Reactions of a Monospecific Antiserum to Ferredoxin-NADP$^+$-Reductase with Chloroplast Preparations

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A monospecific antiserum to ferredoxin-NADP$^+$-reductase inhibits the diaphorase activity of soluble ferredoxin-NADP$^+$-reductase from chloroplasts. Two states of the molecular structure of the lamellar system have been observed, one of which is the state described earlier by Berzborn. Stroma-freed chloroplasts in this condition are not agglutinated by the antiserum, although a specific adsorption of antibodies to reductase onto the lamellar system was demonstrated by the Coombs test. However, a second type of chloroplast preparations gives direct agglutination upon addition of the antiserum. Apparently, agglutination in this state is not sterically hindered by neighboring protein structures. This type of chloroplast preparations appears swollen under the light microscope, but exhibits high rates of electron transport.

Chloroplasts of three types of tobacco have been used which differ in the morphology of their lamellar systems. The green type contains a normal ratio of grana and intergrana regions whereas the other two types have extended intergrana regions with either only small grana or no partitions at all. Comparison of the maximal degree of inhibition of the NADP$^+$-reduction in chloroplasts from these types of tobacco by the antiserum, leads to the conclusion that ferredoxin-NADP$^+$-reductase is located in the grana and the intergrana regions of the lamellar system, in the outer surface of the thylakoid membrane.

Ferredoxin NADP$^+$-reductase was shown to be located in the outer surface of the thylakoid membrane [1, 2]. Berzborn was able to show that spinach and *Antirrhinum* chloroplasts were not directly agglutinated by antibodies to ferredoxin-NADP$^+$-reductase [3]. However, after the addition of rabbit anti-γ-globulins [4], or upon addition of soluble reductase in the "mixed antigen agglutination" according to Uhlenbruck [5] agglutination was observed. This was interpreted to mean that ferredoxin-NADP$^+$-reductase was located in depressions of the thylakoid membrane or between coupling factor molecules [6]. The steric hindrance of agglutinations was relieved by washing with $5 \cdot 10^{-4}$ M EDTA. As this EDTA concentration was the optimal concentration for the removal of the Ca^{2+}-dependent ATPase (coupling factor) from chloroplast membranes it was proposed that ferredoxin-NADP$^+$-reductase was located between protruding protein molecules namely the coupling factor [6].

In the present paper we report on a condition of the lamellar system in chloroplasts from normal green tobacco and two tobacco mutants in which our monospecific antiserum to ferredoxin-NADP$^+$-reductase agglutinates these chloroplasts directly.

From earlier investigations we know that the used tobacco mutant chloroplasts contain a simplified lamellar system with extended intergrana regions [7, 8].

In grana-containing chloroplasts only partial inhibition of the DPIP/ascorbate-mediated NADP$^+$-reduction by the antiserum is observed whereas the inhibition is complete in the absence of grana. From this it is concluded that ferredoxin-NADP$^+$-reductase is located in the grana and the intergrana regions of the lamellar system.

Materials and Methods

Chloroplast preparations: Stroma-freed chloroplasts from green *N. tabacum* var. John William's Broadleaf, from the *aurea* mutant Su/su^2 and from *Antirrhinum majus* strain 50 were prepared according to Kreutz and Menke [9]. The second type of chloroplast preparations from *N. tabacum* var. John William's Broadleaf, the tobacco aurea mutant Su/su^2 and from yellow leaf patches of the variegated tobacco mutant from *N. tabacum* var. NC 95 was

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Abbrevations: DPIP, 2,6-dichlorophenol-indophenol; TPIP, 2,3,6-trichlorophenol-indophenol; DCMU, 3,4-dichlorophenyl, 1,1′-dimethylurea; PMS, phenazine methosulphate; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid).

prepared in a buffer containing 0.4 M sucrose, 0.05 M tris, pH 7.8, 0.01 NaCl, serum albumin and pectinase according to Homann and Schmid [7].

The variegated tobacco mutant NC 95 is described in an earlier publication [10]. The tobacco aurea mutant Su/su^2 is a dominant aurea mutant which originated from a seed population of the earlier described Su/su [8, 11]. The mutant is a more pronounced type of regular Su/su. Its properties will be described elsewhere.

$NADP^+$-reduction in chloroplasts with the DPIP/ascorbate couple as the electron donor was carried out as described earlier [7]. In order to show the effect of Fig. 3, chloroplasts prepared according to this preparation procedure were subsequently suspended in a buffer containing 0.05 M tricine pH 7.3 and 0.15 M sucrose. After 5 min dark the chloroplasts were spun down and suspended in 0.05 M tricine containing 0.05 M methylamine pH 7.3. After 5 min in the dark the chloroplasts were spun down (3 min table centrifuge) and suspended in the same buffer to which the desired amounts of antiserum or control serum were added. Incubation with the serum was done in the dark for 5 min. After incubation, the chloroplasts were spun down again, washed, (in the dark) with 2 ml 0.05 M tricine, 0.05 M methylamine and resuspended in 2.4 ml of the assay buffer containing 50 mM tricine, 50 mM methylamine 1.3 mM $NADP^+$, 2.5 mM oxidized glutathion. To this buffer 0.1 ml $3.5 \cdot 10^{-5}$ M DCMU, 0.1 ml purified ferredoxin (equivalent to 70 μg), 0.1 ml 2.58 mM DPIP, 0.2 ml 0.2 M ascorbate and 0.1 ml glutathion reductase (Boehringer, Mannheim, equivalent to 50 μg) were added. The chloroplasts were removed with a Sartorious ultra filter. The glutathion reduced in the light was taken as a measure of $NADP^+$-reduction. The reduced glutathion was determined with DTNB according to Ellman [12]. Chloroplasts from normal green tobacco equivalent to 20 – 50 μg of chlorophyll were used per assay and chlorophyll was determined for each individual assay. Su/su^2 chloroplasts equivalent to 3 – 10 μg of chlorophyll were used per assay. The same procedure in the absence of methylamine or $MgCl_2$ (just in tricine buffer) gives full inhibition by the antiserum. Partially purified ferredoxin freed from reductase which had to be added in this reaction was prepared from the respective tobacco types according to Bendall *et al.* [13].

Pigment analyses and light conditions are described and referred to in an earlier publication [14].

Protein determinations were carried out according to the Lowry procedure [15].

Measurements of diaphorase activity: Ferredoxin-$NADP^+$-reductase exhibits diaphorase activity [16]. This activity was measured as absorbance decrease at 623 nm caused by the reduction of TPIP (2,3,6-trichlorophenol-indophenol) in the presence of NADPH and enzyme according to Avron and Jagendorf [16]. The test mixture contained 1.5 ml 0.1 M tris pH 7.5; 0.1 ml TPIP to give an extinction value of approx. 1; 0.2 ml NADPH equivalent to 0.2 mg; 0.1 ml 10^{-3} M FAD if necessary, suitable amounts of the diaphorase fractions and water to give a final volume of 3.2 ml.

Measurements were made with a digital Zeiss Spectrophotometer type PM2D.

Electrophoresis: The polyacrylamide gels were prepared, run and removed for protein analysis according to the Bloemendal procedure [17]. Samples contained 20 to 100 μg of protein. Tris-glycine buffer pH 8.5 served as electrolyte. Staining was achieved with amido black in acetic acid (7% v/v) for 3 – 4 hours followed by electrophoretic removal of excess amido black.

Immunization of rabbits with ferredoxin-$NADP^+$-reductase was carried out as described for plastocyanin by Schmid and Radunz *. The reductase preparations used for immunization and the obtained antisera from 2 rabbits were tested by means of immunoelectrophoresis. The tests were carried out in 0.8 per cent agarose and 0.06 M Sørensen buffer pH 7.8. After the electrophoresis of the antigen (90 min at 1 V/cm^2) and the diffusion of the antiserum (≅ 16 h), the agarose plates were treated with 1.7% sodium chloride in order to wash out the not precipitated proteins. The plates were then washed in water, dried and stained with amido black.

Double diffusion tests were carried out according to Ouchterlony [19].

Preparation of ferredoxin-$NADP^+$-reductase from green N. tabacum var. John William's Broadleaf and from spinach: Two types of procedures were used: One kind of preparation was obtained according to the slightly modified method of Shin, Tagawa, and Arnon [20]. Starting material was 3 – 4 kg of tobacco leaves. The end product was only occasionally pure when tested with a complex antiserum to chloroplasts, containing often traces of immunologically detectable ferredoxin. Therefore, a second type of preparation procedure was prefered:

Chloroplasts from 4 kg of green leaves of *N. tabacum* var. John William's Broadleaf or spinach were suspended in 0.4 M sucrose containing 50 mM tris buffer pH 8 and 10 mM NaCl according to Vambutas and Racker [21]. The suspension was di-

* In preparation.

vided into 4 equal parts and each part (120 ml) was dropped into 2.4 l of cold (−10 °C) acetone. From this acetone sediment the large part of the acetone was decanted and the remaining thick suspension was centrifuged at 600 × g for 10 min in a centrifuge (W. Stock, Maschinenbau KG, Marburg/L., W.-Germany). The sediment was dried on filter papers as described by Vambutas and Racker [21]. This dried acetone preparation, exhibiting still a slight acetone odour, was homogenized in a Braun type 853202 homogenizer (Braun, Melsungen, W.-Germany) with 900 ml of 0.05 M tris buffer pH 8. The heavy suspension was stirred at room temperature for 45 min and then centrifuged at 40 000 × g. The supernatant was collected. The sediment was resuspended in 600 ml additional 0.05 M tris buffer and stirred again for 45 min at room temperature. The suspension was respun at 40 000 × g for 20 min and the combined reddish-brown supernatants (1550 ml) were precipitated between 0 − 20% saturation with $(NH_4)_2SO_4$ (11 g/100 ml). The solution was allowed to stand over night at 5 °C and was filtered through a Buchner funnel. The clear filtrate was precipitated with $(NH_4)_2SO_4$ between 20 − 45% saturation (additional 15 g/100 ml) and stirred for 30 min. Then the sediment was spun down at 40 000 × g for 20 min. The supernatant was dialyzed over night (Visking cellulose dialysis bags, 38 mm diameter) against several changes of 10 mM phosphate buffer.

The dialysed supernatant (2.5 l) contains plastocyanin and a large part of the chloroplast ferredoxin-NADP⁺-reductase. The solution was entirely loaded on a DEAE-cellulose column (⌀ 3 cm, 25 cm high, Whatmann DE 23). The passing solution when loading the column turns yellow after some colourless liquid has run through and starts smelling ammonia. Plastocyanin stays on the column. The yellow eluate (1150 ml) is neutralized and fractioned with $(NH_4)_2SO_4$ between 0 − 70 and 70 − 85 per cent saturation. The fraction with the highest specific diaphorase activity, usually the 0 − 70% fraction is further purified (Table I). The fraction was dialyzed over night against 0.03 M tris pH 7.8 and the resulting solution (24 ml) was applied to a Sephadex G-200 column (⌀ 2.5 cm, 85 cm high) equilibrated with 0.03 M tris pH 7.8, eluted with the same buffer and 9.5 ml fractions (150 drops) collected. The fraction numbers 12 − 21 were recombined and concentrated with 52 g $(NH_4)_2SO_4$/100 ml (80% saturation) and dialyzed over night against 0.03 M tris. This new protein solution (6.2 ml) was applied to a small Sephadex G-100 column (⌀ 3 cm, 25 cm high) equilibrated with the same buffer and elution done also with the same buffer. Occasionally at this point the preparation was immunologically pure. However, sometimes a small impurity as detected in the immunoelectrophoresis (as a slower migrating component) against a complex antiserum to broken chloroplasts may require the following additional procedure. The fraction from the Sephadex G-100 column was applied to a Sephadex G-75 column (⌀ 3 cm, 34 cm high). Equilibration and elution buffer being always 0.03 M tris. The fraction numbers 20 − 23 (5.45 ml/fraction) were concentrated over a small DEAE-cellulose column (⌀ 3 cm, 4 cm high). The fractions were loaded in 0.03 M tris onto the column and eluted with 0.03 M tris pH 7.8 containing 0.5 M NaCl. This step can be repeated several times with a certain purification effect as demonstrated by immunoelectrophoresis. From the preparative point of view the yields are low but quite sufficient to give the necessary amounts of protein for the injection into rabbits. Also, in the course of the prolonged procedure the specific diaphorase activity decreases even though immunoelectrophoresis shows further purification (Table I). Also, the enzyme looses by the DEAE-treatment and the repeated dialysis part of its coenzyme (Table I). The purification procedure works for spinach and tobacco and is sketched for spinach in Table I.

Immunological Characterization of the Ferredoxin-NADP⁺-Reductase Preparation, Used for Injection

After purification of the reductase to the point where only one single protein band was observed in the polyacrylamide electrophoresis, we regularly observed that such preparations, when tested against complex antisera to the lamellar system of *Antirrhinum* chloroplasts or to broken chloroplasts [22] from *Antirrhinum* or spinach, showed more than one immunoprecipitation band in the Ouchterlony test or in the immunoelectrophoresis. We, therefore, purified our reductase preparation further until only one band was visible in immune assays with these complex antisera (Figs 1 a, b *). It should be noted that such a reductase preparation is not necessarily optimal with respect to specific diaphorase activity, since enzyme activity is obviously lost in the course of the long purification procedure. The later fractions of the purification procedure show an absolute requirement for FAD in the diaphorase assay (Table I). In Figs 1 c and d

* Figs 1 a − d see Table on page 388 a.

Table I. Preparation of serologically pure ferredoxin-NADP$^+$-reductase from spinach chloroplasts.

Steps	Volume [ml]	Protein [mg/ml]	Specific Diaphorase Activity [ΔExt_{623} · (mg protein)$^{-1}$ · min^{-1})
Dialyzed supernatant after the 20—45% $(NH_4)_2SO_4$ fractionation	1470	0.556	4.1
Part of the dialyzed supernatant which passed when loading the DE 23 column	1120	0.39	5.3
$(NH_4)_2SO_4$ fractionation between 0—70% saturation	24	5.94	6.9
Sephadex G-200 and precipitate between 0—80% saturation of $(NH_4)_2SO_4$	6.2	5.75	11.6
Sephadex G-100 Fraction numbers			
110	8.2	1.95	3.4
111	8.2	1.92	7.7
112	8.2	1.08	2.5
Sephadex G-75 Fraction numbers			
20	5.4	0.26	2.3
21	5.4	0.38	9.5
22	5.4	0.65	11.5
23	5.4	0.57	7.8
1st DEAE cellulose column Fraction numbers			
13	6.5	0.81	20.7
14	6.5	0.64	11.6
2nd DEAE cellulose column Fraction numbers			
32	5.0	0.142	2.1
33	5.0	0.308	—
34	5.0	0.218	7.4
34+0.1 ml 10^{-4} M FAD			14.3

the monospecificity of the antisera to tobacco and spinach reductase are documented. Only one immunoprecipitation band is observed when the obtained antisera are run against a complex protein mixture from which the pure preparation originated (Fig. 1 d) or only one immunoprecipitation band is observed when a Triton-treated [23] lamellar system from *Antirrhinum* is tested against our antisera in the double diffusion test, whereas in the same assay multiple bands are seen when the complex antisera are used instead (Figs 1 c and d). Consequently, as a difference to Berzborn's work [3] our antisera are truly monospecific. The antisera inhibit the diaphorase activity of a soluble preparation obtained by ammonium sulphate fractionation (Fig. 2).

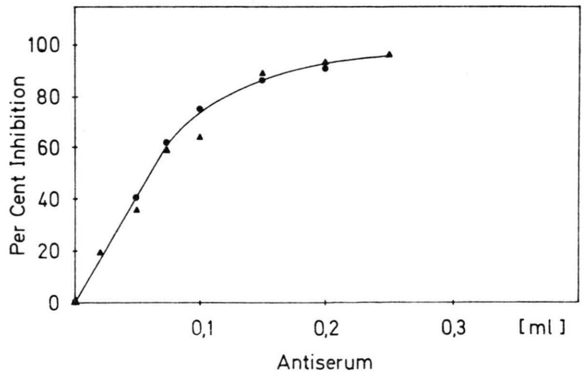

Fig. 2. Inhibition of the diaphorase activity: ▲—▲ by the monospecific antiserum to tobacco ferredoxin-NADP$^+$-reductase, ●—● by a complex antiserum to the *Antirrhinum* lamellar system containing antibodies to ferredoxin-NADP$^+$-reductase. The inhibition titer of this complex antiserum was adjusted to that of the monospecific one.

Serological Reactions of Different Types of Chloroplast Preparations with the Monospecific Antiserum to Ferredoxin NADP$^+$-Reductase

Berzborn observed that chloroplasts from *Antirrhinum* and spinach were not directly agglutinated by an antiserum which contained antibodies to ferredoxin-NADP$^+$-reductase [3]. Only in the Coombs test [4] agglutination occurred. From this it was concluded that reductase is located in depressions of

Table II. Agglutination reactions of the antiserum to ferredoxin-NADP$^+$-reductase from tobacco with chloroplasts from two types of *N. tabacum* and with chloroplasts from *Antirrhinum majus*.

Type of Chloroplast Preparation	*N. tabacum* var. John William's Broadleaf	*N. tabacum* Aurea Mutant Su/su^2	*Antirrhinum majus* Strain 50
Chloroplast with swollen thylakoids [7]	agglutination	agglutination	agglutination
Stroma-freed chloroplasts [9]	agglutination	very strong agglutination	no agglutination
Amount of stroma-freed chloroplasts in mg by which 1 ml antiserum to reductase appears exhausted	10	4.2	15

Chloroplasts were prepared according to reference [7] and [9].

the thylakoid membrane. By the fact that washing with EDTA resulted in direct agglutination it was suggested that reductase should rather be located between protruding protein structures on the surface of the thylakoid membrane. Many authors have observed that EDTA removes the coupling factor from the thylakoid membrane surface [21, 24, 25]. This condition of the thylakoid membrane is apparently the one in Table II in which it is shown that stroma-freed chloroplasts from *Antirrhinum majus* are not directly agglutinated by our antiserum to reductase which is in agreement with Berzborn [3]. As described by Berzborn in the Coombs test agglutination occurs which demonstrates specific adsorption of antibodies onto the surface of the lamellar system. However, a different condition of the lamellar system in *Antirrhinum majus*, green *N. tabacum* var. John William's Broadleaf and the tobacco aurea mutant Su/su^2 allows direct agglutination (Table II). Such chloroplasts have been prepared according to Homann and Schmid [7] in 0.4 M sucrose, 0.05 tris pH 7.8, 0.01 M NaCl in the presence of serum albumin and pectinase. No osmotic shock treatment as in the stroma-freed *Antirrhinum* chloroplasts was used. Hence, the difference is a consequence of the different preparation procedures. However, this is not fully conclusive, since stroma-freed chloroplasts from *N. tabacum* are also directly agglutinated (Table II). From the agglutinability we conclude that ferredoxin-NADP$^+$ is accesible to antibodies in these preparations and that a steric hindrance of agglutination eventually caused by the coupling factor, as observed by Berzborn [3] is not present in this special type of chloroplast preparation. The special state of the thylakoid membrane in our preparation might be characterized by a swelling of the thylakoids due to which, structures, otherwise located in depressions of the membrane surface, are pushed more towards the outside (compare Fig. 1 a in ref. 18). Consequently, direct or indirect agglutinability depends on the condition in which the thylakoid membrane is in.

Effect of the Antiserum to Ferredoxin-NADP$^+$-Reductase on Photosynthetic Electron Transport

NADP$^+$-reduction with the donor couple DPIP/ascorbate in the presence of DCMU is inhibited by 80% in chloroplasts from green *N. tabacum* var. John William's Broadleaf and is completely inhibited in the tobacco aurea mutant Su/su^2 (Table

Table III. Maximal inhibition of NADP$^+$-reduction in tobacco chloroplasts by the antiserum to tobacco ferredoxin-NADP$^+$-reductase.

	N. tabacum var John William's Broadleaf	*N. tabacum* aurea Mutant Su/su^2
	[μmoles NADP$^+$-reduced · (mg chlorophyll^{-1} · h^{-1}]	
Control (no additions)	23	187.5
0.05 ml antiserum to reductase	4.3	12.9
0.1 ml antiserum to reductase	4.4	0
0.01 ml normal rabbit serum	19.8	–
% Maximal inhibition	81	100

Chloroplasts were prepared according to Homann and Schmid [7] and were in the state in which direct agglutination occurred. The reaction was carried out at room temperature with DPIP/ascorbate as the electron donor, and in the presence of DCMU.

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a b c d

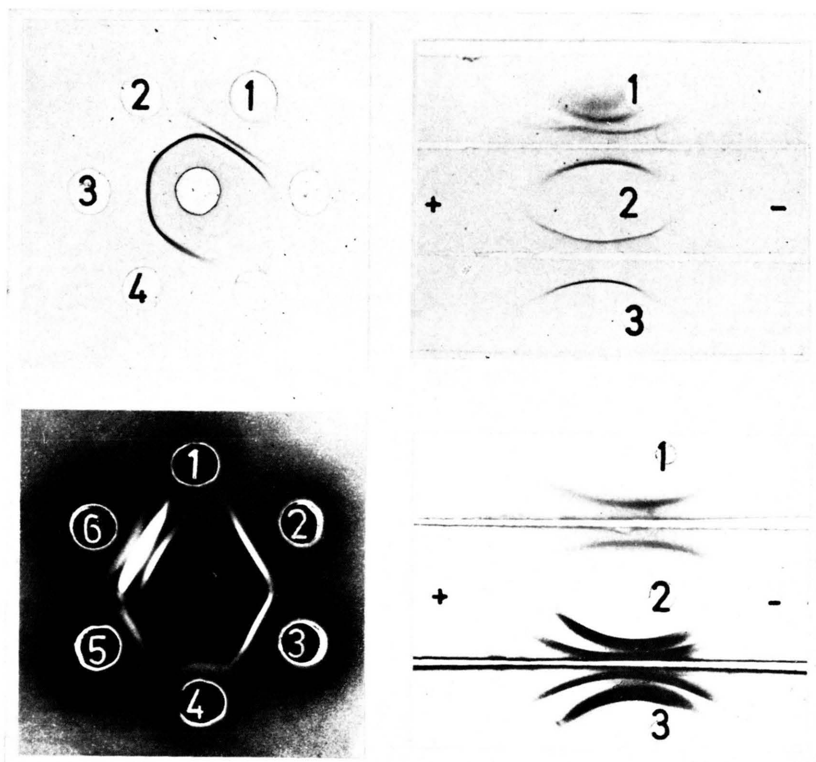

Fig. 1. a. Serological test for purity of the reductase preparation from tobacco according to Ouchterlony [19]. The center well contains a complex antiserum to the lamellar system of *Antirrhinum* (1) $(NH_4)_2SO_4$ fraction between 0—70% saturation, (2) — (4) pure fractions of tobacco reductase. The identical picture was obtained with a complex antiserum to broken chloroplasts from *Antirrhinum*.

b. Test for purity of the reductase preparation from tobacco by means of immunoelectrophoresis (1) $(NH_4)_2SO_4$ fraction between 0—70% saturation, (2) — (3) pure fractions. Well between (1) and (2) contains an antiserum to broken chloroplasts from *Antirrhinum*, the well between (2) and (3) contains an antiserum to the lamellar system of *Antirrhinum*.

c. Test for monospecificity of the antiserum to reductase from tobacco and spinach in the double diffusion test [19]. The center well contains stroma-freed *Antirrhinum* chloroplasts treated with 1% Triton [23]. (1) Control serum; (2) antiserum to tobacco reductase (2nd blood withdrawal); (3) antiserum to tobacco reductase (8th blood withdrawal); (4) antiserum to spinach reductase (2nd blood withdrawal); (5) antiserum to spinach reductase (3rd blood withdrawal); (6) late antiserum to the *Antirrhinum* lamellar system.

d. Test for monospecificity of the antiserum to spinach reductase by means of immunoelectrophoresis, (1), (2) and (3) contains a crude reductase preparation obtained by $(NH_4)_2SO_4$ fractionation between 35 and 65 per cent saturation. The well between (1) and (2) contains the monospecific antiserum to spinach reductase the well between (2) and (3) complex antiserum to broken chloroplasts from spinach [2].

III). This means that all the reductase that plays a role for photosynthetic electron transport is accessible to antibodies in chloroplast preparations which were prepared according to Homann and Schmid [7]. However, in the presence of methylamine or $MgCl_2$ the maximally achievable inhibition by the antiserum is in general about 35 – 40 per cent in the green type chloroplasts from tobacco, but may go up to 60 per cent (Fig. 3). Under the same condition the maximal inhibition of $NADP^+$-reduction in chloroplasts from the aurea mutant Su/su^2 is only about 70 per cent in comparison to full (100%) inhibition without methylamine or $MgCl_2$ (Table III). From earlier morphological studies, we know that chloroplasts from the tobacco aurea mutant Su/su contain a simplified lamellar system with low stacked grana and very extended intergrana regions [7, 26]. In addition, from investigations by Izawa and Good it is known that methylamine causes shrinkage of a swollen lamellar system and leads to partition-, *i. e.* grana-formation [27, 28]. Radunz and Schmid have confirmed this observation [18]. The observations of Table III and Fig. 8 together with the observation of Izawa and Good were taken to mean that antibodies do not enter into well preserved partitions and that in the presence of methylamine the amount of accessible reductase has decreased. It can obviously mean that ferredoxin-$NADP^+$-reductase is located in the grana and the intergrana regions of the lamellar system. In order to verify this view we have tested the antiserum against a chloroplast preparation from the yellow leaf patches of the variegated *N. tabacum* var.

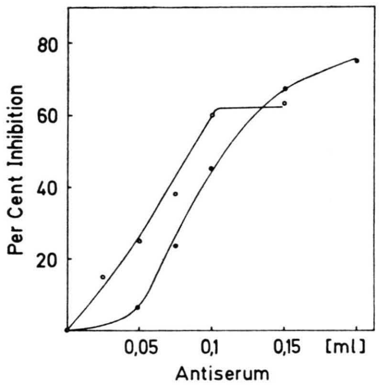

Fig. 3. Relative inhibition of photosystem-I mediated photoreduction of $NADP^+$ with the electron donor couple DPIP/ascorbate by the monospecific antiserum to tobacco reductase in the presence of $5 \cdot 10^{-2}$ M methylamine: ○—○ chloroplasts from normal green *N. tabacum* car. John William's Broadleaf, ●—● chloroplasts from the tobacco aurea mutant Su/su^2. The chloroplasts were prepared according to Homann and Schmid [7]. Control rates at zero inhibition in the presence of 0.1 ml normal rabbit serum were 58.9 (○) and 190 (●) μmol $NADP^+$-reduced × (mg chlorophyll)$^{-1}$ × h^{-1}; 2 min of illumination with 100,000 lx white light through 10 cm of water at 22 °C. Buffer 0.05 M tricine, 0.05 M CH_3NH_2 pH 7.3 in the presence of 10^{-6} M DCMU.

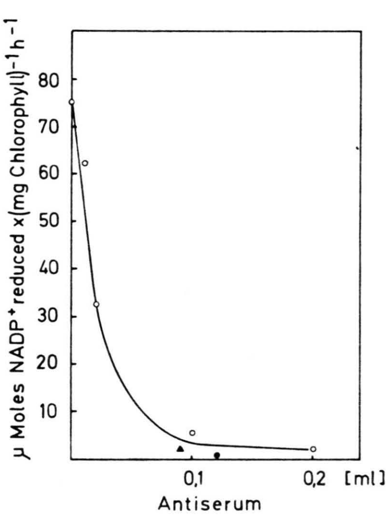

Fig. 4. Dependence of the degree of inhibition of the DPIP/ascorbate driven $NADP^+$ reduction by the antiserum to tobacco reductase in chloroplasts from yellow leaf patches of the variegated *N. tabacum* NC95 ○—○ in the presence of $5.2 \cdot 10^{-2}$ M methylamine, (●) in the presence of $2.6 \cdot 10^{-2}$ M $MgCl_2$, (▲) no additions just 0.05 M tricine pH 7.8.

Table IV. Effect of the antiserum to tobacco reductase on photophosphorylation in chloroplasts from *N. tobacum* var. John William's Broadleaf.

	Cyclic PMS-mediated	Pseudocyclic $H_2O \rightarrow$ Methylviologen	Non-cyclic [1] $H_2O \rightarrow NADP^+$
	[μmol ATP formed · mg chlorophyll^{-1} · h^{-1}]		
Control	208 ± 6	13.8	41.3 ± 10
Control minus mediator or acceptor	32	corrected for	31.5
Control plus 0.2 ml antiserum to reductase	210	13	61
Control plus 0.2 ml null serum	194	11	48.5
Control in the presence of DCMU	194	0	40

[1] The assay was run on purpose in the presence of 150 μg ferredoxin/assay; under the assay conditions photophosphorylation is almost independent of the acceptor $NADP^+$, indicating that it is of the cyclic type.

NC 95. These chloroplasts contain single thylakoids and practically no partitions [7, 10]. In this case inhibition should not be dependent on the degree of swelling which amongst other effects might open the partitions [18, 27, 28]. And indeed, this is shown to be the case (Fig. 4). Clearly, there is no influence on the presence or absence of methylamine or $MgCl_2$ and the $NADP^+$-reduction with the DPIP/ascorbate couple is inhibited by almost 100 per cent.

No effect of the antiserum on PMS-mediated cyclic photophosphorylation is observed in agreement with Bothe and Berzborn [29]. The slight stimulation of the ferredoxin mediated cyclic photophosphorylation in the presence of $NADP^+$ by the antiserum can be explained that electron leakage out of the cyclic electron flow is prevented by blocking linear electron flow from the reductase to $NADP^+$ by the antiserum (Table IV).

Discussion

Ferredoxin-$NADP^+$-reductase [2, 3, 6], ferredoxin [30] and the coupling factor [31] are located in the outer surface of the thylakoid membrane apparently in such a close spatial relationship that the occurence of serological reactions may be hindered for steric reasons. In this paper we have reported on a special condition of the thylakoid membrane in which this steric hindrance of agglutination with respect to reductase does not exist. This is evidenced by the fact that these chloroplast preparations are directly agglutinated by our monospecific antiserum obtained from tobacco chloroplasts.

Concomitantly, the photosystem-I-dependent $NADP^+$-reduction in these chloroplasts is inhibited to a large degree by the antiserum (Table III). In another state of the thylakoid membrane which is represented by the preparation termed in Table II stroma-freed chloroplasts the condition already described by Berzborn seems to be realized [3].

The reason for this difference might be a swelling of the lamellar system in our type of chloroplast preparation by which the thylakoid membrane is stretched, pushing components which are located in depressions more toward the outer surface but on the other hand also smoothing out protruding protein structures from the surface (compare Fig. 1 a in ref. 18). A similar conclusion was reached for the first time in our laboratory in context with unfinished experiments by Kannangara and van Wyk. At this point we would like to note that it seems to be a general property of the tobacco chloroplasts prepared according to Homann and Schmid [7] to become agglutinated under conditions when stroma-freed chloroplasts from *Antirrhinum* only specifically adsorb the respective antibodies [14, 18, 32, 33] and consequently are not agglutinated. It should be emphasized, however, that this is not necessarily a species-dependent difference. Stroma-freed chloroplasts from *Antirrhinum* appear very compact and rather well preserved under the light microscope. However, our explanation for the absence of the steric hindrance of agglutination might not necessarly describe the entire situation because stroma-freed chloroplasts from green tobacco are also directly agglutinated by the antiserum (Table II). In the condition where the lamellar system appears swollen a further phenomen takes place: former partition regions become obviously exposed to the surrounding medium (compare Fig. 1 a, Radunz and Schmid [18] or Fig. 1 case 3, Izawa and Good [28]). In this case almost all the reductase seems to become accessible to antibodies and the $NADP^+$-reduction is eventually fully impaired. However, upon addition of substances like methylamine or $MgCl_2$ which cause reformation of partitions and grana (compare Fig. 4 in ref. 18 or Fig. 1 case 4 in ref. 28) the green type chloroplasts from tobacco give much less than 100 per cent inhibition (Fig. 3). This is most conveniently explained by the assumption that antibodies do not enter into partitions. In this situation only the reductase in the intergrana regions in the outer surfaces of the grana is accessible to antibodies and only this type of reductase can be inhibited by the antiserum. If this was indeed correct, a lamellar system with extended intergrana regions like the one in the tobacco aurea mutants [7], should yield a higher inhibition than that of green tobacco chloroplasts in the presence of methylamine, since the partitions are not accessible to antibodies. Indeed, this was found to be so as shown in Fig. 3. With antisera of totally different specificities we have repeatedly made observations which are best explained by the assumption that antibodies do not enter into partitions. This point has been discussed in another context by Menke [34].

Furthermore, mutant chloroplasts the lamellar system of which barely contains any partitions, but mostly single unfolded thylakoids [10, 7], show full inhibition of the $NADP^+$-reduction in the presence *and* absence of methylamine or $MgCl_2$ (Fig. 4). From these observations it is inferred that $NADP^+$-reductase is located in both the grana and the inter-grana regions of the chloroplast lamellar system in the outer surface of the thylakoid membrane.

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