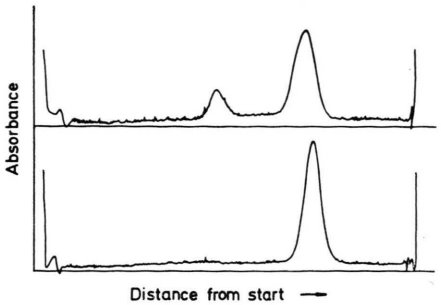

Fig. 2. Optical scan of the dodecyl sulfate polyacrylamide gel electropherogram of the isolated cytochrome f. Two bands are seen if the preparation is not incubated long enough with mercaptoethanol-containing sodium dodecyl sulfate (upper curve).

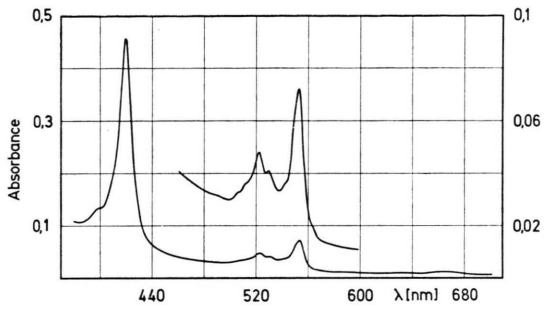

Fig. 3. Spectrum of the pure cytochrome f used for the injection into the rabbits.

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

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 [10, 14] agglutination is observed with stroma-free swellable chloroplasts [9] 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 [8] from tobacco and *Antirrhinum* specifically adsorb the antibodies but do not agglutinate. The specific adsorption is demonstrated by a positive Coombs test [16] and a mixed antigen agglutination with cytochrome f according to Uhlenbruck [17]. 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 I. Serological reactions of the antiserum to cytochrome f with chloroplast preparations from *Antirrhinum majus* and *N. tabacum*.

Chloroplast type	Reaction	Amount of chloroplasts in mg dry weight by which 1 ml of antiserum is exhausted
Stroma-freed chloroplasts from *Antirrhinum*	specific adsorption (no agglutination)	40
Stroma-free swellable *Antirrhinum* chloroplasts [9]	agglutination	38
Ultrasonic sediment from stroma-freed *Antirrhinum* chloroplasts	agglutination	15
Stroma-freed chloroplasts from wild type tobacco [8]	specific adsorption (no agglutination)	45
Stroma-free swellable chloroplasts from wild type tobacco [9]	agglutination	43
Stroma-free swellable chloroplasts from the tobacco mutant NC95 [9]	agglutination	—
Ultrasonic sediment from stroma-freed wild type tobacco chloroplasts	agglutination	25

Chloroplasts were prepared according to refs 8 and 9.

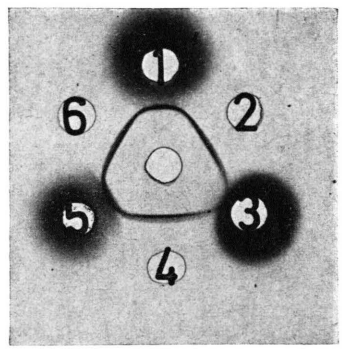

Fig. 1 a. Serological test to show the monospecificity of the antiserum to cytochrome f. The center well contained the antiserum. (1) Chloroplasts from parsley; (2) chloroplasts from tobacco; (3) french pressed *Chlorella*; (4) tobacco chloroplasts; (5) chloroplasts from *Antirrhinum majus*; (6) tobacco chloroplasts. The chloroplast preparations according to Homann and Schmid [9], were dissolved in 1% Triton.

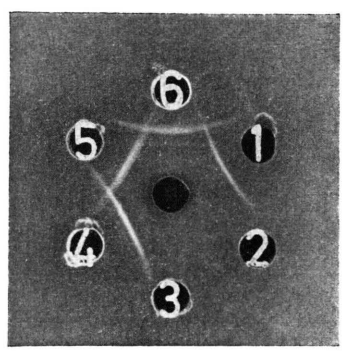

Fig. 1 b. The center well contained chloroplasts from tobacco, dissolved in 1% Triton. (1) Antiserum to cytochrome f; (2) control serum; (3) antiserum to plastocyanin; (4) antiserum to ferredoxin $NADP^+$-reductase; (5) antiserum to cytochrome f and (6) antiserum to the lamellar system of *Antirrhinum 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 Ouchterlony 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 → 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-

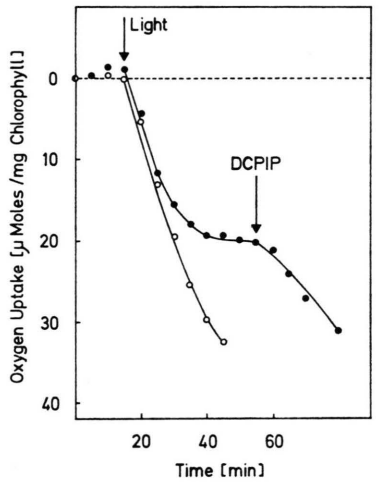

Fig. 4. Effect of the antiserum to cytochrome f on electron transport in unsonicated tobacco chloroplasts in the electron transport system tetramethyl benzidine/ascorbate → anthraquinone-2-sulfonate. The inhibition is relieved upon addition of DCPiP. Concentrations in the assay 0.14 mM DCPiP and 30 mM ascorbate. (○) Assay in the presence of control serum; (●) assay in the presence of antiserum.

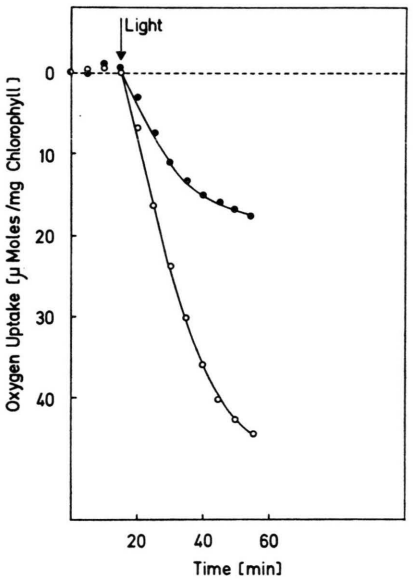

Fig. 5. Effect of the antiserum to cytochrome f on electron transport in tobacco chloroplasts which were sonicated for 5 sec in the presence of antiserum. Electron transport system tetramethyl benzidine/asc. → anthraquinone-2-sulfonate. (○) Assay in the presence of control serum; (●) assay in the presence of antiserum.

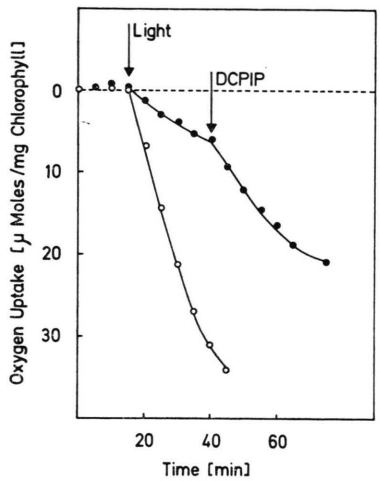

Fig. 6. Inhibition of electron transport in tobacco chloroplasts which were sonicated for 5 sec in the presence of antiserum to cytochrome f. After sonication in addition antiserum to plastocyanin was added. Electron transport system as in Figs 4 and 5. (○) Assay in the presence of control serum; (●) assay in the presence of antiserum.

tion can be fully relieved upon addition of DCPiP (Fig. 6). The observation shows that plastocyanin and cytochrome f might substitute for each other in electron transport *. The localization of the inhibitory action of the antiserum to cytochrome f on the donor side of photosystem I is demonstrated by the inhibition of electron transport in the system diaminodurene/tetramethyl *p*-phenylene diamine → anthraquinone-2-sulfonate in the presence of DCMU. Diaminodurene is supposed to donate electrons directly to plastocyanin [19]. The relative degree of inhibition of electron transport in chloroplasts from wild type tobacco which were briefly (5 sec) ultrasonicated in the presence of antiserum is 50% which is the same degree of inhibition as in the longer electron transport piece (Fig. 7). Only upon addition of antiserum to plastocyanin to the chloroplasts, inhibited already by the antiserum to cytochrome f, can the degree of inhibition be increased to over 70 per cent (Fig. 7).

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 [11]. 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 [9] 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

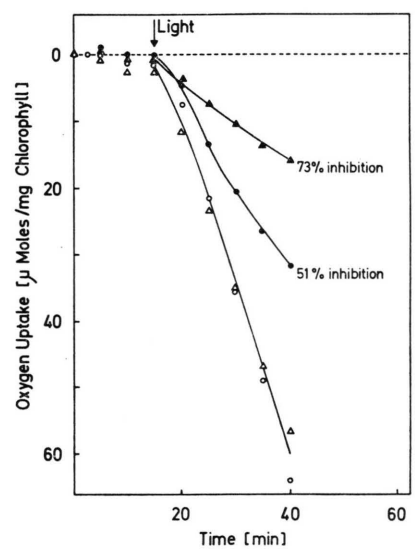

Fig. 7. Localization of the inhibition site of the antiserum to cytochrome f; electron transport system diaminodurene (DAD) tetramethyl *p*-phenylene diamine (TMPD)/acsorbate → antraquinone-2-sulfonate in the presence of 10^{-6} M DCMU in the assay. (○) Tobacco chloroplasts sonicated for 5 sec in the presence of control serum; (●) tobacco chloroplasts sonicated for 5 sec in the presence of antiserum to cytochrome f. (△) Assay as (○) but *after* sonication in the presence of the control serum to cytochrome f in addition the control serum belonging to the antiserum of plastocyanin has been added. (▲) Assay as (●) but after sonication antiserum to plastocyanin has been added.

* 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 P_{700} [25]. Our results can be interpreted in the same way.

Reaction	Ultra-sonication [sec]	Photophosphorylation [μmol ATP formed $\times$mg Chl^{-1} h^{-1}]	Inhibition [%]
PMS+antiserum	0	433	7
PMS+control serum	0	467	
PMS+antiserum	5	134	23
PMS+control serum	5	174	
PMS+antiserum	15	49	17
PMS+control serum	15	60	
Benzidine/ascorbate → MV +antiserum	5	5.2	47
Benzidine/ascorbate → MV +control serum	5	9.8	

Table II. Effect of the antiserum to cytochrome f on photophosphorylation reactions in chloroplasts from wild type tobacco.

Illumination for 2 min with 120000 lx white light at 20 °C. PMS-concentration 3.3×10^{-5} M.

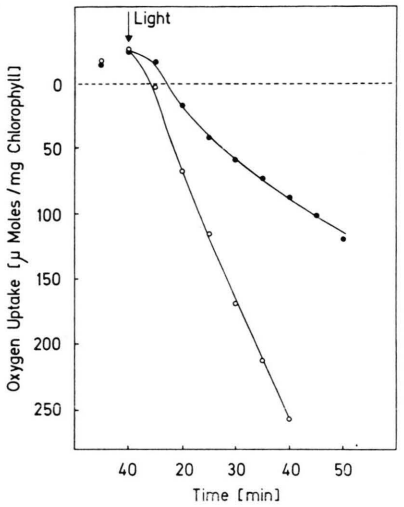

Fig. 8. Effect of the antiserum to cytochrome f on unsonicated chloroplasts from the variegated tobacco mutant NC95 in the electron transport system DAD/TMPD → anthraquinone-2-sulfonate. (○) Assay in the presence of control serum; (●) assay in the presence of antiserum to cytochrome f.

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 [9]. 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.* [7] 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 [22]. 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 [22].

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.

Reaction	Ultra-sonication [sec]	Photophosphorylation [μmol ATP formed $\times$mg Chl^{-1} h^{-1}]
PMS	0	303
PMS+antiserum	0	360
PMS+control serum	0	235
PMS+antiserum	5	37
PMS+control serum	5	76

Illumination for 2 min 120000 lx white light at 20 °C. PMS-concentration 3.3×10^{-5} M.

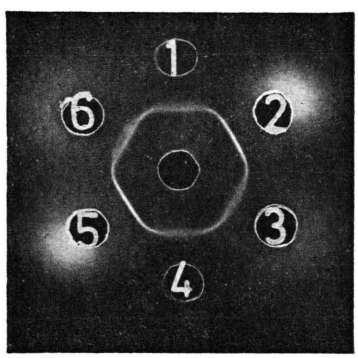

Fig. 9. Serological test to show the presence of cytochrome f in the lamellar system of chloroplasts of the variegated tobacco mutant NC95. The centerwell contained antiserum to cytochrome f. (1) Cytochrome f preparation; (2) chloroplasts from wild type tobacco; (3) chloroplasts from the yellow leaf 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 [23]. 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 P_{700} [25]. Furthermore, from the fact that the inhibitory action of the antiserum to cytochrome f and the combined inhibition by the antisera 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.* [7]. However, ultrasonication might induce reaction artifacts and according to Rumberg and Witt high concentrations of PMS react directly with P_{700} [26]. 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.* [24]. Consequently, it appears that cytochrome f is situated in two different sites in the thylakoid membrane and that cytochrome f in these different locations is involved in different functions. On the other hand, it also appears possible that the 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 [20, 21, 27]. 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 [15]. Also, the interpretation of an observed cooperative effect, requires that the cytochrome f molecule is composed of several polypeptide chains.

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