Binding of Antibodies onto the Thylakoid Membrane. V. Distribution of Proteins and Lipids in the Thylakoid Membrane

Alfons Radunz

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut), Abt. Menke, D-5000 Köln-Vogelsang

Z. Naturforsch. **34 c,** 1199 – 1204 (1979); received July 6, 1979

Chloroplasts, Thylakoid Membrane Surface, Proteins, Lipids, Antibodies

Binding of antibodies to proteins and lipids onto fragments of the thylakoid membrane was studied and compared with the binding of antibodies by stroma-freed chloroplasts. The membrane fragments were prepared from stroma-free chloroplasts by ultrasonication and fractional centrifugation. The fragments have an average diameter of 200 Å. Their thickness corresponds to that of the thylakoid membrane. The membrane fragments adsorb out of an antiserum to lipids approximately the same amount of antibodies as out of an antiserum to proteins. In comparison to this, stroma-free chloroplasts bind 4 times more antibodies to proteins than to lipids. From this it follows that the major part of the lipids is located in the membrane surface which is directed towards the inside or is located inside the membrane. As the chemical analysis has shown these results are not caused by an altered chemical composition of the membrane fragments.

Despite the fact that membrane proteins bind considerably less protein antibodies than stroma-free chloroplasts, the antibody binding in membrane fragment might be considerably increased for certain proteins such as a polypeptide with an apparent molecular weight 24000 and cytochrome f. Antibodies to the major components of the lipid mixture, such as to monogalactosyl diglyceride, trigalactosyl diglyceride, sulfoquinovosyl diglyceride and phosphatidyl glycerol are 3 to 4 times more bound by membrane fragments than by stroma-free chloroplasts. From these results it is concluded that the thylakoid membrane surface directed towards the inside is preponderently composed of lipids whereas the surface directed towards the outside consists only by 10 to 15% of lipids.

In earlier investigations it was found, that stroma-free chloroplasts are able to bind onto the outer surface 4 times more antibodies to proteins than antibodies to lipids [1 – 4]. From this it can be estimated that the outer surface of the thylakoid membrane consists of 85 – 90% proteins and 10 – 15% lipids. For this estimation we had to consider that bound antibodies also overlap not homologous antigens. As the thylakoid membrane is composed of equal amounts of lipids and proteins [5] these serological studies lead to the result that the major amount of the lipids is localized in the surface directed towards the inside of the membrane. Adsorption studies had further shown that EDTA washed stroma-free chloroplasts are able to bind twice the amount of antibodies to monogalactolipid than the untreated chloroplasts [6]. This means that removal of the coupling factor of photophosphorylation exposes lipid antigens.

Our investigations up to-now permit only conclusions concerning the outer surface of the thylakoid membrane. In order to be able to obtain some information on the molecular architecture of the thylakoid membrane surface directed towards the inside, the present publication deals with antibody binding onto stroma-free chloroplasts after their fragmentation by ultrasonic treatment. In order to obtain a uniform starting material only the small membrane fragments of the ultrasonic supernatant were used [7].

Reprint requests to Dr. Alfons Radunz.

0341-0382/79/1200-1199 $ 01.00/0

Methods

1. Preparation of the antibodies

The antisera to the lipids listed in the tables [8 – 11], the proteins of photosynthetic electron transport [7, 3, 12, 13], the polypeptide with the apparent molecular weight 24000 [1, 13] and stroma-free chloroplasts [8] were obtained according to earlier described methods by immunisation of rabbits. From the antisera the immune globulines were separated by chromatography on diethylamino-cellulose (DEAE-anion exchanger from Whatman) according to J. L. Fahey [14]. The antibody fractions were subsequently adjusted to the starting volume of the antisera by diafiltration over membrane filters (Amicon XM 50) in physiological NaCl-solution.

2. Preparation of membrane fragments of the thylakoid membrane (ultrasonic supernatant)

Stroma-free chloroplasts of Antirrhinum were obtained according to Kreutz and Menke [15]. For further purification the chloroplasts were centrifuged twice over a sucrose density gradient and washed subsequently 6 times with water. The stroma-free chloroplasts were sonicated according to van Wyk *et al.* [7] 8 times 30 sec and subsequently centrifuged for 2 h at 33000 × *g*. The supernatant of this centrifugation contains fragments of the thylakoid membrane. With these membrane fragments quantitative precipitation reactions were carried out.

3. Quantitative determination of the lipids

The lipid contents in stroma-free chloroplasts of *Antirrhinum majus* was taken from work by Koenig [5]. The amount of galactolipids in the membrane fragments was determined according to Heinz [16] and according to methods described in earlier publications [11, 17]. The determination of the phosphatides was carried out according to Debuch and coworkers [18] and Koenig [5]. Carotenoids were determined according to Hager and Meyer-Berthenrath [19] as well according to Hager and Stransky [20].

4. Quantitative determination of the adsorbed antibodies onto membrane fragments

According to the quantitative precipitation reactions described by Heidelberger and Kendall [22, 21] increasing amounts of membrane fragments were added to a constant volume of antiserum. The concentration of the membrane fragments varied between 4 to 40 µg per assay. The reaction volume was filled up to 2 ml with 0.8% NaCl and was shaken at room temperature for 6 h and kept for 16 h at 5 °C. Thereafter the sediments were centrifuged and washed 5 times with 0.8% NaCl and the nitrogen content determined with Neßler's reagent as described in part I [1]. The amount of protein was determined by multiplication of the nitrogen content with the factor 6. As small sediments were also obtained with the control serum, the protein content of the control experiments was substracted from the protein content of the precipitates.

The point of equivalence of the antisera listed in Table II was with the following amounts of sera: Antiserum to stroma-free chloroplasts (0.02 ml), to total proteins (0.1 ml), to coupling factor (0.1 ml), to cytochrome f (0.2 ml), to polypeptide 24000 (0.1 ml), to monogalactosyl diglyceride (0.2 ml), to trigalactosyl diglyceride (0.1 ml), to sulfoquinovosyl diglyceride (0.35 ml) and to phosphatidyl glycerol (0.3 ml).

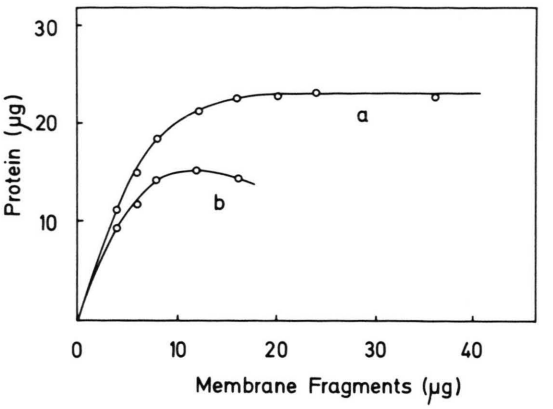

Fig. 1. Quantitative precipitation reaction between an antiserum to stroma-free chloroplasts (0.02 ml) and fragments of the thylakoid membrane of *Antirrhinum majus* chloroplasts (4 µg to 36 µg). Curve a, protein amount of the precipitate, plotted as a function of the added membrane fragments. Curve b, protein amount of bound antibodies, plotted as function of the added membrane fragments.

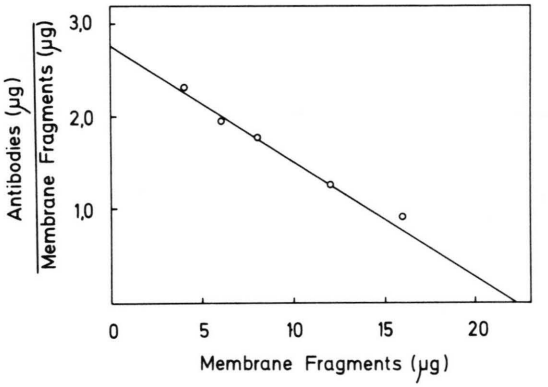

Fig. 2. Ratio of the amount of bound antibodies out of an antiserum to stroma-free chloroplasts and the amount of precipitated membrane fragments as function of the added membrane fragments.

If one plots the protein amount of the precipitate against the amount of antigen added, a curve is obtained which increases in the region of excess antibodies but which does not decrease again behind the zone of equivalence (Fig. 1 a). If one subtracts of the amount of protein in the precipitate the known amount of protein of the added membrane fragments and if one plots the obtained values against

the amount of antigen, a curve is obtained which as shown by Heidelberger and Kendall [21, 22] exhibits a maximum (Fig. 1 b). The maximum of the curve is identical with the region of equivalence. As in the equivalence region all membrane fragments and all present antibodies are precipitated, the maximum of curve b (Fig. 1 b) permits the determination of the amount of bound antibodies to the lipids and proteins listed in Table II.

Furthermore, the point of equivalence was calculated according to the equation of Heidelberger and Kendall. For this purpose the quotient of the amount of antibodies and antigens in the respective precipitate was plotted as a function of the added antigen (Fig. 2). In agreement with Heidelberger and Kendall [21, 22] and Kabat [23] a straight line is obtained. The calculated amount of the precipitated antibodies is identical within ±3% with the value obtained from the point of equivalence in Fig. 1 b. The values in the plot (Fig. 1a and b) and those used for the calculation are averages of 12 individual determinations.

Results

Characterization of the fragments of the thylakoid membrane

If antibody binding onto membrane fragments is to be compared with the binding onto the outer surface of stroma-free chloroplasts, the prerequisite is an identical chemical composition of fragments and chloroplasts. Electron micrographs show that the fragments which have been obtained by ultrasonication and subsequent fractional centrifugation, exhibit an average diameter of approximately 200 Å [7]. Their thickness corresponds to that of the thylakoid membrane. As with stroma-free chloroplasts they contain approximately 50% proteins [24]. The lipids, however, are lower by 10% when compared to chloroplasts (Table I). With the exception of chlorophyll a, phosphatidyl inositol and trigalactosyl diglyceride all other lipids and pigments are present in lower amounts in the membrane fragments. Moreover, the ratio chlorophyll a/b and the ratio of chlorophyll to xanthophylls is bigger than in the chloroplasts. And differences exist also in the polypeptide composition as earlier studies have shown [24]. In the membrane fragments polypeptides with higher molecular weights prevail.

Table I. Chemical composition of stroma-free chloroplasts and fragments of the thylakoid membrane in per cent dry weight.

	Stroma-free chloroplasts	Fragments of the thylakoid membrane
Proteins	50.0 ±1	51.0 ±1
Lipids	43.4 ±0.8	37.9 ±0.9
Monogalactosyl diglyceride	11.0 ±0.3	8.3 ±0.3
Digalactosyl diglyceride	10.1 ±0.3	6.9 ±0.3
Trigalactosyl diglyceride	0.9 ±0.02	1.1 ±0.1
Sulfoquinovosyl diglyceride	2.0 ±0.2	1.5 ±0.1
Phosphatidyl glycerol	1.85 ±0.06	1.6 ±0.06
Phosphatidyl cholin	0.13 ±0.01	0.11 ±0.07
Phosphatidyl inositol	0.14 ±0.04	0.28 ±0.01
Chlorophyll a	9.3 ±0.4	10.2 ±0.4
Chlorophyll b	3.3 ±0.4	3.0 ±0.4
Lutein	0.88 ±0.03	0.45 ±0.01
Neoxanthin	0.30 ±0.01	0.24 ±0.01
Violaxanthin	0.29 ±0.01	0.22 ±0.01
β-Carotene	0.62 ±0.02	0.38 ±0.02

Adsorption of antibodies onto membrane fragments

The amount of antibodies to lipids and proteins bound onto membrane fragments, was determined according to the methods of Heidelberger and coworkers [21 – 23] and can be depicted from Table II. The values are referred to 1 g antigen. In this table the earlier found values for stroma-free chloroplasts were also incorporated [2, 4]. It is clearly seen that the membrane fragments bind approximately the same amount of antibodies out of an antiserum to lipids or out of an antiserum to protein. These comprise only 60% of the amount of antibodies which are bound out of an antiserum to stroma-free chloroplasts. The antiserum to the lipids is a mixture of antisera to the major components of the lipid mixture of the thylakoid membrane listed in Table I with the exception of chlorophyll. The protein antiserum was obtained by immunization with a protein preparation of the thylakoid membrane, the preparation of which was described earlier [13]. The antiserum to stroma-free chloroplasts contains antibodies to lipids as well as to proteins of the thylakoid membrane [8, 3, 11]. Concerning the monospecific antisera it was found that antibodies to the glycolipids and phosphatidyl glycerol are bound in an amount which corresponds to 40 to 50% of the amount of antibodies which membrane fragments can totally bind. As to the antibodies to coupling factor, cytochrome f and to the polypeptide with the appa-

Table II. Maximal binding of antibodies to lipids and proteins onto fragments of the thylakoid membrane and onto stroma-free chloroplasts of *Antirrhinum majus*.

Antiserum to	Membrane fragments g antibodies / g antigen	Stroma-free chloroplasts g antibodies / g antigen
Stroma-free chloroplasts	1.27	1.05
Total proteins	0.78	1.02
Total lipids	0.75	0.24
Polypeptide 24 000	0.36	0.10
Coupling factor	0.46	0.40
Cytochrome f	0.24	0.05
Monogalactosyl diglyceride	0.58	0.16
Tri- and digalactosyl diglyceride	0.49	0.17
Sulfoquinovosyl diglyceride	0.58	0.12
Phosphatidyl glycerol	0.60	0.13

rent molecular weight 24000, the antibody binding is 19 – 36 per cent of the maximal binding. The polypeptide 24000 has been described earlier and has the amino terminal sequence Ala-Ala-Gly-Lys-Pro-Thr-Asp [3, 25]. The antiserum to this polypeptide does not influence photosynthetic electron transport [25].

A direct comparison of the antibody binding by membrane fragments with that by chloroplasts is not possible since the determination of the maximal binding was done following differing methods. With the membrane fragments the amount of antibodies bound was determined according to the quantitative precipitation method in the region of equivalence and with chloroplasts the determination was made in the region of antibody excess. Whereas in the region of excess the antibodies react only in a monovalent manner with the chloroplasts, membrane fragments on the other hand react in the region of equivalence preponderantly in a bivalent manner. With stroma-free chloroplasts the entire surface is covered with antibodies, which should not be the case with the precipitate. Therefore, the amount of antibodies, which is bound by the membrane fragments out of an antiserum to chloroplasts is only slightly higher than with stroma-free chloroplasts, despite the fact that the fragment surface referred to a given amount is several fold higher than the surface of stroma-free chloroplasts which is accessible to antibodies (Table II). However, the values for stroma-free chloroplasts and membrane fragments become comparable if one normalizes the values to the amounts of antibodies, which are maximally bound out of an antiserum to chloroplasts. In doing so, it is assumed, that in the precipitate proportionality exists between the number of the different antigenic determinants present and the number of antibodies bound.

The relative values obtained are summarized in Table III. Roughly the same amounts of antibodies were bound out of a lipid antiserum, which contained antibodies to the major lipid components and out of a protein antiserum which contained only antibodies to membrane proteins. This means that with membrane fragments the lipid and protein areas accessible to antibodies have approximately the same dimensions. In contrast, the outer surface of stroma-free chloroplasts binds roughly 4 times more protein antibodies than antibodies to lipids [2]. This means that by ultrasonication the surface composed of lipids was increased considerably in comparison to the surface composed of proteins and this despite the fact that membrane fragments contain 10% less lipids than the chloroplasts (Table I).

It should be mentioned again that the quantitative serological studies with stroma-free chloroplasts have shown that only 10 – 15% of the thylakoid membrane surface is composed out of lipids [2]. If one considers that the thylakoid membrane is composed of equal parts per volume of proteins and li-

Table III. Antibody binding onto membrane fragments and stroma-free chloroplasts.

Antiserum to	Membrane fragments	Stroma-free chloroplasts	Factor
Stroma-free chloroplasts	1.00	1.00	1.0
Total proteins	0.61	0.97	0.6
Total lipids	0.59	0.23	2.6
Polypeptide 24 000	0.28	0.10	2.8
Coupling factor	0.36	0.38	0.9
Cytochrome f	0.19	0.05	3.8
Monogalactosyl diglyceride	0.46	0.15	3.1
Tri- and digalactosyl diglyceride	0.39	0.16	2.4
Sulfoquinovosyl diglyceride	0.46	0.11	4.2
Phosphatidyl glycerol	0.47	0.12	3.9

The values are normalized to the amount of antibodies which are maximally bound by the preparations out of a serum to stroma-free chloroplasts.

pids, and if one considers further the thickness of the thylakoid membrane and the size of the molecules one is led to think that the surface directed towards the inside is composed of 85 to 90% of lipids. The results of the investigation do not contradict this conclusion, because when compared with stroma-free chloroplasts the amount of the maximally bound protein antibodies to membrane fragments decreases considerably, whereas the relative amount of the maximally bound lipid antibodies increases. Quantitative data concerning the molecular composition of the inner membrane side cannot be given since with these membrane fragments not only the inner membrane surface becomes accessible, but also considerable parts of the inner membrane. In addition it must be borne in mind that during fragmentation a rearrangement of proteins and lipid molecules might have occured. Despite these objections the results speak in favor of the notion that the inner surface of the thylakoid membrane is preponderantly built up of lipids. In a completely independent way experiments using small angle X-ray scattering with stroma-free chloroplast have led to the same result [26 – 29].

Concerning the occurence of lipids inside the thylakoid membrane the following should be pointed out: if a bimolecular lipid film is involved in the composition of the membrane and if the outer surface consists of only 10% lipids, it follows logically that a considerable portion of the lipid molecules of the thylakoid are inaccessible to antibodies both from the outside and from the inside. Only after the removal of coupling factor a portion of these lipid molecules can be reached by antibodies. As earlier studies have shown, 1 g stroma-free chloroplasts bind 0.16 g antibodies to monogalactolipid [1]. After washing the chloroplast with 0.7 mM EDTA the binding of monogalactolipid antibodies is 0.36 g per g chloroplasts [3, 6].

Concerning the results with monospecific antisera, it should be noted, that despite the fact that fragmentation lowers considerably the binding of antibodies to proteins the binding of antibodies to cytochrome f increases four fold (Table III). From this it must be concluded that cytochrome f is either enriched in the membrane fragments or that the major part of the cytochrome f molecule is located inside the thylakoid membrane. It should be noted that this antiserum inhibits photosynthetic electron transport by maximally 50% [12]. The same is valid for the binding of antibodies to the polypeptide 24 000 which is increased 2.8 fold. The barely appreciable decrease of the binding of antibodies to coupling factor may have different reasons and shall be discussed if the necessary data are available. For the major components of the membrane lipids listed in Table III which participate with 23 per cent in the thylakoid membrane composition it can be stated that the maximally bound amount of antibodies is increased between 2.4 to 4.2 fold by ultrasonication.

Finally we should like to mention, as found with serological methods, that all proteins involved in photosynthetic electron transport with only one exception, are detectable in the outer surface of the thylakoid membrane. Antibodies to these polypeptides inhibit photosynthetic electron transport to different degrees [30 – 37]. Thus, different experimental results lead to the concept that proteins are located preponderantly in the outer thylakoid membrane surface, whereas the major part of the lipid molecules are located at the inner membrane surface or inside the membrane.

Acknowledgements

The author thanks Prof. W. Menke for discussions and Prof. Uhlenbruck, Abt. Immunbiologie of the Medical Clinic of the University of Cologne for helpful suggestions. He also thanks Prof. G. H. Schmid, C. E. N. de Cadarache, Service de Radioagronomie, Saint-Paul-Lez-Durance for critical reading of the manuscript.

[1] A. Radunz, Z. Naturforsch. **30 c,** 484 (1975).
[2] A. Radunz, Z. Naturforsch. **32 c,** 597 (1977).
[3] A. Radunz, Z. Naturforsch. **33 c,** 731 (1978).
[4] A. Radunz. Z. Naturforsch. **33 c,** 941 (1978).
[5] F. Koenig. Z. Naturforsch. **26 b,** 1180 (1971).
[6] A. Radunz, unpublished.
[7] G. Kannangara, D. van Wyk, and W. Menke, Z. Naturforsch. **25 b,** 613 (1970).
[8] A. Radunz and R. Berzborn, Z. Naturforsch. **25 b,** 412 (1970).
[9] A. Radunz. Z. Naturforsch. **26 b,** 916 (1971).
[10] A. Radunz, Z. Naturforsch. **27 b,** 821 (1972).
[11] A. Radunz, Z. Naturforsch. **31 c,** 589 (1976).
[12] G. H. Schmid, A. Radunz, and W. Menke, Z. Naturforsch. **32 c,** 271 (1977).
[13] W. Menke and F. Koenig, Methods in Enzymology, Part C, in preparation.
[14] J. L. Fahey, Methods in Immunobiology and Immunochemistry **I,** (C. A. Williams and J. W. Chase, eds.), p. 321–332, Academic Press, New York and London 1967.

[15] W. Kreutz and W. Menke, Z. Naturforsch. **15 b,** 402 (1960).
[16] E. Heinz, Biochim. Biophys. Acta **144,** 333 (1967).
[17] A. Radunz, Hoppe-Seyler's Z. Physiol. Chemie **350,** 411 (1969).
[18] H. Debuch, W. Mertens, and M. Winterfeld, Hoppe-Seyler's Z. Physiol. Chemie **319,** 898 (1968).
[19] A. Hager and T. Meyer-Berthenrath, Planta **69,** 198 (1966).
[20] A. Hager and H. Stransky, Arch. Mikrobiol. **71,** 132 (1970).
[21] M. Heidelberger and F. E. Kendall, J. Exp. Med. **62,** 697 (1935).
[22] M. Heidelberger, Bacteriol. Rev. **3,** 49 (1949).
[23] E. H. Kabat, Kabat and Mayer's Experimental Immunochemistry, 2nd ed. Springfield (Ill.), Charles C. Thomas Publ. 1961.
[24] A. Radunz, G. H. Schmid, and W. Menke, Z. Naturforsch. **26 b,** 435 (1971).
[25] G. H. Schmid, W. Menke, A. Radunz, and F. Koenig, Z. Naturforsch. **33 c,** 723 (1978).
[26] W. Kreutz and W. Menke, Z. Naturforsch. **15 b,** 483 (1960).
[27] W. Kreutz and W. Menke, Z. Naturforsch. **17 b,** 675 (1962).
[28] W. Menke, Brookhaven Symp. Biol. **19,** 328 (1966).
[29] W. Kreutz, Advances Bot. Res. **3,** 54 (1970).
[30] G. H. Schmid and A. Radunz, Z. Naturforsch. **29 c,** 348 (1974).
[31] G. H. Schmid, A. Radunz, and W. Menke, Z. Naturforsch. **30 c,** 201 (1975).
[32] F. Koenig, A. Radunz, G. H. Schmid, and W. Menke, Z. Naturforsch. **33 c,** 529 (1978).
[33] W. Menke, F. Koenig, A. Radunz, and G. H. Schmid, FEBS Lett. **49,** 372 (1974).
[34] F. Koenig, G. H. Schmid, A. Radunz, B. Pineau, and W. Menke, FEBS Lett. **62,** 342 (1975).
[35] G. H. Schmid, W. Menke, F. Koenig, and A. Radunz, Z. Naturforsch. **31 c,** 304 (1976).
[36] F. Koenig, W. Menke, A. Radunz, and G. H. Schmid, Z. Naturforsch. **32 c,** 817 (1977).
[37] W. Menke, F. Koenig, G. H. Schmid, and A. Radunz, Z. Naturforsch. **33 c,** 280 (1978).