ACYL AND PIGMENT LIPID COMPOSITION OF TWO CHLOROPHYLL-PROTEINS

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A differential distribution of acyl and pigment lipids among chlorophyll-proteins of spinach, obtained by SDS-polyacrylamide gel electrophoresis of solubilized thylakoids, was demonstrated by thin-layer chromatography. Light-harvesting chlorophyll *a/b*-protein 2 and *P*-700 chlorophyll *a*-protein 1 were isolated and analysed for their lipid class composition including the fatty acid distribution within each lipid class. The light-harvesting chlorophyll *a/b*-protein 2 was rich in galactolipids and phosphatidylglycerol containing a high amount of 3-*trans*-hexadecenoic acid. In contrast, the *P*-700 chlorophyll *a*-protein 1 lacked these two lipid classes but was associated with sulfoquinovosyldiglyceride and phosphatidylinositol containing a comparatively large proportion of saturated fatty acids. β -carotene was preferentially associated with the chlorophyll *a*-protein whereas xanthophylls were restricted to the chlorophyll *a/b*-proteins. The present data support lateral heterogeneity in the distribution of pigment and acyl lipids within the photosynthetic lamellae.

1. INTRODUCTION

The chlorophyll-proteins obtained by fractionation of thylakoids solubilized in detergents have been extensively studied (2, 4, 10, 15, 18), but the amount and composition of acyl lipids

associated with the individual chlorophyll-proteins is unknown. Acyl lipids are prominent membrane constituents involved in the molecular organization of the membrane as well as in the activity of certain membrane-bound enzymes

Abbreviations: Chl = chlorophyll; Chl-P = chlorophyll-protein; Cl₂Ind = 2,6-dichlorophenol-indophenol; NADP⁺ = nicotinamide adenine dinucleotide phosphate; PS = photosystem; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(13, 26). Two chlorophyll-proteins, namely the *P*-700 chlorophyll *a*-protein 1 and the light-harvesting chlorophyll *a/b*-protein 2 were prepared from spinach leaves and analysed for their content of acyl and pigment lipids.

2. MATERIALS AND METHODS

2.1. Plant Material

Spinacia oleracea (var. Nobel) was grown in a growth chamber in hydroponic culture for 14 weeks at approx. 20 °C and a relative humidity of 70–90%. A photoperiod of 10 hours light (6000 lux) and 14 hours dark was used (20).

2.2. Analyses of chlorophyll-proteins prepared by electrophoresis

Isolation of chloroplasts and of thylakoids showing little or no evidence of lipid hydrolysis and/or transformations was performed as described earlier (10). The thylakoids were solubilized with 1% SDS, 2% Triton X-100 (SDS/chlorophyll ratio 10:1 w/w) without prior lipid extraction. Electrophoretic separation of the chlorophyll-proteins was carried out at 4 °C in the dark using a 2.4% stacking and 8% analysing gel. After separation, the gels were immediately sectioned and the individual chlorophyll-proteins electrophoretically eluted into dialysis tubing. SDS was removed by precipitation with CaCl₂. Aliquots were separated by thin-layer chromatography on silica plates with hexane/acetone (60:40, v/v) as solvent. After separation the plate was sprayed with H₂SO₄:ethanol:H₂O (34/33/33) and charred to reveal the acyl lipids.

2.3. Isolation of chlorophyll-proteins

Light-harvesting chlorophyll *a/b*-protein 2 was prepared as in (8) and the *P*-700 chlorophyll *a*-protein was obtained by hydroxyapatite column chromatography of Triton X-100 solubilized membranes as described by WILLIAMS-SMITH et al. (27). The preparations could be stored in liquid N₂ (77K) without any noticeable effect upon the subsequent analyses.

2.4. Photochemical measurements

Electron flow reactions involving H₂O → Cl₂Ind (PSII), Ascorbate/Cl₂Ind → NADP⁺ (PSI) and H₂O → NADP⁺ (PSII + PSI) were carried out as in (10).

2.5. Lipid analyses

Light-harvesting chlorophyll *a/b*-protein 2 (equivalent to 8.5 mg total chlorophyll) was extracted twice with chloroform/methanol according to BLIGH and DYER (6) and finally with *n*-butanol. All organic fractions were pooled, brought to dryness under a N₂ atmosphere and redissolved in a small volume of chloroform. The *P*-700 chlorophyll *a*-protein 1 (equivalent to 2.5 mg chlorophyll) was first treated with Bio-Beads (Bio-Rad, Richmond, Calif.) to remove most of the Triton X-100 (12) and then extracted three times with chloroform/methanol as in (6). The organic phases were pooled, brought to dryness and redissolved in a small volume of chloroform.

Lipids of the light-harvesting chlorophyll *a/b*-protein 2 were applied to a 70–325 mesh silicic acid column (4 × 1 cm) and sequentially eluted with 35 ml chloroform (fraction A, pigments) and 50 ml methanol (fraction B, acyl lipids). The entire lipid extract of the *P*-700 chlorophyll *a*-protein 1 was separated on a silica plate (Merck, Darmstadt, W. Germany) with chloroform/methanol/H₂O (65:25:4, v/v) as solvent. Fraction B was also separated on a silica plate with chloroform/methanol/acetic acid/H₂O (85:15:10:3, v/v) as solvent. The lipid bands were visualized on the plates with an alkaline bromothymol blue spray reagent (16), isolated

Figure 1. Analyses of acyl and pigment lipids in different chlorophyll-proteins.

The chlorophyll-proteins were separated by SDS-PAGE (A) and the lipids associated with each pigmented band were analyzed by thin-layer chromatography. The pigment lipids are shown in (B) and acyl lipids after charring in (C). Lipid bands are: 0 (origin) = acyl lipids; 1 = acyl-monogalactolipid; 2 = neoxanthin; 3 = violaxanthin; 4 = sterols; 5 = lutein; 6 = chlorophyll *b*; 7 = chlorophyll *a*; 8, 9 = phaeophytins; 10 = unidentified carotenoid; 11 = β-carotene.

and derivatized by methylation. The fatty acid methyl esters thus obtained were analysed by gas-liquid chromatography (16).

2.6. Pigment analyses

2.6.1. Carotenoid composition

Aliquots of fraction A (see section 2.5), or aliquots of the *P*-700 chlorophyll *a*-protein 1 lipid extract were saponified (5) and the non-saponifiable fractions separated by thin-layer chromatography with dichloromethane/ethyl acetate (80:20, v/v) as developing solvent. The individual carotenoid bands were scraped into tubes fitted with teflon-lined stoppers and extracted with suitable volumes of diethylether to permit spectral characterization. Quantitative determinations of carotenoids were carried out using the principal peak of each spectrum and assuming an average $E_{1\text{cm}}^{1\%}$ of 2500 (14).

2.6.2. *P*-700 determination

P-700 was determined from the ascorbate versus ferricyanide difference spectrum (650–720 nm) using a molar extinction coefficient for *P*-700 of 64,000 (11).

2.6.3. Chlorophyll determination

Chlorophylls were determined in 80% acetone and quantified according to ARNON (3).

3. RESULTS

3.1. Analyses of chlorophyll-proteins prepared by electrophoresis

Detergent-solubilized thylakoids were electrophoretically resolved into seven pigmented bands (Figure 1A) which have been designated according to MACHOLD et al. (15). Figure 1B shows the differential distribution of major carotenoids among these pigmented bands. β -carotene is preferentially associated with Chl_a -P1 and Chl_a -P2, whereas violaxanthin and neoxanthin are exclusively present in the chlorophyll *a/b*-proteins. Lutein is associated with all chlorophyll-proteins. All four major carotenoids were observed in the free pigment band. The same thin-layer chromatographic plate is also shown after charring to reveal acyl lipids in

Figure 1C. Acyl lipids are associated with all chlorophyll-proteins and the free pigment band. With the solvent system used acyl-monogalactosyldiglyceride migrated ahead of the more polar lipids found close to the origin. The chlorophyll *a*-protein 1 is deficient in acyl-monogalactosyldiglyceride.

3.2. Analyses of isolated chlorophyll-proteins

The light-harvesting chlorophyll *a/b*-protein prepared by phase partitioning had a chlorophyll *a/b* ratio of about 1.3. The red absorption maximum of chlorophyll *a* was at 675 nm with a shoulder at 652 nm and Soret peaks were found at 436 and 470 nm. The preparation was inactive in all photochemical measurements.

The *P*-700 chlorophyll *a*-protein prepared by hydroxyapatite column chromatography migrated as a single pigmented band in SDS-PAGE with a mobility higher than the corresponding Chl_a -P1 band. Like the Chl_a -P1 band it had a chlorophyll *a/b* ratio greater than 7. The red absorption maximum of chlorophyll *a* was found at 674–675 nm with a Soret peak at 436 nm, whereas chlorophyll *b* was judged absent by the lack of the characteristic absorbance at 652 and 470 nm. The chlorophyll/*P*-700 ratio was about 100, and the preparation was photochemically active in photoreduction of NADP^+ using ascorbate- Cl_2 Ind as electron donor (Table I). Additions of ferredoxin- NADP^+ -reductase and/or plastocyanin stimulated this activity. No PSII mediated electron flow could be measured using either H_2O or 1,5-diphenylcarbazine as electron donors.

Table II shows the carotenoid composition of the *P*-700 chlorophyll *a*-protein and the light-harvesting chlorophyll *a/b*-protein. In agreement with the qualitative analysis (3.1) β -carotene is the prominent carotenoid in chlorophyll *a*-protein 1 while violaxanthin and neoxanthin are exclusively found in the chlorophyll *a/b*-proteins. Lutein was in this analysis only present in the chlorophyll *a/b*-protein. The absence of xanthophylls from the Chl_a -P1 could reflect either that these pigments are not bound to this protein in vivo or their preferential extraction during isolation. Loss of carotenoids from the light harvesting $\text{Chl}_{a/b}$ -P2 may explain the high ratios of chlorophyll to carotenoids in this protein.

Table I.

Electron transport activity of *P*-700 chlorophyll *a*-protein obtained after hydroxyapatite column chromatography.

Conditions	μ moles NADP ⁺ reduced mg chl ⁻¹ h ⁻¹
1. Basic medium	121
2. Basic medium + ferredoxin-NADP ⁺ -reductase	232
3. Basic medium + plastocyanin	214
4. Basic medium + ferredoxin/NADP ⁺ reductase and plastocyanin	221

3.3. Acyl lipid composition of the isolated chlorophyll-proteins

The lipid class and fatty acid composition of the isolated light-harvesting chlorophyll *a/b*-protein and of the chlorophyll *a*-protein 1 is presented in Tables III and IV, respectively. While the Chl_{*a/b*}-P contained galactolipids and four ionic lipid classes the Chl_{*a*}-P1 yielded only sulfoquinovosyldiglyceride and phosphatidylinositol. Comparing the fatty acid composition of phosphatidylinositol from both proteins a large difference in the degree of saturation is noticeable. The phosphatidylinositol from Chl_{*a/b*}-P contains 46 % linolenic acid [18:3] while that of Chl_{*a*}-P1 has only 6 % of this polyunsaturated fatty acid. A similar tendency of greater saturation of the fatty acids in the Chl_{*a*}-P1 is apparent for the sulfoquinovosyldiglyceride.

Besides the dominating content of monogalactosyldiglyceride and digalactosyldiglyceride, the Chl_{*a/b*}-P contains as a major component phosphatidylglycerol with a high content of 3-*trans*-hexadecenoic acid [16:1(3t)] (Table III). Comparing the content of ionic lipids in the Chl_{*a/b*}-P

preparation with that of the thylakoids (17) a 20 % enrichment is observed. This is attributable to a 2-fold increase in phosphatidylglycerol and a 3-fold increase in sulfoquinovosyldiglyceride in the preparation of Chl_{*a/b*}-P. The ratio of galactolipids to chlorophyll is lower in the isolated Chl_{*a/b*}-P than in total thylakoids. The presence of lyso-derivatives (lyso-acyl-monogalactosyldiglyceride, lyso-phosphatidylcholine and lyso-phosphatidylinositol) appears to be a result of aging as their relative amounts in thylakoids are dependent upon the physiological state of the preparation (22).

4. DISCUSSION

Lipids detected in isolated chlorophyll-proteins were earlier considered to originate from artifactual exchange processes involving the detergent and membrane lipids (23). The reproducibility of the differential acyl lipid and carotenoid composition among the isolated chlorophyll-proteins indicates specific associations.

The proposal of ANDERSON (1) that the light-

Table II.

Comparison of the molar ratios of chlorophyll to carotenoid in two isolated chlorophyll-proteins with the ratios in thylakoids.

	Thylakoids (19)	Light-harvesting chlorophyll <i>a/b</i> -protein	<i>P</i> -700 chlorophyll <i>a</i> -protein
β -carotene	8	200	10
Lutein	7	48	∞
Violaxanthin	9	333	∞
Neoxanthin	14	1000	∞

Table III.

Lipid analyses of light-harvesting chlorophyll *a/b*-protein.

Lipid	Amount		In moles % of total fatty acid in the lipid molecule								
	$\mu\text{mole}/\mu\text{mole chlorophyll}$	moles %	16:0	16:1	16:1(3t)	16:2	16:3	18:0	18:1	18:2	18:3
monogalactosyldiglyceride	0.157	32.7	1.7	0.6	—	tr	21.5	1.0	0.5	1.4	73.7
digalactosyldiglyceride	0.134	27.9	5.6	tr	1.9	tr	3.3	1.7	1.4	1.2	84.9
phosphatidylglycerol	0.072	15.0	7.5	—	40.6	—	—	1.1	0.7	1.9	48.3
sulfoquinovosyldiglyceride	0.059	12.3	32.8	—	—	—	1.7	1.8	2.1	8.1	52.6
phosphatidylcholine	0.030	6.2	13.0	—	—	—	3.9	3.1	3.0	9.7	67.2
lyso-acyl-monogalactosyldiglyceride	0.014	2.9	6.0	—	—	—	18.4	1.8	tr	tr	73.4
phosphatidylinositol	0.008	1.7	40.0	—	—	—	—	4.1	1.4	8.6	45.5
lyso-phosphatidylcholine	0.006	1.2	—	—	—	—	—	—	—	—	—

Fatty acids are described by the number of carbon atoms:number of double bonds.

Table IV

Lipid analyses of *P*-700 chlorophyll *a*-protein.

Lipids		In moles % of total fatty acid in the lipid molecule						
		16:0	16:1	16:1(3t)	18:0	18:1	18:2	18:3
sulfoquinovosyldiglyceride	34.5*	16.5	0.4	0.2	10.0	4.0	5.0	32.0
	72.2 ⁺	23.6	0.5	0.4	15.6	6.5	7.9	45.5
phosphatidylinositol	13.3*	9.6	—	—	12.2	1.5	1.6	1.5
	27.8 ⁺	36.3	—	—	45.9	5.8	6.1	5.8

Sometimes traces of phosphatidylcholine and lyso-phosphatidylinositol were detected.

* = nanomoles lipid/ μ mole chl.

⁺ = moles %

Fatty acids are described by the number of carbon atoms:number of double bonds.

harvesting chlorophyll *a/b*-protein participates in membrane stacking and that anionic lipids are mediators in this process is in agreement with the high content of phosphatidylglycerol and sulfoquinovosyldiglyceride in light-harvesting chlorophyll *a/b*-protein.

TUQUET (24) has reported the disorganization of the grana stacks upon treatment with snake venom phospholipase A₂, which specifically hydrolyses phosphatidylcholine and phosphatidylglycerol. Phosphatidylglycerol molecules esterified at the C-2 position of the glycerol with 3-*trans*-hexadecenoic acid were found preferentially associated with the light-harvesting chlorophyll *a/b*-protein (7). Phosphatidylglycerol molecules containing the 3-*trans*-hexadecenoic acid have also been implicated in the formation of appressed regions in chloroplasts (9, 25). The present lipid analyses of the Chl_{*a/b*}-P2 conform to these observations.

The strongly polar lipids with a high content of saturated fatty acids (40 % in sulfoquinovosyldiglyceride and 82 % in phosphatidylinositol) in the *P*-700 chlorophyll *a*-protein were not removed even after extensive treatment with 0.5 % Triton X-100. This may be due to polar electrostatic interactions between the sulfoquinovosyl and phosphoryl-inositol head groups and certain polar amino acid residues in the protein moiety, together with hydrophobic interactions between the acyl chains and non-polar amino acid residues. The phosphatidylinositol and sulfoquinovosyldiglyceride molecules associated with the *P*-700 chlorophyll *a*-protein could not be hydro-

lysed by phospholipase A₂, by *Rhizopus arrhizus* lipase or by potato lipolytic acyl hydrolase suggesting the unavailability of these lipids to the enzymes. The strong resistance of photosystem I electron transport towards aging in vitro, compared to that of photosystem II (21) may be related to this strong lipid-protein interaction. The organization of these acyl lipids in a boundary layer around the protein core seems unlikely from their low molar ratios (chlorophyll/sulfolipid/phosphatidylinositol 1000:35:13) (cf. 1).

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