Binding of Antibodies onto the Thylakoid Membrane
I. Maximal Antibody Binding and Adsorption of Antibodies to Lipids

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Dedicated to Prof. Dr. W. Menke *on the Occasion of His 65th Birthday*

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Chloroplasts, Lipids, Proteins, Antibodies, Membrane Structure

The binding of antibodies onto the lamellar system of *Antirrhinum majus* was determined in dependence on the serum addition. The unspecific adsorption of serum proteins was taken into account or eliminated. The binding of antibodies as a function of the amount of serum added is seen from a saturation curve. From an antiserum obtained by hyperimmunization with stroma-freed chloroplasts, the chloroplasts bind maximally 1 gram antibodies per gram stroma-freed chloroplasts. From an antiserum to the proteins of the thylakoid membrane prepared in the same way an equal amount of antibodies is adsorbed. It is assumed that with this amount the surface of the lamellar system accessible to antibodies is completely covered by antibodies. For an antiserum to monogalactosyl diglyceride a maximal antibody binding of 0.16 g, for sulphoquinovosyl diglyceride 0.12 g and for phosphatidyl glycerol 0.13 g of antibodies per gram stroma-freed chloroplasts are obtained. The significance of these results with respect to the molecular surface structure of the thylakoid membrane is discussed.

X-ray diffraction studies have led to the conclusion that the thylakoid membrane is assymetric and consists of a monomolecular protein layer directed towards the outside and a bimolecular lipid film directed towards the inside [1–6]. Serological investigations with antibodies to proteins of the thylakoid membrane initially seemed to confirm this idea. Antisera to the structural protein obtained by formic acid treatment [7, 8], to the coupling factor of photophosphorylation [9], to ferredoxin [10] and carboxydismutase [9] agglutinated stroma-freed chloroplasts. However, antibodies to ferredoxin-NADP-reductase [11–14] and plastocyanin [15] did not agglutinate stroma-freed chloroplasts, but were specifically adsorbed. Furthermore, it was recently observed that antisera to 4 polypeptides from the thylakoid membrane agglutinated chloroplasts and inhibited photochemical reactions [16]. This infers that antigenic determinants of the mentioned proteins are located on the outer surface of the thylakoid membrane. However, antigenic determinants of proteins were also detected in the surface directed towards the inside. By treatment with deoxycholate solution and subsequent gel filtration a chlorophyll containing protein fraction with a molecular weight of 600 000 was isolated [17]. This fraction photoreduced methylviologen with dichlorophenol indophenol/ascorbate as the electron donors. Despite the fact that antibodies to this fraction were specifically adsorbed by stroma-freed chloroplasts, they did not inhibit the mentioned photochemical reactions. However, after disrupture of the thylakoids by ultrasonication the photoreduction of methylviologen was fully blocked by the antiserum. Moreover, it was demonstrated, that contrary to the concept developped from X-ray diffraction studies, also lipid molecules are accessible to antibodies from the outside, which is valid for antibodies to monogalactosyl diglyceride [18], sulphoquinovosyl diglyceride [19] and phosphatidyl glycerol [20]. In addition, antibodies to chlorophyll [21–23] and to the xanthophylls lutein [24] and neoxanthin (Radunz unpublished) are adsorbed onto the thylakoid membrane.

With respect to these results it is doubtful whether the X-ray diffraction studies were correctly interpreted [25]. However, it should be borne in mind, that the agglutination reactions used as a test are very sensitive. An agglutination of particles may already occur after the reaction of only a small number of antigen molecules with antibodies. The question arises, whether these antigenic determinants belong to the integrated elements of the outer surface structure of the thylakoid membrane, or whether they got to the surface due to disturbancies of the surface structure. In fact, it must be considered,

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that all chloroplast preparations contain few disrupted thylakoids. However, the uncertainty, resulting from this for the localization of antigens, can be eliminated by determination of the saturating amounts of adsorbed antibodies for the respective antigens and comparing this data with the amount of antigen present in the thylakoid membrane. From the thus obtained results even established concepts concerning the surface structure of the thylakoid membrane should be refined. In comparison to other methods, investigations with antibodies have the advantage that antisera are highly specific reagents which due to their high molecular weight, cannot permeate through the thylakoid membrane.

For the investigations stroma-freed chloroplasts from *Antirrhinum* strain 50 were used because these chloroplasts are very well preserved during the isolation procedure as shown by light and electron microscopy. For the interpretation of the following results it must be taken into consideration, that only part of the thylakoid surface is accessible to antibodies. This part consists of the intergrana regions of the thylakoids and those parts of the thylakoid membrane of the grana, which in intact chloroplasts are in contact with the stroma. The antibodies have access to the thylakoid membranes in the interior of the grana only after the thylakoids have been separated from each other.

Materials and Methods

Isolation of the antigens

Stroma-freed chloroplasts were isolated from leaves of *Antirrhinum majus* strain 50 according to Kreutz and Menke [1]. The chloroplasts were centrifuged twice over a sucrose density gradient and subsequently washed 6 times with destilled water. This way, all water soluble substances are washed out and a morphologically intact lamellar system is obtained. Dry weight of the chloroplasts was determined by weighing aliquots.

The protein preparation was obtained according to a recently described procedure for the preparation of renatured membrane proteins [16].

The isolation of the lipids monogalactosyl diglyceride, sulphoquinovosyl diglyceride and phosphatidyl glycerol was carried out as described earlier [26, 27].

Preparation of the antisera

The antisera to stroma-freed chloroplasts were prepared by a series of intravenous injections as described earlier [19]. Five month after the first immunization series a second series was made, subsequently followed by a third post immunization series. The antisera of the third post immunization series are called hyperimmune sera.

For the preparation of antisera to membrane proteins 1 mg proteins were suspended in 1 ml 0.06 M phosphate buffer, pH 7.4, and emulsified with 1 ml of Freund's adjuvant. This emulsion was subcutaneously injected into the hind leg of a rabbit. After 30 days with a five day interval two intravenous injections of each time 1 mg protein in the aforementioned buffer were made. The first blood withdrawal was made 10 days after the last injection. Control sera were withdrawn from the animals before the respective treatments.

The antisera to monogalactosyl diglyceride [18] and to the anionic lipids sulphoquinovosyl diglyceride [19] and phosphatidyl glycerol [20] were prepared according to earlier described methods. The antisera used for adsorption measurements were heated for 20 min to 56 °C in order to exclude reactions of the complement in the binding of antibodies.

Binding of antibodies onto stroma-freed chloroplasts

In test tubes (10 cm high, diameter 16 mm) 30 μg stroma-freed chloroplasts were incubated with increasing amounts of antiserum. The amount of serum added was 0.05 – 10 ml. The reaction mixtures were incubated for 6 hours at room temperature and subsequently stored for 12 hours at 7 °C. In order to obtain a homogeneous distribution of the stroma-freed chloroplasts in the antiserum solution, the test tubes were vigorously shaken by means of a vibrator at the onset of the experiment, then one hour later and then again after 6 hours. After 18 hours of incubation the stroma-freed chloroplasts were spun down, suspended in 5 ml physiological saline solution by vigorous vibrations and again spun down. This washing was repeated seven times in order to remove all not bound immune globulines as well as other adsorbed serum proteins from the chloroplast preparation. The nitrogen determination was carried out according to the procedure of Lanni and co-workers [28, 29] with Neßler's reagent. Alanine was used for the standard curve. Photometric determinations were carried out with the Zeiss-Photometer PMQ II. The amount of adsorbed proteins was obtained by multiplication of the results with the factor 6. All given values are averages of four individual determinations.

Results

The first objective was to determine how many antibodies can be maximally bound to stroma-freed chloroplasts. Therefore, we determined the binding of antibodies from antisera which had been obtained by immunization of rabbits with stroma-freed chloroplasts. Fig. 1 shows the amount of adsorbed antibodies as a function of the amount of serum added. The adsorption curve shows hyperbolic shape. It became evident, that stroma-freed chloroplasts also adsorb considerable amounts of proteins from the control serum (Fig. 1 a, curve K). Obviously, these have to be subtracted from the proteins adsorbed out of the immune serum. The amount of adsorbed antibodies is seen from Fig. 1 b. In the region of saturation this value is 0.9 g antibodies per gram stroma-freed chloroplasts (Table I). The same result is obtained from a reciprocal plot of the data (Fig. 2). By extrapolation of the straight line obtained by regression calculation the maximally bound amount of antibodies is obtained. This way of presenting the data has the advantage, that values outside the saturation region are also included into the calculation of the maximal binding value. The mentioned serum was obtained by only one series of intravenous injections. From an antiserum, obtained after 3 series of intravenous injections chloroplasts usually bind a somewhat higher amount of antibodies, namely 1 g per g chloroplasts (Table I). Precipitation reactions showed, that the antiserum obtained by hyperimmunization contains antibodies to proteins and lipids, whereas in the first mentioned antiserum only antibodies to proteins were detected. However, the increase in the adsorbed amount of antibodies cannot exclusively be due to additional adsorption of antibodies to lipids. This is concluded from the observation that from an antiserum to a mixture of the membrane proteins also 1 g protein was maximally bound. In investigations of different antisera to chloroplast preparations in no case more antibodies were adsorbed. Therefore, it is concluded that with this value the accessible surface of the stroma-freed chloroplasts is saturated with antibodies. In addition, Table I contains values for the maximal binding of antibodies to the chloroplast lipids monogalactosyl diglyceride, sulphoquinovosyl diglyceride and phosphatidyl glycerol. The saturation values with these monospecific antisera are almost one order of magnitude lower than those described above.

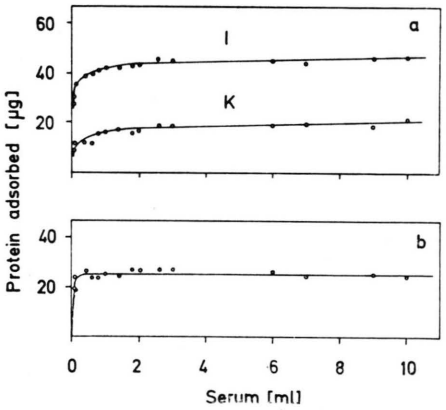

Fig. 1. Adsorption of antibodies and serum proteins onto 30 µg of stroma-freed chloroplasts in dependence on the amount of serum added. a. Control serum (K) and antiserum to stroma-freed chloroplasts (J); b. antibodies bound.

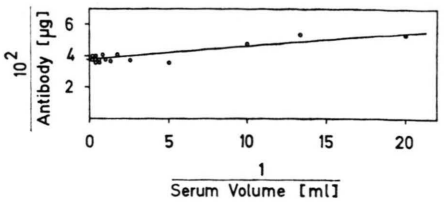

Fig. 2. Dependence of the amount of antibodies adsorbed (Fig. 1 b) as reciprocal plot.

Table I. Maximal binding of antibodies by stroma-freed chloroplasts of *Antirrhinum majus*.

Antiserum to	Reaction of antibodies with stroma-freed chloroplasts	g antibodies bound/ g stroma-freed chloroplasts
Stroma-freed chloroplasts		
First immunization	agglutination	0.87 ± 0.01
2nd post immunization	agglutination	1.05 ± 0.02
Membrane proteins of the lamellar system	agglutination	1.02 ± 0.03
Monogalactosyl diglyceride	agglutination	0.16 ± 0.02
Sulphoquinovosyl diglyceride	adsorption of antibodies	0.12 ± 0.01
Phosphatidyl glycerol	adsorption of antibodies	0.13 ± 0.01

If one compares the number of antibody molecules which are bound by 1 g chloroplasts, to the number of antigen molecules present, it becomes obvious that there is no proportionality concerning the 3 investigated compounds (Tab. II). The concentration

Table II. Comparison the lipid molecules present in Strom-freed chloroplasts to the number of lipid antibody molecules bound.

Antiserum to	Number of lipid molecules/ g stroma-freed chloroplasts	Number of antibody molecules bound/ g stroma-freed chloroplasts
Monogalactosyl diglyceride	850×10^{17}	1×10^{17}
Sulphoquinovosyl diglyceride	140×10^{17}	5×10^{17}
Phosphatidyl glycerol	150×10^{17}	5×10^{17}

The number of antibody molecules was determined according to

$$\frac{\text{Loschmid' number} \times \text{amount of protein bound (g)}}{\text{molecular weight of the antibodies}}.$$

For the molecular weight of the antibodies to the anionic lipids 1.5×10^5 (type IgG) and for the antibodies to monogalactosyl diglyceride 9×10^5 type IgM) was used.

of the two anionic lipids in chloroplasts is approximately the same [30, 27]. Only one antibody molecule is adsorbed per every 28th or 30th antigen molecule, respectively. As to the neutral monogalactolipid, which is present in chloroplasts in a 5-fold higher concentration [30] only 1 antibody molecule per 850 antigen molecules is adsorbed. It should be borne in mind that if antibodies are present in excess the binding is monovalent. From this it follows that monogalactosyl diglyceride is much less accessible to antibodies than the two anionic lipids. This binding difference may be due to the possibilities that the monogalactosyl diglyceride molecules are either covered by protein molecules, or that they are preferentially located in the surface directed towards the inside of the thylakoid or that they are preponderantly located in the grana regions. In addition, the molecules of the monogalactolipid might be located in an accessible location but in such a close relationship to each other that each bound antibody molecule covers up several antigen molecules. That the last mentioned possibility might play a role is seen from the following experiment: To a defined amount of stroma-freed chloroplasts a mixture of equal amounts of antisera to monogalactolipid, sulpholipid and phosphatidyl glycerol was added. In the region of saturation from this serum mixture approximately the same amount of antibody molecules was bound as from the monospecific serum to monogalactosyl diglyceride.

Our experiments allow the conclusion that the outer surface of the thylakoid membrane is not exclusively composed of proteins, but that also lipids take part in this structure. In this context it must be considered that from the described lipids, the antiserum to the monogalactolipid agglutinates chloroplasts directly (Table I) whereas binding of the antibodies to the two anionic lipids is only demonstrated in the Coombs test [31] or in the mixed antigen agglutination [32, 33]. The difference is due to the fact that the agglutination reaction for the anionic lipids is sterically hindered, which might be caused by the possibility that the respective antigenic determinants are located in depressions. A more detailed discussion is postponed to the time when a larger number of monospecific lipid and protein antisera can be compared.

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