Binding of Antibodies onto the Thylakoid Membrane

IV. Phosphatides and Xanthophylls in the Outer Surface of the Thylakoid Membrane

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Antisera to the phosphatides phosphatidyl inositol and phosphatidyl choline as well as to the xanthophylls violaxanthin and zeaxanthin were obtained by immunization of rabbits.

The phosphatidyl choline antiserum did not give cross reactions with thylakoid membrane glycolipids, nor with phosphatidyl glycerol and phosphatidyl inositol. The antiserum to phosphatidyl inositol also did not react with the glycolipids and phosphatidyl choline. However, a cross reaction with phosphatidyl glycerol was observed. Exhaustion tests revealed, that the phosphatidyl inositol antiserum was fully neutralized with the homologous phosphatide, but not with phosphatidyl glycerol.

Antibodies to the phosphatides phosphatidyl inositol and phosphatidyl choline react with stroma-freed, not swellable chloroplasts of *Antirrhinum majus* only in a monovalent manner as demonstrated by means of the anti-γ-globuline consumption test. Sonicated and spun down chloroplasts, however, are agglutinated. Also an antiserum to phosphatidyl inositol treated with exhausting quantities of phosphatidyl glycerol agglutinates this ultrasonic sediment. This type of chloroplast preparation binds approximately double the amount of antibodies to phosphatides. Small membrane fragments of the ultrasonic supernatant in which the outer as well as the inner surface of the thylakoid membrane is accessible to antibodies, are not precipitated. They react with phosphatide antisera just like untreated chloroplasts. Chloroplasts of *Antirrhinum majus* and of *Nicotiana tabacum*, which were isolated in Tris buffer and whose thylakoids are swellable and exhibit high Hill reaction rates, were agglutinated by the phosphatide antisera.

The antisera to violaxanthin and zeaxanthin gave with the described chloroplast preparations the same reactions as the phosphatide antisera. From this it is concluded that antigenic determinants of the phosphatides and xanthophylls are located in the surface of the thylakoid membrane directed towards the outside.

Quantitative investigations on the maximal binding of antibodies showed that 1 g stroma-freed not swellable chloroplasts of *Antirrhinum majus* bind maximally 0.06 g antibodies to phosphatidyl choline and approximately four times the amount of antibodies to phosphatidyl inositol. Antibodies to the xanthophylls were bound in an amount of 0.04 to 0.09 g. The order of magnitude of these values fits the values of the maximal binding of antibodies to the glycolipids and also to proteins, which participate in photosynthetic electron transport. Onto the outer surface of the thylakoid membrane, accessible to antibodies, 1 g of antibodies can be maximally bound by 1 g chloroplasts.

By means of monospecific antisera we have succeeded in localizing the main components of the membrane lipids [1–5] and pigments [6–9] in the thylakoid membrane. From the agglutination reactions we were able to conclude that antigenic determinants of the galactolipids, the anionic lipids sulfoquinovosyl-diglyceride and phosphatidyl glycerol, of chlorophyll and the xanthophylls lutein and neoxanthin are situated in such a location of the outer thylakoid membrane surface that they are accessible to antibodies. The quantitative investigations on the maximal binding of antibodies to lipids and proteins lead to the estimate that the outer surface of the thylakoid membrane is composed to approximately 85% of proteins and only to 10–15% out of lipids [10, 11]. In order to gain some information on the function of lipids and pigments in the thylakoid membrane we have investigated the influence of these antisera on the photosynthetic electron transport in chloroplasts. It was shown that antibodies to chlorophyll [6, 12] and antibodies to the xanthophylls [8, 9, 13] inhibit partial reaction of electron transport in the region of light reaction II. Antibodies to the anionic lipid sulfoquinovosyl diglyceride, however, inhibit the photosynthetic reduction of anthraquinone-2-sulfonate with dichlorophenolindophenol/ascorbate as the electron donors [14].

In the following we report on the reactions of the antisera to phosphatidyl inositol and phosphatidyl

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choline as well as those to violaxanthin and zeaxanthin with chloroplasts and on the distribution of the phosphatides and the xanthophylls in the outer thylakoid membrane surface.

Methods

1. Isolation of the antigens

Phosphatidyl inositol was obtained from the seed lipids of *Glycine hispida*. As starting material served a phosphatidyl inositol fraction of the firm Nattermann, Cologne. This fraction contained as an impurity a trigalactolipid and a further unknown galactolipid. The trigalactolipid was removed by anion exchange chromatography on DEAE cellulose [15, 16]. The phosphatidyl inositol was subsequently isolated via thin layer chromatography on silica gel-H-layers in the solvent, chloroform, methanol, acetic acid, water (85, 15, 10, 3.5) and a subsequent rechromatography on silica gel. The purity of the phosphatidyl inositol was demonstrated by means of a two-dimensional thin layer chromatography on silica gel-H-layers. The solvent in the first direction consisted of chloroform, methanol, water (65, 25, 4) [17] and the solvent in the second direction was the above described acid solvent system.

Inositol and fatty acids were analyzed by gas chromatography with the earlier described methods [15, 18]. Inositol was identified as trimethylsilylether on chromosorb columns which were impregnated with SE 30 (Silicane Gum Rubber SE 30, methyl) as stationary phase [19]. The fatty acids were investigated as methyl esters also on chromosorb columns, which were impregnated with 10% succinyl ethyl ester.

All further lipids used for cross reactions such as monogalactolipid, di- and trigalactolipid as well as the anionic lipids sulfoquinovosyl diglyceride. Phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine were isolated from leaves of higher plants and algae according to earlier described procedures by means of a combined column and thin layer chromatography [4, 15, 18]. All obtained lipids were dissolved according to Folch in chloroform/methanol 2/1 (v/v) and washed several times with 0.4% saline [20]. The staining material used for the chromatographic detection were the reagents described in earlier papers [4]. Violaxanthin was isolated according to an earlier described method by means of column chromatography on cellulose [21] and aluminium oxide [9] and subsequently with the thin layer chromatography system described by Hager and co-workers [22].

2. Quantitative determination of the phosphatides

The quantitative determination of the phosphatides in Antirrhinum chloroplasts was carried out according to the thin layer chromatographic methods described by Debuch [23] and Koenig [24]. For the calculation of the molecular weights the average fatty acid composition of the phosphatides was taken, which resulted from gas chromatographic determination (Radunz, unpublished). The molecular weight was for phosphatidyl glycerol 740, phosphatidyl inositol 845 and for phosphatidyl choline 745 [24].

3. Preparation of the antisera

24 mg phosphatide and 6 mg methylated bovine serum albumin were emulgated in 3 ml 0.06 mol phosphate buffer, according to Sörensen, pH 7.8 and in 3 ml adjuvant (Freund's adjuvant, complete, Difco, Michigan, USA). The emulsion was subcutaneously injected into the back skin of 3 rabbits at 2 different spots. Six weeks later an emulsion of 12 mg phosphatidyl inositol and 3 mg methylated bovine serum albumin in 6 ml 0.06 mol phosphate buffer, pH 7.8 was intraveneously injected into the animals every other day. The emulsion was prepared as follows: 12 mg phosphatides were dissolved in 10 ml ethanol and subsequently precipitated by the addition of 10 ml water. Subsequently, ethanol and water were distilled off until the final volume was 1 ml. Thereafter, 3 ml of an aqueous 0.1% methylated bovine serum albumin solution was added and filled up to 6 ml with 2 ml of 0.36 mol phosphate buffer. This emulsion was vigorously shaken and sonicated four times for 30 sec.

The antisera to the xanthophylls violaxanthin and zeaxanthin were equally prepared by immunization of rabbits according to earlier described methods [8, 9, 21].

4. Detection of the antibodies

Antibodies to the phosphatides were characterized by means of the earlier described passive heme agglutination test [3]. Furthermore, the presence of phosphatide antibodies could be demonstrated by the precipitation reaction of a phosphatide chicken albumin emulsion. This emulsion was prepared as

described above. Instead of the methylated bovine serum albumin, we used oval albumin (Serva Preparation No. 11840) in order to avoid a cross reaction with the methylated bovine serum albumin. Antibodies to xanthophylls could not be demonstrated with these methods. The agglutination reactions of these antisera with different chloroplast preparations and their influence on photosynthetic electron transport were described in earlier publications [8, 9, 21].

5. Demonstration of the specific binding of antibodies onto the thylakoid membrane

The monovalent binding of phosphatide antibodies was determined by means of the direct antiglobulin consumption test [25 – 27]. To 10 mg stroma-freed chloroplasts suspended in 2 ml phosphate buffer 2 ml antiserum to phosphatides were added. After 12 hours the chloroplasts were washed 12 times with physiological saline and adjusted with 0.06 mol phosphate buffer to a final concentration of 1.5 mg chloroplasts per ml. In a dilution series antiserum against the rabbit-γ-globulines was diluted. The dilution factor was 1/1.5. To each tube of this dilution series the same amount of stroma-freed chloroplasts loaded with antibodies to phosphatides was added. After 12 hours the tubes were viewed. As a clear positive agglutination the tubes with the dilution were taken, in which the sedimentated chloroplasts showed inverted borders.

The amount of the monovalently bound phosphatide and xanthophyll antibodies was determined according to an earlier described method via a nitrogen determination [10, 11]. From the obtained nitrogen values, the protein content of the bound antibodies was obtained by multiplication with the factor of 6.

Results

1. Characterization of the phosphatide antisera

An antiserum to phosphatidyl inositol was obtained by immunization of rabbits with phosphatidyl inositol from the seed lipids of *Glycine hispida* *. The antiserum reacted in the passive heme agglutination test with erythrocytes, which were sensibilized with the homologous phosphatide with an agglutination titer of 1:16. Also phosphatidyl inositol of Antirrhinum chloroplasts and a phosphatidyl inositol of other plants from Serva (Preparation No. 32513) yielded agglutination. In investigations with the chloroplast glycolipids and phosphatides a cross reaction was only observed with phosphatidyl glycerol. An earlier described antiserum to phosphatidyl glycerol did not react with the phosphatidyl inositol [2]. In order to characterize the specificity of the obtained antiserum closer, exhaustion tests were carried out. This led to the observation that the antiserum could be neutralized with a 0.08 mol suspension of the homologous phosphatide. If to the phosphatidyl inositol antiserum phosphatidyl glycerol was added, it was shown, that with even double the concentration the activity of the antiserum was only diminished by 80%. Thus, always a remaining activity is observed. It should be mentioned that a 0.08 mol inositol solution led to a 30% exhaustion of the antiserum.

For the preparation of the antiserum to phosphatidyl choline a synthesized lecithin from Fluka (D,L,β-γ-di-palmitoyl-α-lecithin, Preparation No. 42557) was used. The antiserum reacted with the homologous lecithin only with a titer of 1:8. With the same activity it also showed a reaction with a synthesized Dioleoyl-L-α-lecithin (Preparation No. 27629 from Serva), as well as with phosphatidyl choline of *Antirrhinum majus*, of *Dryopteris felix* mas, of seeds of *Glycine hispida* and also with an egg lecithin. No cross reactions were observed with the phosphatidyl glycerol and phosphatidyl inositol of chloroplasts or with the glycolipids mono-, di-, trigalactolipid and sulfoquinovosyl diglyceride. From this observation it is concluded that the lecithin antiserum is monospecific.

2. Reactions of the phosphatide antisera with chloroplasts

The reaction of the phosphatidyl inositol and phosphatidyl choline antibodies with chloroplasts depends on the condition in which the thylakoid membrane is in. Chloroplasts isolated over a sucrose density gradient and washed with water are not swellable chloroplasts anymore and are not agglutinated. However, as shown by means of the antiglobulin test, antibodies are specifically adsorbed onto the thylakoid membrane [27, 28]. Only upon addition of antibodies to rabbit-γ-globulines stroma-freed chloroplasts loaded with antibodies to lecithin or phos-

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phatidyl inositol were agglutinated. Quantitative investigations on the maximal binding of antibodies have shown, that besides serum proteins also immunoglobulines are unspecifically absorbed onto the thylakoid membrane [10]. For the demonstration of the specific monovalent binding of antibodies the antiglobuline consumption test was carried out [25 – 27]. As the test summarized in Table I shows, those stroma-freed chloroplasts which were treated with antiserum to phosphatidyl choline and phosphatidyl inositol, are able to bind more antibodies to rabbit-γ-globuline than chloroplasts treated only with the corresponding control sera. This means, that from the phosphatide antisera besides unspecifically adsorbed serum proteins and gamma globulines also antibodies to lecithin as well as to phosphatidyl inositol are specifically bound onto the thylakoid membrane. From the results obtained with the antiglobuline consumption test it further concluded, that the antibodies to phosphatidyl choline and phosphatidyl inositol belong to the gamma globuline type IgG and not to the type IgM. Chloroplasts, which in parallel experiments were treated with control sera and the active sera, reacted with the antisera to rabbit-γ-globulines of the IgM type with the same dilution factor (Table I).

Stroma-freed swellable chloroplasts, which were isolated according to the methods of Homann and Schmid [29] in 0.02 mol Tris-buffer-sucrose solution were agglutinated by the antisera to the phosphatides with a titer of 1:32. With these chloroplasts, due to the swellability of the thylakoids the sterical hindrance of the antibodies to phosphatidyl inositol and phosphatidyl choline is relieved, which otherwise react only monovalently. The bivalent reaction leads to an agglutination of the chloroplasts.

Table I. Antiglobuline consumptions test-demonstration of monovalent binding of antibodies to phosphatides onto the thylakoid membrane.

Stroma-freed not swellable chloroplasts were treated with	Agglutination of the serum treated chloroplasts was observed until the indicated tube of the dilution series. The numbers in brackets are the titer value of the antiglobuline dilution offered for consumption	
	Anti-rabbit-γ-globuline	
	type IgG	type IgM
Antiserum to phosphatidyl inositol	4th tube (1:5)	3rd tube (1:3.4)
Control serum	6th tube (1:11)	3rd tube (1:3.4)
Antiserum to phosphatidyl choline	5th tube (1:7.6)	3rd tube (1:3.4)
Control serum	7th tube (1:17)	4th tube (1:5)

The inhibition of the antibody reaction to react bivalently can be relieved by ultrasonication of the stroma-freed not swellable chloroplasts. An ultrasonic sediment obtained by subsequent centrifugation, consists of small thylakoid stacks and disrupted thylakoids and is directly agglutinated by both phosphatide antisera. Ultrasonication exposes more antigen determinants of the phosphatides, because neutralization experiments have shown that the exhaustion of the phosphatide antisera requires approximately twice the amount of intact untreated chloroplasts than thylakoid fragments of the ultrasonic sediment. Furthermore, it was shown that an antiserum to phosphatidyl inositol exhausted with phosphatidyl glycerol agglutinates the ultrasonic sediment. The membrane fragments of the ultrasonic supernatant, which have a diameter of approximately 100 Å and which correspond to the thickness of the thylakoid membrane [30] are not precipitated neither by the antiserum to phosphatidyl inositol nor by that to phosphatidyl choline. They react apparently just like untreated stroma-freed chloroplasts. The here described antisera to phosphatidyl inositol and phosphatidyl choline react with stroma-freed chloroplasts and chloroplast fragments just as the earlier described antisera to the anionic lipids phosphatidyl glycerol [2] and sulfolipid [1] and the antiserum to chlorophyll a [6, 7].

Schmid and Lehmann-Kirk [31] investigated the influence of the phosphatide antisera on the photosynthetic electron transport in tobacco chloroplasts. They observed that only the phosphatidyl choline antiserum influences electron transport reactions. Orientating experiments showed, that the photoreduction of anthraquinone-2-sulfonate with water as the electron donor was stimulated by 55%.

3. Reactions of the xanthophyll antisera with chloroplasts

The antisera to violaxanthin and zeaxanthin were also obtained by immunization of rabbits [8, 9, 21]. The antigen violaxanthin was isolated according to earlier described methods from the lipids of *Urtica dioica* [21]. The zeaxanthin was synthesized by Hoffmann-La Roche (Basel). The UV-absorption spectra of the xanthophylls are described in previous papers [21].

The reactions of the obtained xanthophyll antisera with chloroplasts are, as shown for the above described phosphatide antisera, also dependent on the state of the thylakoid membrane. Stroma-freed chloroplasts which have swellable thylakoids, prepared according to Homann and Schmid [29] were directly agglutinated. The antiserum to violaxanthin inhibits photosynthetic electron transport in the region of light reaction II up to 30%. The antiserum to zeaxanthin shows the same influence on photosystem II. Only the inhibitory effect was smaller. From this we conclude that antigenic determinants of the violaxanthin and zeaxanthin are located in the outer surface of the thylakoid membrane.

With chloroplasts isolated by a sucrose density gradient and washed with water, which, as said before, have no swellable thylakoids, the antibodies to the xanthophylls were only monovalently bound. An agglutination was only obtained after the addition of anti rabbit-γ-globulines. The monovalent binding of the antibodies was demonstrated by means of the quantitative antiglobulin consumption test. It was shown that chloroplasts, which were treated with xanthophyll antisera were able to bind twice the amount of antibodies to rabbit-γ-globulines, than chloroplasts that were only treated with the corresponding control sera.

4. Maximal binding of antibodies to phosphatides and xanthophylls onto the thylakoid membrane

In order to obtain some information on the distribution of lipids and pigments in the outer membrane surface, the maximal binding of antibodies to the above described phosphatides and xanthophylls was determined. The amount of antibodies bound plotted as a function of added serum volume, yielded as in the case of the maximal binding of antibodies to the galactolipids [11] and to the proteins involved in electron transport [32] a curve with saturation region. The values found for the maximal binding of antibodies are summarized in Table II. In this table we have for comparison purpose included the earlier determined binding values for the antibodies to lutein, neoxanthin and to phosphatidyl glycerol [11]. As shown by these results, the amount of antibodies to phosphatidyl choline and to the xanthophylls is approximately the same and is 4 – 9% of the amount of antibodies which chloroplasts can maximally bind. Out of the antiserum to phosphatidyl inositol approximately twice the amount of antibodies was bound as out of the antiserum to phosphatidyl glycerol and four times the amount as out of the phosphatidyl choline antiserum. The high binding value for phosphatidyl inositol antibodies is apparently due to the cross reaction of the phosphatidyl inositol with phosphatidyl glycerol.

Table II. Maximal binding of antibodies to phosphatides and xanthophylls by stroma-freed chloroplasts of *Antirrhinum majus*.

Antiserum	Mode of reaction of the antibodies with stroma-freed chloroplasts	g Antibodies bound / g stroma-freed chloroplasts
Phosphatidyl choline	monovalent	0.06 ± 0.01
Phosphatidyl inositol	monovalent	0.21 ± 0.02
Phosphatidyl glycerol	monovalent	0.13 ± 0.01
Violaxanthin	monovalent	0.09 ± 0.02
Zeaxanthin	monovalent	0.04 ± 0.01
Lutein	monovalent	0.09 ± 0.02
Neoxanthin	monovalent	0.08 ± 0.02

In Table III the number of bound antibody molecules is compared with the antigen molecules present in the same amount of stroma-freed chloroplasts. This comparison showed that with the antigens which are present in low concentrations such as phosphatidyl choline, violaxanthin, zeaxanthin and neoxanthin every 4th to 7th antigen binds an antibody molecule. With the lipids present in higher amounts, such as lutein, phosphatidyl inositol and phosphatidyl glycerol only every 12th to 26th antigen molecule binds one antibody molecule. Whereas the number of antigen molecules differs by a factor of 16, the difference of the bound antibody molecules is only fourfold.

In Fig. 1 the number of antibody molecules is plotted against the number of phosphatide and xanthophyll molecules present in the thylakoid membrane. We have also included the values described in part II of this publication for the maximal binding of antibodies to the galactolipids, anionic lipids, sulfolipid and phosphatidyl glycerol as well as to sitosterol and plastoquinone [11]. It was shown, that the earlier found proportionality between the number of bound antibodies and the number of antigen molecules present which exists for antigens which occur in low concentrations, is also valid for phosphatides and xanthophylls.

Table III. Comparison of the number of antigen molecules present in the thylakoid membrane with the number of bound antibody molecules.

Antiserum	Number of antigen molecules / g stroma-freed chloroplasts	Number of antibody molecules bound / g stroma-freed chloroplasts
Phosphatidyl choline	10×10^{17}	2×10^{17}
Phosphatidyl inositol	160×10^{17}	8×10^{17}
Phosphatidyl glycerol	140×10^{17}	5×10^{17}
Violaxanthin	29×10^{17}	4×10^{17}
Zeaxanthin	29×10^{17}	2×10^{17}
Lutein	94×10^{17}	4×10^{17}
Neoxanthin	30×10^{17}	3×10^{17}

The values refer to 1 g stroma-freed chloroplasts of *Antirrhinum majus*. The number of xanthophyll molecules present in Antirrhinum chloroplasts was calculated from the concentration values given by Koenig [24]. The amount of zeaxanthin was assumed to be the same as that of violaxanthin as, according to Hager and co-workers [33] a reversible process zeaxanthin $\rightleftarrows$ antheraxanthin $\rightleftarrows$ violaxanthin takes place in the thylakoid membrane. The given concentration of the p. inositol is composed of the obtained value for p.glycerol and p.inositol, as the p.inositol antiserum cross reacts with p.glycerol.

The values summarized in Tables II and III were obtained, if antibodies were adsorbed onto the thylakoid membrane out of monospecific antisera. If, however, antibodies were adsorbed out of mixed antisera, which contained antibodies to the four described xanthophylls in equal amounts, then the number of the maximally bound antibody molecules corresponded to the amount which was already bounded out of a monospecific antiserum to one of these xanthophylls. The adsorption of phosphatide antibodies out of mixed phosphatide antisera yielded the same results as with the mixed xanthophyll antisera. As on the other hand in the thylakoid membrane surface of 1 g stroma-freed chloroplasts maximally 42×10^{17} antibody molecules can be accomodated, the low values for the binding of antibodies to xanthophylls and phosphatides lead to the conclusion that the antigenic determinants of the three phosphatides as well as those to the xanthophylls are not randomly distributed in the thylakoid membrane surface but arranged in domaines. These domaines can have only a small diameter in which already the binding of a single antibody molecule to one antigen covers several homologous and non-homologous antigens. From the obtained values we know now that the antibodies to phosphatides can cover 20% and the antibodies to xanthophylls 10% of the thylakoid membrane surface which is accessible to antibodies.

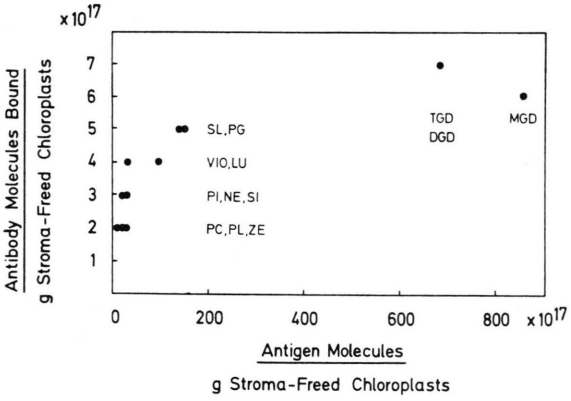

Fig. 1. Dependence of the number of bound antibody molecules on the number of lipid and xanthophyll molecules present in the thylakoid membranes of 1 g stroma-freed chloroplasts of *Antirrhinum majus*. MGD, monogalactosyl glyceride; TGD and DGD, tri- and digalactosyl diglyceride; PI, phosphatidyl inositol; PC, phosphatidyl choline; PL, plastoquinone; SI, sitosterol; LU, lutein; Vio, violaxanthin; NE, neoxanthin; ZE, zeaxanthin; PG, phosphatidyl glycerol; SL, sulfoquinovosyl diglyceride.

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