STUDY OF THE SUPRAMOLECULAR ORGANIZATION OF LIGHT-HARVESTING CHLOROPHYLL PROTEIN (LHCP)

Conversion of the oligomeric form into the monomeric one by phospholipase A_2 and reconstitution with liposomes

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Received 30 November 1981

1. Introduction

The photosynthetic membranes are composed mainly of lipids and proteins arranged in lipo–protein complexes. Among these complexes, the light-harvesting chlorophyll protein (LHCP) represents 40–50% of the total chlorophyll found in the membrane. Consequently, it is supposed to play a fundamental role in the structure of thylakoids, e.g., in grana stacking (reviews [1–4]). Due to its heterogeneous composition including pigments, lipids and polypeptides, it cannot be established with certainty which of its constituents plays the key role in the stacking process.

However, recently, the stacking phenomenon has been studied using a membrane model system constituted of isolated LHCP incorporated into lipid vesicles [5]. It has been shown that trypsinated LHCP, having lost a 2000 kd polypeptide, can no longer induce a cation-mediated aggregation of LHCP vesicles and it was deduced that this protein may be responsible for stacking. This view has also been supported by [6] using lipids extracted from leaves for the preparation of vesicles. In these experiments, lipids were only considered as a passive support. In [7] a more functional role of lipids was proposed and it was suggested that specific arrangements of components necessary for the functioning of the light reactions of photosynthesis can be regulated to a large extent by the lipid content of the membrane [7].

The lipid composition of the main chlorophyll protein complexes (CP_1 and LHCP) has been investigated by different groups [6,8,9]. Although different methods of purification were used, there is an agreement that LHCP is specifically enriched in PG containing *trans*-hexadecenoic acid. We have reported that an oligomeric form of LHCP ($LHCP_1$ according to the nomenclature in [9]) purified by electrophoresis contains a 3–4-times higher amount of PG than all other complexes and the monomeric form ($LHCP_3$). This oligomeric form first described in [10] and then purified and studied by us seems more representative of the in vivo LHCP and has been characterized as a dimer of LHCP [11]. Here, we demonstrate the role of lipids and especially that of PG in the structural organisation of the oligomeric form of tobacco LHCP:

(i) By its disappearance after a treatment of the thylakoids with phospholipase A_2;
(ii) By its reconstitution from the monomer using liposomes obtained from different tobacco leaf lipids.

Abbreviations: chl, chlorophyll; CP_1, P700 chl *a*–protein; C16:1-*trans*, 3-*trans*-hexadecenoic acid; C16:0, palmitate; C18:0, stearate; C18:2, linoleate; C18:3, α-linolenate; DGDG, digalactosyldiacylglycerol; GLC, gas–liquid chromatography; $LHCP_1$, oligomeric form of light-harvesting chl *a*/*b* protein; $LHCP_3$, monomeric form of light-harvesting chl *a*/*b* protein; MGDG, monogalactosyldiacylglycerol; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PG, phosphatidyldiacylglycerol; SDS, sodium dodecyl sulfate; SL, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography

2. Materials and methods

2.1. *Isolation of chl–protein complexes*

These were isolated from tobacco leaves, *Nicotiana*

Published by Elsevier Biomedical Press
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tabacum L. cv. *Xanthi*, by preparative PAGE as in [9,11]. Extensive dialysis by ultrafiltration under N_2 pressure in an Amicon cell fitted with a Diaflo membrane UM-10 was done to remove, as much as possible, the SDS from the chl–protein complexes.

2.2. *Lipid analysis*

Lipids were extracted from the different fractions by chloroform:methanol as in [12]. Lipid classes were analyzed by TLC as in [13].

2.3. *Lipase treatment*

Phospholipase A_2 from *Vipera russelli* (1.06 Sigma unit) was added to a suspension of thylakoids containing ~400 μg chl in 1 ml as in [14] except that 50 mM Tris–HCl buffer (pH 8) was used.

The reaction mixture was incubated at 22°C in the dark for 2 h. Then, the suspension was washed 4 times with the same buffer. Between each washing, thylakoids were sedimented by centrifugation for 2 min in an Eppendorf microcentrifuge 5412. A control sample without lipase was treated in the same way.

2.4. *Preparation of liposomes*

This was done from lipids of tobacco leaves prepurified by TLC as in [13]. The purity of lipids was checked by TLC and their fatty acid composition analyzed by GLC. Diacyl lipids in chloroform were evaporated to dryness under N_2, then resuspended at 3 mg/ml in 50 mM Tris–HCl (pH 8). The suspension was cooled on ice and sonicated for 20 min under N_2.

2.5. *Reconstitution of the oligomeric form of LHCP*

This was simply realized by mixing the monomeric form of $LHCP_3$ with liposomes. The reaction mixture giving the best results was composed of 150 μg lipids (PG, PC, DGDG or MGDG), $LHCP_3$ corresponding to 45 μg chl and 180 μg protein suspended in a total volume of 100 μl 50 mM Tris–HCl buffer (pH 8). After 1 h of vigorous shaking at room temperature in sealed Eppendorf centrifuge tubes, the resulting mixture was directly analyzed by SDS–PAGE.

2.6. *SDS–PAGE analysis of chl–protein complexes*

This was done in cylinder tubes containing a 10% acrylamide gel in Tris–borate buffer (pH 8) as in [11].

For the electrophoresis of chl–protein complexes after lipase treatment, thylakoids were SDS-solubilized at 21°C with a ratio of SDS/chl of 8 and electrophoresed immediately after the addition of SDS. For the electrophoresis of LHCP–liposomes, no previous solubilization was done; proteoliposomes were directly loaded on the gels because the SDS content (10%, w/v) of the electrophoresis buffer contained in the upper tank of the apparatus was sufficient to allow complete penetration of proteoliposomes in the gel.

3. Results

3.1. *Lipids distribution in the gel after preparative separation of chl–protein complexes*

Since [9], the question of a possible contamination of the isolated complexes by diffuse lipids in the gel has remained unanswered.

Fig.1 shows a classical separation of chlorophyll protein complexes on a preparative gel (5 cm long × 1 cm diam.) and the different slices numbered from 1–5 from which chl–protein complexes and lipids were extracted.

The main lipid class distribution in the different fractions of the gel is reported in fig.2. It is clear that

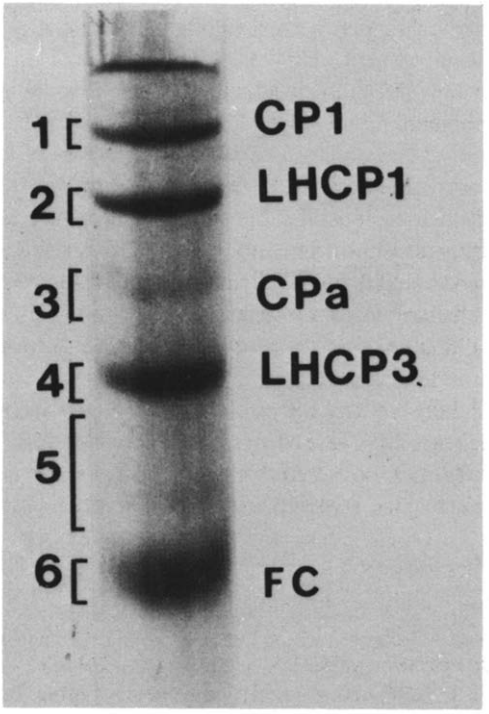

Fig.1. Preparative PAGE showing the different chl–protein complexes separated from tobacco thylakoids. Numbers in the left indicate the different fractions in which lipids were analyzed.

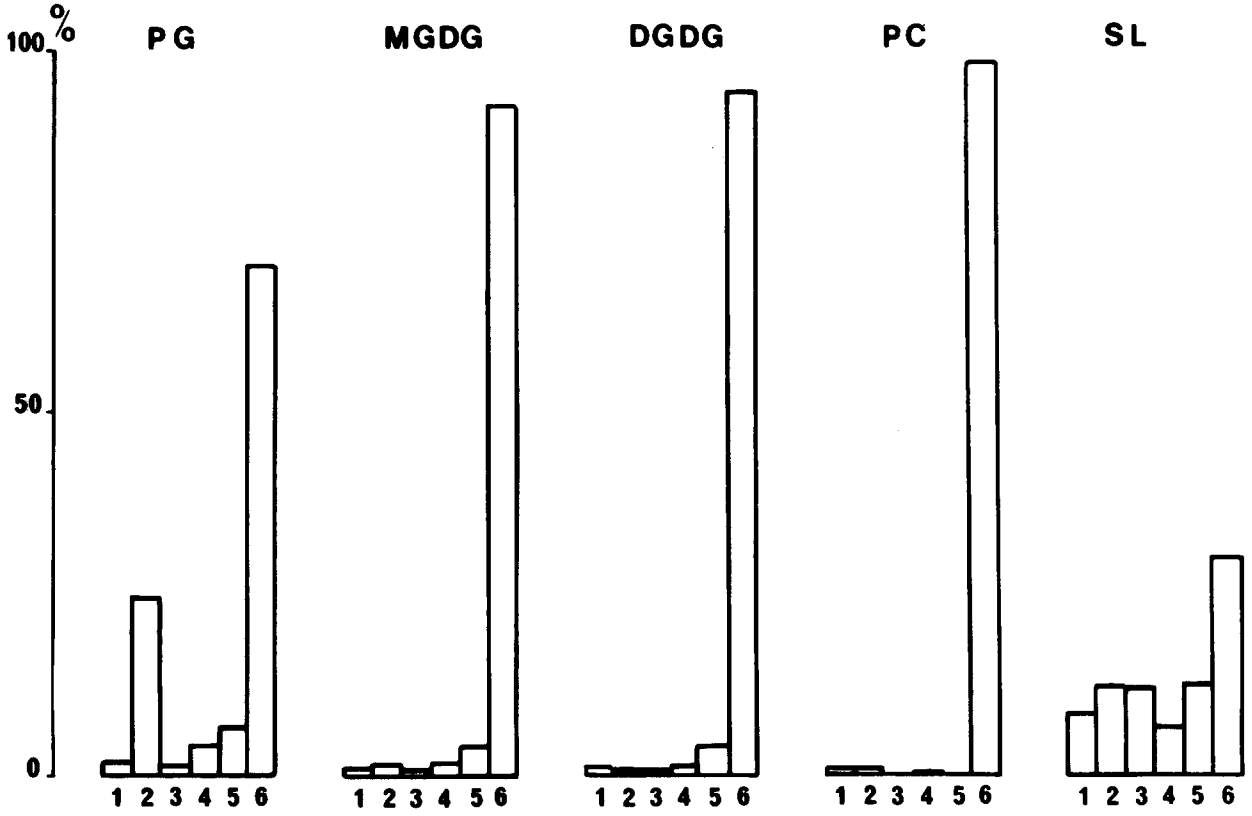

Fig.2. Repartition of lipidic classes along a preparative gel. Each lipid class (PG, MGDG, DGDG, PC and SL) is expressed in % of its total amount in the gel. Numbers on the abscissa correspond to different parts of a gel, as represented in fig.1.

most of the lipids migrate in front of the gel with the free chlorophyll. The lipid content in fraction 6 is so high that it is difficult to decide as to whether the lipids found in $LHCP_3$ (fraction 4) are really associated with this complex or result from a contamination by training lipids as those found in fraction 5 which is devoid of chlorophyll.

However, among the different chl-protein complexes, only $LHCP_1$ (fraction 2) retains a significantly higher content of lipids (especially in PG) than the lipid background in the gel. In table 1 is reported the lipid content found in each fraction expressed per mg of total chl loaded on the gel. From this table, we can estimate that ~15% of the total PG found in the gel remains associated with $LHCP_1$ in fraction 2. With, respectively, 0.5% and 1.7% of their total amount in the gel, DGDG and MGDG are far less retained in this complex. PC is found as traces along the gel and migrates almost completely with the free chlorophyll, while the sulfolipids seem to be more equally distributed in the different fractions of the gel.

3.2. *Effect of phospholipase* A_2 *treatment*

Fig.3 shows that lipase treatment of the thylakoids decreases largely the amount of $LHCP_1$, with a concomitant increase in $LHCP_3$ and free chlorophyll. This experiment demonstrates that lipase releasing

Table 1
Lipid composition of different fractions of a preparative gel. Between brackets are reported the C16:1-*trans*-hexadecenoic acid content of PG

Gel fractions	PG	MGDG	DGDG	PC	SL
1	2 (1)	8	3	1	9
2	21 (7)	27	5	2	10
3	2 (0.5)	7	1	–	10
4	6 (2)	35	12	2	7
5	10 (4)	68	35	–	13
6	97 (40)	1438	876	256	49

Lipid content is expressed in μg/mg of total chlorophyll found in the gel

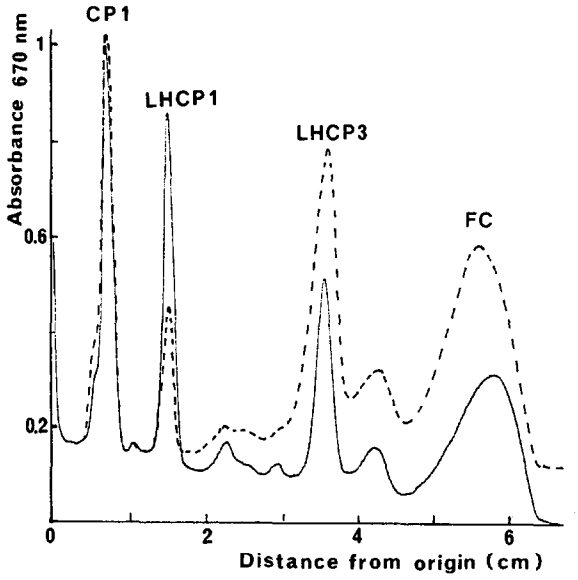

Fig.3. Densitometric tracings at 670 nm of PAGE analysis showing the effect of phospholipase A_2 treatment on chlorophyll–protein complexes from tobacco thylakoids: (——) control; (– – –) effect of lipase.

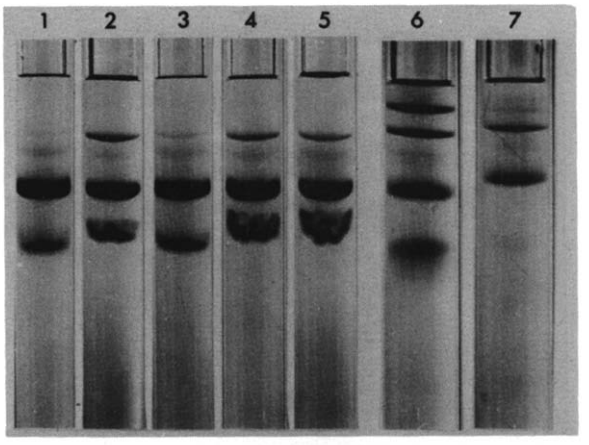

Fig.4. Chlorophyll-containing bands after SDS–PAGE analysis of different mixtures of liposomes and tobacco $LHCP_3$: (1) control with $LHCP_3$ alone; (2) mixture PG–$LHCP_3$; (3) mixture MGDG–$LHCP_3$; (4) mixture DGDG–$LHCP_3$; (5) mixture PC–$LHCP_3$; (6) control with tobacco thylakoids; (7) control with $LHCP_1$.

most of PG and $C_{16:1}$-*trans* [14] splits the oligomeric form $LHCP_1$ into the monomeric one.

3.3. *Reconstitution experiments with $LHCP_3$ liposomes*

The purity and integrity of lipids used for proteoliposome preparations is shown by the characteristic fatty acid composition of these lipids (table 2).

As mentioned in section 2, liposomes and $LHCP_3$ were mixed at a weight ratio lipids/proteins of ~1. The SDS–PAGE analysis of proteoliposomes for each lipid class is given in fig.4.

In lanes 1, 6 and 7, the controls are shown. Other controls, for instance with free chlorophyll mixed into liposomes (not shown), also do not yield reconstituted $LHCP_1$. Lane 1 corresponds to $LHCP_3$ without liposomes but treated exactly as $LHCP_3$ mixed with liposomes. Lanes 6 and 7 correspond respectively to SDS-treated thylakoids and isolated $LHCP_1$ re-electrophoresed without subsequent solubilization.

It is clear that PG, DGDG and PC (lanes 2, 4, 5) are able to reaggregate $LHCP_3$ and give $LHCP_1$ while this is not the case for MGDG. From densitometric tracings at 670 nm of such gels we calculate that the peak area of the PG-reconstituted $LHCP_1$ represents 15% of the total chlorophyll while it is only 8% and 7%, respectively, for DGDG and PC. The presence of a free chlorophyll band in all experiments where liposomes are mixed with $LHCP_3$ can be easily explained by the well-known detergent effect of such lipid dispersion.

It is noticeable that the reconstituted $LHCP_1$ migrates exactly at the same place as its equivalent form obtained with thylakoids (lane 6) or with re-electrophoresed $LHCP_1$.

Table 2
Percentage of fatty acids in the lipids used for liposomes preparation. Traces correspond to <0.5% of fatty acid

	C16:0	C16:1-*trans*	C16:3	C18:0	C18:1	C18:2	C18:3
PG	28	27	–	3	7	15	20
DGDG	16	–	–	Trace	Trace	6	78
MGDG	3	–	17	Trace	Trace	4	76
PC	29	–	–	3	3	31	34

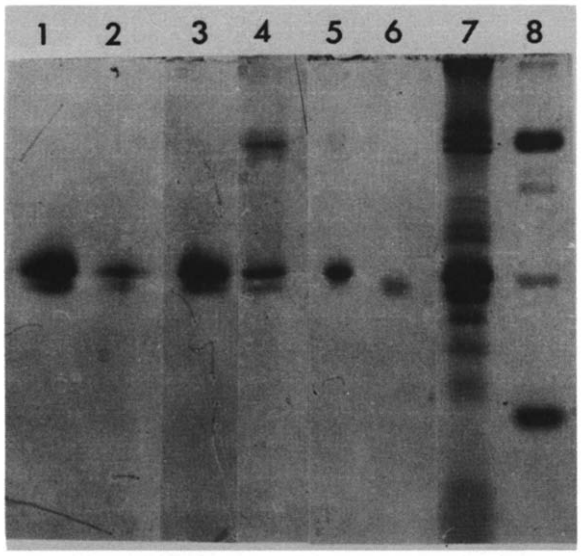

Fig.5. Slab PAGE of Coomassie blue-stained polypeptides: (1) $LHCP_3$ (sliced from a gel similar to that in fig.3, lane 2); (2) PG reconstituted $LHCP_1$ (sliced from a gel similar to that in fig.3, lane 2); (3) isolated $LHCP_3$ used for reconstitution experiments; (4) isolated $LHCP_1$; (5) control with the isolated 25 kd apoprotein of $LHCP_3$; (6) control with the isolated 23 kd apoprotein of $LHCP_3$; (7) control with thylakoid polypeptides; (8) protein markers: serum albumin 67 kd; ovalbumin 43 kd; chymotrypsinogen 25 kd; RNase 13 kd.

However, to gain more evidence that the reconstituted $LHCP_1$ band corresponds really to the $LHCP_1$ obtained directly from thylakoids, analysis of their respective apoproteins was done. This is reported in fig.4 which shows that the reconstituted complex (lane 2) possesses exactly the same 25 and 23 kd apoproteins which were also found in the different controls. As reported in [11] it is noticeable that the isolated $LHCP_1$ used as control is slightly contaminated by a 58 kd polypeptide corresponding to the α-subunit of the coupling factor. This is simply due to the fact that $LHCP_1$ in its native form comigrates with this polypeptide. From densitometric tracing at 600 nm, we calculate that the reconstituted $LHCP_1$ has the same proportions of the 25 and 23 kd polypeptides as the $LHCP_1$ used as control.

4. Discussion

These experiments show clearly the important role of lipids in the molecular organization of chl–protein complexes. We had shown that in $LHCP_1$ lipids represent 25% of the chlorophyll and that the quantitatively most important lipid in $LHCP_1$ is PG containing the *trans*-hexadecenoic acid, since it represents ~40% of the total polar lipids. Here, the effect of phospholipase digestion as well as the better reconstitution of $LHCP_1$ obtained with PG indicate that this lipid may play a key role in the molecular structure of $LHCP_1$. However, we cannot overlook the reconstitution possibilities with PC and DGDG and the lack of reconstitution with MGDG. If we consider the fatty acid composition of lipids which were used for the studies (table 2) it is striking to note that the common feature of positive reconstitution experiments is the relatively high content of the used liposome in $C_{16:0}$ while the unsuccessful experiment corresponds to a very low content in $C_{16:0}$.

This observation points out the interest of studying more precisely the role of fatty acids especially palmitate and *trans*-hexadecenoate in the supramolecular organization of LHCP.

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