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Spin label EPR study of lipid solvation of supramolecular photosynthetic protein complexes in thylakoids

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Abstract

Lipid-protein association in the chloroplast membrane and its various thylakoid fractions from higher plants, namely pea and maize, rich in Photosystem I (PSI) and Photosystem II (PSII), respectively, were studied using EPR spectroscopy of spin-labelled lipid molecules. All the EPR spectra consisted of two spectral components corresponding to bulk fluid lipids and solvation lipids motionally restricted at the hydrophobic surface of membrane proteins. Spin-labelled stearic acid and phosphatidylglycerol exhibited marked selectivity towards the supramolecular protein complexes of both PSI and PSII although to different extent. In addition, lipid-protein titration experiments are described for partially delipidated PSII-enriched membrane fractions of pea chloroplasts, incorporating unlabelled egg phosphatidylcholine prior to or after the incorporation of spin-labelled lipids. Two sets of solvation sites were resolved by timed labelling experiments and a significant result of these studies was that a well-defined population of solvation sites (approx. 100 mol lipids/820 kDa protein) was rapidly exchanged by laterally diffusing membrane lipids, while other solvation sites (approx. 50 mol lipids/820 kDa protein) were exchanged much slower or not exchanged at all.

Keywords: EPR; Thylakoid; Lipid–protein interaction

1. Introduction

The thylakoid membrane of higher plant chloroplasts is a highly specialized assembly of protein and pigment molecules which are ultimately designed for the efficient absorbance of light and its conversion into chemical energy [1–3]. Its functional constituents are organized into four major supramolecular protein complexes: Photosystem I (PSI), Photosystem II (PSII), cytochrome b/f, and ATP synthase complexes [4,5]. The distribution of these four protein complexes in various parts of chloroplast thylakoids is well-defined; more specifically, the appressed regions are highly enriched in PSII, while the non-appressed regions contain predominantly PSI and ATP synthase complexes, and cytochrome b/f complexes are distributed more homogeneously [2]. The bilayer structure of the thylakoid membrane is maintained by a set of diverse lipids and this structural determinant has been suggested to have an essential functional role in stabilizing the protein complexes [6–8].

From the viewpoint of molecular dynamics, the supramolecular protein complexes, lipids, and pigments, the three major constituents of thylakoid, have rather different motional properties owing to their different hydrodynamic sizes. Hence, protein-lipid associations in the membrane result in motional coupling between fluid lipids and low mobility proteins leading to the formation of at least one shell of motionally restricted lipids, the so-called solvation

Abbreviations: 14-PCSL and 14-PGSL, 1-acyl-2-(13-(4,4-dimethyl-3-oxy-2-butyl-2-oxazolidinyl)tridecanoyl)-*sn*-glycero-3-phosphocholine and -phosphoglycerol, respectively; 14-SASL, 14-(4,4-dimethyl-oxazolidine-*N*-oxyl)stearic acid; EPR, electron paramagnetic resonance; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; PSI and PSII, Photosystem I and II; LHCI and LHCII, light harvesting Complex I and II.

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layer, at the hydrophobic surface of proteins. Amongst the available spectroscopic methods, spin label EPR spectroscopy has an optimal time-scale to resolve such motionally restricted solvation lipids from fluid lipid molecules which undergo rapid unrestricted tumbling in protein-free membrane domains [9,10].

In this work, we present spin label EPR experiments on the dimorphic chloroplast system of maize, in addition to confirmatory results on pea chloroplasts [11,12]. A novel approach which consists of lipid supplementation experiments on PSII-enriched pea thylakoid fractions allowed the determination of the stoichiometry and timed selectivity of lipid-protein association. The alteration of the lipid/(protein + chlorophyll) ratio requires the incorporation of exogenous unlabelled lipids into the chloroplast membrane and phosphatidylcholine has been chosen since this is a ubiquitously occurring phospholipid towards which no selectivity has been found in the case of any of the previously studied integral membrane proteins [9,10]. Moreover, a timed labelling procedure is proposed in order to study the dynamics of the lipid incorporation within different thylakoid membranes.

2. Materials and methods

Chloroplasts were isolated from pea seedlings according to standard procedures [13] with the exception that ascorbic acid was excluded from the medium to avoid unwanted spin label reduction [14,15]. The appressed and non-appressed thylakoid membrane fractions, i.e., the grana and stroma membranes, respectively, were isolated according to the method of Sane et al. [16]. A key step in this protocol is mechanical disruption of chloroplasts through an Aminco French pressure cell followed by fractionation at $1000 \times g$ for 10 min, $10000 \times g$ for 30 min, and $70000 \times g$ for 60 min. The second and third fractions were used as appressed and non-appressed membrane preparations (i.e., grana and stroma membranes), respectively. It should be noted that in previous experiments chloroplast fractionation was done with a detergent extraction method [11,12]. In the case of maize chloroplasts, the differential grinding method of Bjorkman and Gauhl was applied for the preparation of mesophyll and bundle-sheath membranes [17]. The electron transport activities of the various thylakoid membrane fractions are given in Table 1. The purity of the thylakoid membrane preparations was checked spectrophotometrically [18] by calculating the ratio of the Chl *a*/Chl *b* and in the case of appressed membranes this diagnostic ratio was < 2.5, while for non-appressed membranes high values, typically > 4.0, were obtained. This brief testing was further substantiated previously by parallel morphological and protein compositional analyses [19,20].

Functional PSII-enriched thylakoid membrane fractions (BBY particles) were prepared by detergent extraction according to the method of Berthold et al. [21] using 25 mg Triton X-100/mg Chl. After this incubation in detergent, the PSII-enriched membrane fractions were pooled by centrifugation at $40000 \times g$ for 30 min. The O_2 evolving capacity was measured by using a Clark-type oxygen electrode as described in Ref. [22]. Lipid supplementation of PSII-enriched thylakoid membrane fractions was done by adding pre-formed liposomes of egg yolk lecithin (Sigma, Germany) and facilitating the incorporation of lipid molecules into the thylakoid membrane by incubation at 30°C in a water bath shaker (New Brunswick, USA) for 2 h. During lipid incorporation the detergent concentration was lowered by adding Amberlite XAD-2 beads. Protein-free lipid vesicles and Triton/lipid mixed micelles were separated by differential centrifugation ($25000 \times g$ for 40 min over a 15 wt%–0 wt% sucrose concentration boundary). The lipid supplemented vesicles were further analysed by continuous sucrose density gradient centrifugation (10–40 wt%, $100000 \times g$ in a SW 40 swing-out rotor for 3 h) and a well-defined band was obtained the position of which was consistent with the amount of lipid added.

Total lipids were extracted from thylakoids by a modified method of Folch et al. [23] and quantitatively analysed by thin-layer chromatography; the acyl chain composition was determined by gas chromatography adding appropriate amount of (17:0) fatty acids as an internal standard [24]. The phospholipid contents of phosphatidylcholine supplemented vesicles was determined by the method of Eibl and Lands [25]. The protein content of thylakoid membranes was measured by the method of Markwell et al. [26].

For spin labelling, the thylakoid membranes (0.5 mg Chl *a*) were suspended in 0.5 ml buffer and, prior to or

Table 1
Characteristic protein and pigment compositions of various thylakoid fragments

Fragment	Chl *a*/Chl *b* (mg/mg)	Prot/Chl (mg/mg)	Lipid/Chl (mg/mg)	Electron transport $H_2O \rightarrow$ pBQ (mmol O_2/mg Chl per h)
Maize chloroplast				
Mesophyll cells	3.2(3)	10.5	0.8	150
Bundle-sheath cells	5.9	17.0	2.1	11
Pea chloroplast				
Appressed region	2.5(2)	10.5	1.0	163
Non-appressed reg.	4.4(2)	19.0	3.1	15
PSII rich fraction	2.1(1)	6.5	0.62	205

after the incorporation of unlabelled lipids (PC), 10 μl of 1 mg/ml spin-label solution in ethanol was added while vortexing the sample. The sample was incubated for 60 min in the dark at room temperature and repeatedly vortexed. The spin-labelled samples were then filled into 1 mm I.D. EPR sample capillaries and concentrated in a bench top centrifuge.

The EPR spectra were recorded using a Bruker ECS 106 (9 GHz) spectrometer with rectangular TE_{102} cavity and equipped with a computer controlled nitrogen gas flow thermostat (ER 411VT). Typical spectrometer settings: microwave power, 10 mW; scan range, 100 Gauss; modulation amplitude, 1.2 Gauss; Gain, $2 \cdot 10^5$; A/D resolution, 16 bit. Spectral subtraction was done using the software provided by ECS 106 software and spectral files were transmitted to IBM compatible environment by Bruker software Doswrite. Further spectral analyses and plotting were performed on IBM machines using software written by one of us (L.I.H.).

3. Results

The various thylakoid membrane fractions have different protein, pigment, and lipid compositions. In particular, as summarized in Table 1, the appressed regions are rich in PSII complexes and the associated light-harvesting protein molecules (LHCII), giving a low protein/chlorophyll ratio [27]; essentially similar trends were observed for mesophyll thylakoids [28]. Non-appressed regions, on the other hand, contain a significantly smaller amount of light-harvesting complexes (LHCI) and, hence, their protein/chlorophyll ratio is almost 2-times higher. The major lipid classes in pea chloroplasts are the galactolipids (monogalactosyl-, MGDG, 41 wt%, and digalactosyldiacylglycerol, DGDG, 32 wt%, and lesser amount of sulfoquinosovosyldiacylglycerol, SQDG, 9 wt%) and the sole phospholipid species are phosphatidylglycerol (PG, 10 wt%) and a trace amount of phosphatidylinositol (PI, < 1 wt%). The amount of plastidial phosphatidylcholine (PC, 7 wt%) in the thylakoid membrane is still a matter of controversy [29–31]. Maize chloroplasts had a rather similar lipid composition: MGDG, 44 wt%; DGDG, 37 wt%; SQDG, 4 wt%; PG, 9 wt%, and PC, 6 wt%. Clearly, the acyl chain compositions in the two membranes follow the same pattern: the majority of linolenic acid (18:3) is acylated to galactolipids, while the phospholipids are more saturated, the dominating species being palmitic (16:0) and oleic acid (18:1). Accordingly the double bond index, $I_{C=C}$, was found to be 2.6 in appressed membranes and mesophyll cells, while 2.4 and 1.7 in non-appressed regions and bundle-sheath cells, respectively.

EPR spectra of C-14 positional isomer of spin-labelled phosphatidylglycerol (14-PGSL) in various thylakoid membrane fractions of pea and maize chloroplasts are shown in Fig. 1. Similarly to previous experiments [11] all

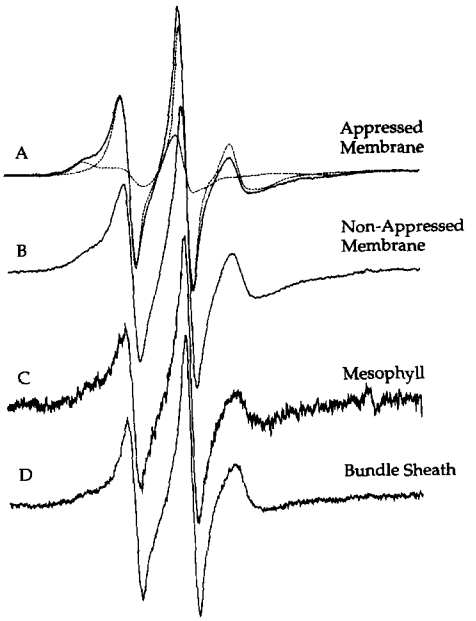

Fig. 1. EPR spectra of spin-labelled phosphatidylglycerol (14-PGSL) in thylakoid fractions of pea and maize chloroplasts. Appressed membrane (A) and non-appressed membrane (B) isolated from pea chloroplast; mesophyll (C) and bundle-sheath membranes (D) isolated from maize chloroplast. The two-component EPR spectrum recorded in pea appressed membranes (solid line, trace A) is shown together with appropriate amounts of rigid limit line shape observed in PSII-enriched membrane fractions and motionally averaged 3-line spectrum recorded in aqueous dispersions of the total lipid extracts (dashed lines, trace A).

these spectra consist of two components: a motionally averaged 3-line spectrum and a second component which arises due to motionally restricted spin labels with an approximate rigid limit line shape; the two components are shown (with dashed lines) together with the two-component EPR spectrum of pea appressed membranes. The former line shape is almost identical with the EPR spectra of these spin probes in protein-free vesicles of the lipid extracts and, hence, will be referred to as the bulk fluid lipid component. The latter line shape is qualitatively similar to those observed in other protein/lipid systems and is assigned to motionally restricted solvation lipids at the hydrophobic interface of integral membrane proteins (for reviews see Refs. [9,10]). As expected, the EPR spectrum of the unfractionated chloroplast is a weighted average of the appressed and non-appressed fractions.

Essentially similar trends were observed in the case of pea and maize chloroplasts; the quantitative evaluation was done by difference spectroscopy. On labelling the different thylakoid fragments, the relative spectral intensities of the two components varied rather significantly (Fig. 1) which was consistent with the lipid/(protein + chlorophyll) ratios of these membranes. Comparison of EPR spectra of spin-labelled stearic acid (14-SASL), phosphatidylglycerol (14-PGSL), and phosphatidylcholine (14-PCSL) in both pea and maize thylakoids has revealed that there is a

pronounced selectivity towards acidic lipids (spectra not shown).

A particular effect of lipid selectivity was highlighted in timed labelling experiments in which the spin label incorporation was done prior to or after (unlabelled) lipid supplementation to partially delipidated PSII-enriched membrane fractions. As seen in Fig. 2, on adding spin-labelled phosphatidylcholine (14-PCSL) to PSII-enriched membrane fractions an approximate slow motion EPR line shape was obtained (trace A in Fig. 2) indicating that the remaining lipids, after Triton treatment, occupy solvation sites. This extreme lipid/(protein + chlorophyll) ratio (< 50 mol/mol) could be altered by supplementing with exogenous unlabelled phosphatidylcholine (PC). However, in spite of the incorporation of 150 mol PC/820 kDa (protein + chlorophyll) the above EPR line shape remained virtually unchanged, provided the spin labels had already occupied solvation sites (trace B in Fig. 2). A basically different pattern was obtained when the timing of the addition of spin labels was changed; on adding 150 mol PC/820 kDa (protein + chlorophyll) first and the spin label afterwards, a clear two-component spectrum could be recorded (trace C in Fig. 2).

This latter timing protocol is suitable for doing combined lipid selectivity and lipid/(protein + chlorophyll) titration experiments as shown in Fig. 3. In these lipid supplementation experiments, unlabelled PC was incorporated into PSII-enriched membrane fractions and protein-free lipid vesicles were separated by differential centrifugation. With the gradual incorporation of unlabelled PC, the intensity of the fluid component, as expected, increased and all the two-component spectra shown in Fig. 3 could be analysed using the single component line shapes of 14-PCSL in PSII-enriched membrane fractions and aqueous dispersions of lipid extracts as described above.

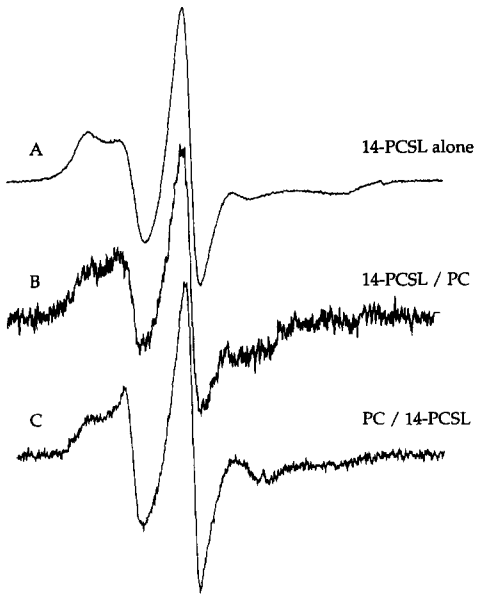

Fig. 2. EPR spectra of spin-labelled phosphatidylcholine (14-PCSL) in timed labelling experiments with pea chloroplasts: PSII-enriched membrane fractions were supplemented with unlabelled phosphatidylcholine (PC) prior to or after the incorporation of spin-labelled lipids into the membrane. (A) 14-PCSL in PSII-enriched membrane fraction alone; lipid/protein ratio is < 50 mol/820 kDa protein. (B) 14-PCSL was added before supplementing the PSII-enriched membrane fraction with 150 mol unlabelled PC/820 kDa protein. (C) The PSII-enriched membrane fraction was first supplemented with 150 mol unlabelled PC/820 kDa protein and after that 14-PCSL was incorporated into the complexes. For a detailed description of timed labelling experiments see Materials and methods.

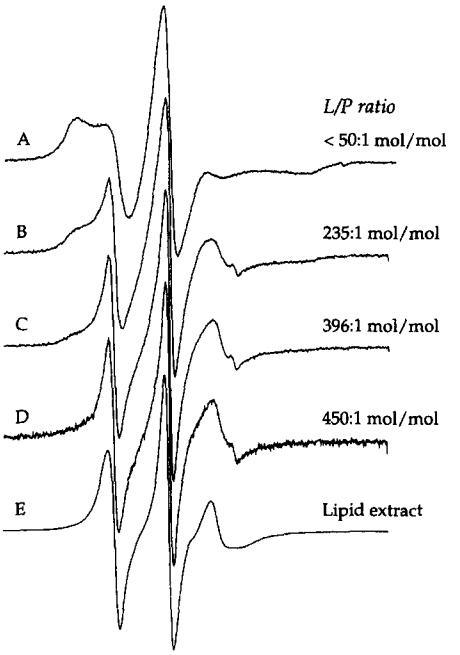

Fig. 3. Lipid/protein titration experiments with lipid supplemented pea chloroplasts: EPR spectra of spin-labelled phosphatidylcholine (14-PCSL) in PSII-enriched membrane fractions supplemented with various amounts of unlabelled phosphatidylcholine (PC). The phospholipid/(protein + chlorophyll) ratio was determined by assaying the phospholipid and protein content as described in Materials and methods. $T = 30°C$; total scan width was 100 Gauss.

4. Discussion

The interpretation of these results is based, as in the case of other membrane protein/lipid complexes, on the assumption that the extent of motional restriction over the lipid pool depends essentially on two factors: the lipid/(protein + chlorophyll) ratio and the lipid selectivity of membrane proteins [9,10,32]. A specific structural feature of chloroplasts, namely the differentiation into appressed and non-appressed regions of thylakoid (grana and stroma), allows the separation of PSII and PSI-enriched membrane fractions. Chloroplast dimorphism, specific for malate type C_4 plants, also allows the separation of two types of chloroplasts with rather different grana and stroma content, and protein compositions, characteristic for PSII

and (PSI + ATP synthase), respectively. Since appressed and non-appressed membranes have different protein/chlorophyll (cf. Table 1) and lipid/protein ratios [2], the average motional properties of lipids exhibit qualitatively similar, but quantitatively different results. This heterogeneity in lipid/protein interaction is particularly dominant in the case of (PSI + ATP synthase)-rich non-appressed membranes.

The present study not only confirms the earlier data for pea [11], but extends the spin labelling approach to another ontogenetically different chloroplast membrane system. The basic characteristics are very similar: the fraction of solvation lipids in mesophyll cells, in agreement with the inherently lower lipid/(protein + chlorophyll) ratio, is significantly higher (Fig. 1). These findings as well as additional data on chloroplasts of various chilling sensitive and resistant plants [12] strongly substantiate the general applicability of the lipid association concept to diverse chloroplast membranes.

The selectivity of the photosynthetic protein complexes is quantified by the relative association constant, K_r, defined as

$$K_r = ([PC][Prot{:}PC^*])/([PC^*][Prot{:}PC]) \quad (1)$$

Here PC and PC* denote unlabelled and spin-labelled PC molecules, and Prot:PC and Prot:PC* denote solvation sites, respectively. Using the above spin-labelled acidic lipids relative association constants of $K_r = 1.3–2.0$ (14-PGSL) and $K_r = 3.5–6.0$ (14-SASL) were obtained.

Strictly speaking the conventional model of two-site exchange can only be applied in the above approximate form provided there is only one class of solvation sites at the protein interface. However, our timed labelling experiments strongly suggest that the uniform-solvation-site model must be an oversimplification (cf. Fig. 2). When spin-labelled lipids were incorporated into the partially delipidated PSII-enriched membrane fraction prior to lipid supplementation, the spin labels occupied a special set of solvation sites from which they were hardly exchanged, at least by phosphatidylcholine. On contrary, supplementation and subsequent labelling gave access to a special set of less tightly bound sites at the hydrophobic surface of proteins which were in continuous and rapid exchange with the laterally diffusing endogenous and supplemented lipids. It should be noted that freeze fracture electron microscopy gave conflicting results as to the homogenous distribution of the incorporated lipids in PSII-enriched membranes [33,34]. The size of the rapidly exchanging solvation lipid pool was determined by lipid/protein titration experiments (cf. Fig. 3) with spin-labelled phosphatidylcholine (14-PCSL) and the titration diagram is shown in Fig. 4. The titration curves can be fitted to the approximate equation for protein-lipid association [9,10,35]

$$n^*_{fluid}/n^*_{solv} = (1/K_r)(n_t/N'_{solv} - 1) \quad (2)$$

where $n^*_{fluid}/n^*_{solv} = (1-f)/f$ is the ratio of the double-integrated intensities of the fluid and solvation components and can be determined from spectral subtractions, n_t is the total phospholipid-to-protein ratio, N'_{solv} is the number of rapidly exchanged solvation sites, and K_r is the association constant. The number of rapidly exchanging solvation sites is determined from the intercept on the abscissa giving $N'_{solv} = 150$ mol/820 kDa protein for spin-labelled phosphatidylcholine (Fig. 4). Of these solvation sites approx. 50 mol/820 kDa protein are occupied by endogenous lipids of probably high selectivity (Horváth, L.I., Ivancich, A., Droppa, M., Horváth, G. and Farkas, T., unpublished results) and so the remaining approximately 100 mol lipids/820 kDa protein is consistent with earlier estimates on the basis of structural and hydrodynamic data ($N'_{hyd} = 86$ mol/820 kDa protein, [2,11]). The relative association constant, as expected, is close to unity indicating that there is no selective association of spin-labelled phosphatidylcholine (14-PCSL) to unlabelled PC at the hydrophobic surface of PSII supramolecular complexes, after extensive delipidation.

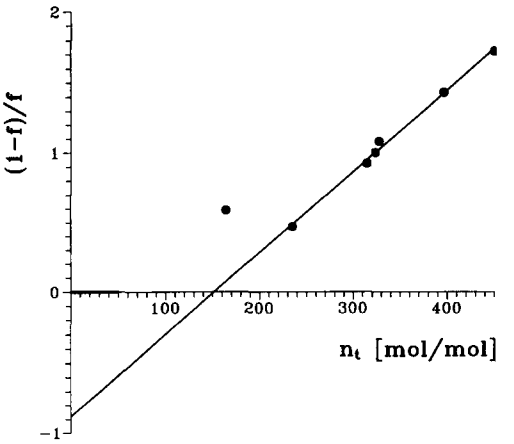

Fig. 4. Lipid-protein titration diagram of PSII-enriched membrane fractions of pea chloroplasts supplemented with unlabelled phosphatidylcholine (PC) using EPR difference spectra of spin-labelled phosphatidylcholine (14-PCSL) at 30°C. $n^*_{fluid}/n^*_{solv} = (1-f)/f$ is the ratio of the double-integrated intensities of the fluid and solvation components and n_t is the lipid/protein ratio. The amount of remaining endogenous lipids after delipidation of PSII-enriched thylakoid membrane fractions was determined by quantitative chromatographic analysis of fatty acids [24] and is estimated to be < 50 mol/820 kDa protein as indicated by the solid bar in the interval $n_t = 0–50$ mol/mol.

In summary, the PSII supramolecular complex possesses at least two sets of solvation sites at its hydrophobic surface, as concluded from timed labelling experiments. One population of solvation sites is rapidly exchanged by laterally diffusing membrane lipids, while the other is exchanged much slower or not exchanged at all.

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