Binding of Antibodies onto the Thylakoid Membrane
III. Proteins in the Outer Surface of the Thylakoid Membrane

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The maximal binding of antibodies to ferredoxin-NADP$^+$-reductase, cytochrome f, plastocyanin, coupling factor of photophosphorylation, carboxydismutase and to a polypeptide with the apparent molecular weight 24 000 onto stroma-freed chloroplasts of *Antirrhinum majus* was determined. The three proteins involved in photosynthetic electron transport bind approximately 0.05 to 0.07 g antibodies per g chloroplasts. The chloroplast preparation itself binds maximally about 1 g antibodies. From an antiserum to carboxydismutase and to a membrane polypeptide with the apparent molecular weight 24 000 approximately double the amount of antibodies namely 0.1 to 0.14 g antibodies per g chloroplasts are bound. Extraction of stroma-freed chloroplasts with 0.02 M Tris buffer pH 7.8 containing 0.7 mM EDTA caused a threefold increase of the amount of bound antibodies in the case of the membrane protein. 40% of the amount of antibodies which can be maximally bound by this chloroplast preparation is adsorbed out of an antiserum to the coupling factor.

Out of an antiserum which contains equal concentrations of antibodies to ferredoxin-NADP$^+$-reductase, cytochrome f and plastocyanin the same amount of antibodies is bound as out of an antiserum directed to only one of these components. This shows that the proteins involved in electron transport are located in a very close relationship to each other in the outer surface of the thylakoid membrane.

Quantitative investigations on the binding of antibodies onto the outer surface of the thylakoid membrane showed that 1 g stroma-freed chloroplasts of *Antirrhinum majus* binds maximally 1 g antibodies [1]. Out of an antiserum to a mixture of all thylakoid membrane proteins the same amount of antibodies was adsorbed. It can be assumed, that in this way the entire thylakoid membrane surface accessible to antibodies is covered with antibody molecules. However, out of an antiserum to chloroplast lipids only 0.25 g antibodies were bound [2]. As the bound antibody molecules, due to their shape and size, cover up homologuous as well as not homologuous antibody molecules, the portion of the thylakoid surface, which is composed of lipids is smaller than 25%. An estimation showed that 85 — 90% of the thylakoid surface is composed of proteins [2]. The lipids just suffice to fill up gaps between the protein molecules or between aggregates of protein molecules. Hence, the major part of the lipids appears to be localized on the surface of the thylakoid membrane which is directed towards the inside. On the other hand also protein molecules were detected on the inner surface [3, 4].

In the following we report on the maximal binding of antibodies to proteins, namely to ferredoxin-NADP$^+$-reductase, cytochrome f, plastocyanin, to the coupling factor of photophosphorylation, carboxydismutase and to a polypeptide with the apparent molecular weight 24 000.

Materials and Methods

1) Preparation of the antisera: Antisera to the proteins listed in Table I namely to ferredoxin-NADP$^+$-reductase, plastocyanin, cytochrome f, coupling factor of photophosphorylation and carboxydismutase were obtained according to the methods described earlier [5 — 7]. The immunization of rabbits required approximately 1 — 2 mg protein. The antiserum to the polypeptide with the apparent molecular weight 24 000, which is insoluble in aqueous media, was also prepared as described earlier [1]. The immunization required in this case 4 mg. The insoluble membrane protein was isolated from Antirrhinum chloroplasts according to the methods described earlier [8].

2) Monospecificity of the protein antisera: The monospecificity of the antisera was demonstrated by double diffusion in agarose according to the method of Ouchterlony [9]. The agarose plates consisted of 0.8% agarose in 0.06 M barbiturate buffer pH 7.8. The diffusion of the antibodies against the antigens required 36 — 72 hours. Subsequently, the not precipitated proteins were washed out of the

agarose plates with 1.7% saline, the precipitation bands being stained with 2% amido black in acetic acid.

3) Serological tests: The agglutination reactions of the antisera with chloroplasts and chloroplast fragments [10], the test for monovalent binding of antibodies onto the thylakoid membrane [11] as well as the determination of antibodies onto stroma-freed not swellable chloroplasts were carried out according to earlier described methods [1, 2].

4) Chloroplast preparations: Stroma-freed chloroplasts were obtained according to Kreutz and Menke via a sucrose density gradient [12]. For the extraction of the coupling factor 100 mg chloroplasts were treated 7 times with 50 ml of 0.02 M Tris buffer pH 8.0 containing 0.7 mM EDTA according to earlier described methods. [13].

Results

The antisera to ferredoxin-NADP+-reductase, plastocyanin and cytochrome f listed in the Table inhibit as earlier investigations have shown photosynthetic electron transport in chloroplasts [5 – 7, 14 – 19]. The antiserum to coupling factor inhibits photophosphorylation reactions practically fully [4, 13]. The polypeptide with the molecular weight 24 000 is a membrane protein which occurs in the thylakoid membrane in relatively large amounts [20]. All antisera to this polypeptide, obtained up to now, did not affect electron transport. Carboxydismutase is the main component of the stroma proteins. The protein is partially adsorbed onto the thylakoid membrane surface directed towards the outside [13].

All antisera tested are monospecific. The antiserum to ferredoxin-NADP+-reductase, cytochrome f, carboxydismutase and to the coupling factor yielded in the double diffussion test against chloroplasts which were treated with 1% Triton X 100 only one precipitation band (Fig. 1 a, b, c, d). The monospecificity of the antiserum to plastocyanin was demonstrated by means of the immune electrophoresis (Fig. 1 f). No cross reactions between the sera were observed (Fig. 1 e). It should be noted that the diffusion in agarose gel showed with the antiserum to coupling factor when tested against a dissociated coupling factor preparation at least four bands. The antigen with the molecular weight 24 000 was wure and had according to Beyreuther [21] an aminoterminal sequence of Ala-Ala-Gly-Lys-Pro-Thr-Asp.

Antibodies to the proteins reductase, cytochrome f and plastocyanin as well as to the polypeptide 24 000 react with stroma-freed chloroplasts of *Antirrhinum majus* only in a monovalent manner, whereas antibodies to the coupling factor and to carboxydismutase show a bivalent reaction [13]. Antigen-antibody reactions are not sterically hindered with coupling factor and carboxydismutase. However, not too much importance should be attached to this observations as far as the surface structure of the thylakoid membrane is concerned, because whether antibodies react mono-or bivalently depends on the condition in which the chloroplasts are in [22 – 25]. With swellable chloroplasts which give higher rates of the Hill reaction, antibodies to all listed proteins react bivalently [5 – 7, 14 – 16, 26].

The amount of antibodies bound plotted as a function of the added serum volume yielded in all

Table I. Maximal binding of antibodies to proteins onto stroma-freed not swellable chloroplasts of *Antirrhinum majus*.

| Antiserum	Mode of reaction of the antibodies with stroma-freed chloroplasts	g Antibodies bound	Number of bound antibodies molecules
		g stroma-freed chloroplasts	g stroma-freed chloroplasts
Ferredoxin-NADP+-Reductase	monovalent	0.07 ± 0.01	2×10^{17}
Plastocyanin	monovalent	0.05 ± 0.01	2×10^{17}
Cytochrome f	monovalent	0.05 ± 0.01	3×10^{17}
Coupling factor of photophosphorylation	bivalent	0.40 ± 0.04	16×10^{17}
Carboxydismutase	bivalent	0.13 ± 0.02	5×10^{17}
Polypeptide 24 000	monovalent	0.10 ± 0.01	4×10^{17}
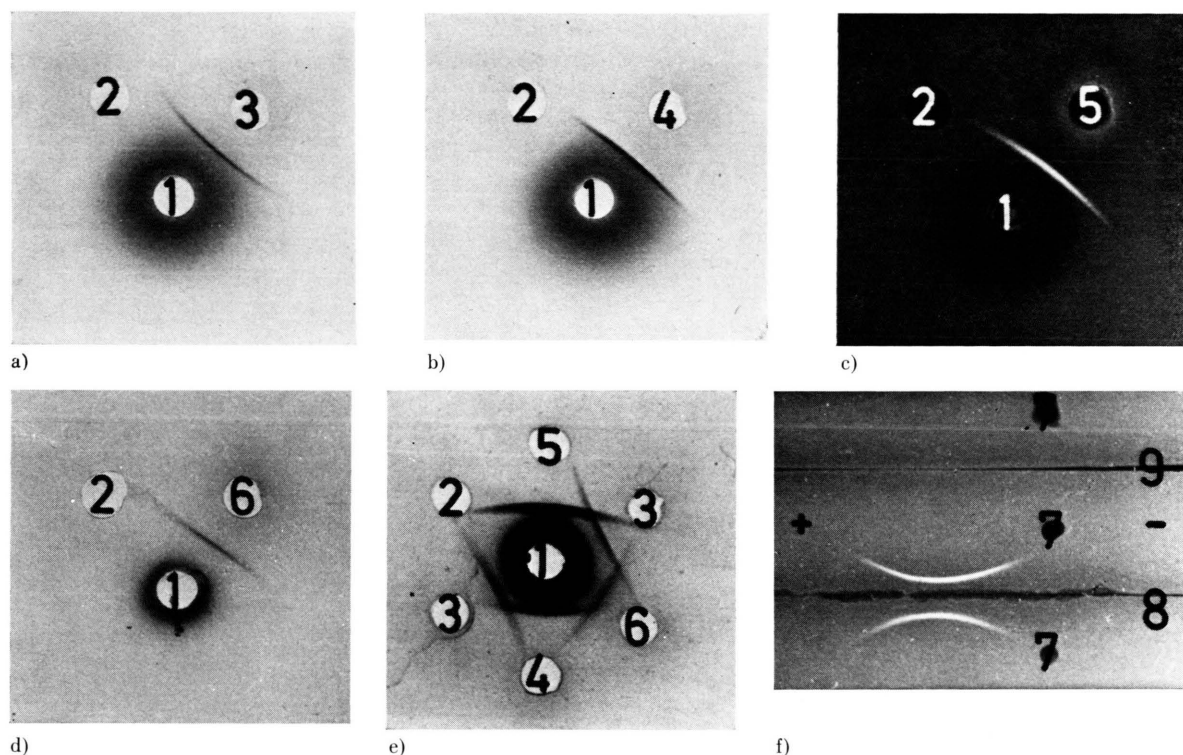
Fig. 1. Double diffusion test in agarose in order to demonstrate monospecificity of the antisera to a) reductase, b) cytochrome f, c) carboxydismutase and d) coupling factor to photophosphorylation.
Hole 1 contained Antirrhinum chloroplasts treated with 1% Triton X-100. a) Hole 3 contained antiserum to reductase; hole 2 control serum. b) Hole 4 contained antiserum to cytochrome f; hole 2 control serum. c) Hole 5 contained antiserum to carboxydismutase; hole 2 control serum. d) Hole 6 contained antiserum to coupling factor; hole 2 control serum. e) Hole 1 contained Antirrhinum chloroplasts treated with 1% Triton X-100; holes 3—6 contained the antisera to the corresponding proteins; hole 2 corresponding control serum to 3. f) Immune electrophoresis in agarose in order to show monospecificity of the plastocyanin antiserum. Holes 7 contained plastocyanin preparation, well (8) antiserum to plastocyanin, well (9) antiserum to stroma-freed chloroplasts.

cases a saturation curve [1]. If the amount of the maximally bound antibodies is determined, it is of no importance whether antibodies react mono- or bivalently as antibodies in the saturation region are only monovalently bound. The values for the maximally bound antibodies are summarized in Table I. It is seen that antibodies to reductase, cytochrome f and plastocyanin are bound in approximately equal amounts. The concentrations of these three antigen molecules in the thylakoid membrane is according to the literature of the same order of magnitude [27]. In view of the controversal reports concerning the localization of plastocyanin and cytochrome f at the inner or outer thylakoid membrane surface, it is noteworthy, that antibodies to plastocyanin and cytochrome are obviously bound in approximately the same amount as antibodies to reductase [5 — 7, 14 — 16, 18, 19, 26 — 28]. Anti-bodies to the polypeptide 24 000 and to carboxydismutase are adsorbed in approximately double the amount as the proteins involved in electron transport. After the washing of stroma-freed chloroplasts with 0.02 M Tris buffer containing EDTA which leads amongst other things to the removal of part of the coupling factor molecules [29 — 31, 13], the amount of antibodies bound to the polypeptide 24 000 is increased threefold. This leads to the conclusion that the coupling factor molecules cover partially other molecules of the membrane protein. Antibodies to the coupling factor are bound in a considerably higher amount. They comprise approximately 40% of the amount of antibodies which stroma-freed chloroplasts can totally bind.

It is tempting to calculate the surface portion which the individual antigens cover on the surface of the thylakoid membrane directed towards the

outside. With this it must be borne in mind, however, that the number of bound antibodies is not only dependent on the number of antigen molecules situated in the surface, but that with antigens of high molecular weights, one antigen molecule might bind several antibody molecules. With this uncertainty in mind it would be not reasonable to make a quantitative evaluation.

The antigenic determinants of the proteins ferredoxin-NADP$^+$-reductase, plastocyanin and cytochrome f are located in the outer thylakoid membrane surface in such a close relationship that binding of one antibody molecule of one of these antigens hinders the other two to react with their antibodies. Out of a mixed antiserum, which contains equal concentrations of antibodies to the three mentioned proteins, 1 g chloroplasts does not bind as expected 7×10^{17} antibodies but only 2×10^{17} antibodies. This number corresponds to the amount of antibodies which are adsorbed out of a monospecific antiserum to one of these protein.

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