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FLUORESCENCE POLARIZATION AND SPIN-LABEL STUDIES OF THE FLUIDITY OF STROMAL AND GRANAL CHLOROPLAST MEMBRANES

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The lipid fluidity of thylakoid membrane regions separated by Yeda press and sonication methods has been investigated using diphenylhexatriene fluorescence polarization measurements and rotational correlation times derived from the ESR spectra of the spin-labels 5-doxyldecane and 12-doxylstearate. According to both techniques, stromal lamellae vesicles with essentially only Photosystem I activity were more fluid than the granal membranes. The differences in lipid fluidity between the two fractions were interpreted in terms of the ratio of the amounts of protein compared to lipid in the membranes. Stromal lamellae fractions contained lower protein/lipid ratios compared with the granal membranes.

Introduction

The thylakoid membranes of higher plant chloroplasts normally consist of appressed membrane regions (grana) with interconnecting lamellae which are not appressed (stromal lamellae). The two regions can be separated by mechanical and detergent treatments [1], and are distinguished by the pigment-protein complexes which they contain. Stromal lamellae are enriched in PS I proteins while the appressed membranes seem to contain predominantly PS II proteins [2,3]. For electron flow to occur between PS II and PS I, mobile redox carriers are required and diffusion of plastoquinone within the lipid matrix of the membrane seems one likely way that this is accomplished [3–5]. The idea of lateral heterogeneity in the distribution of the photosystems and the existence of appressed and non-appressed membranes indicate that there are likely to be differences in the physical and chemical environments for the PS II and PS I proteins. Indeed, the role of surface electrical charges has already been discussed in depth in relation to the organization of the two photosystems [6,7]. Also, the link between fluidity and the overall functioning of the thylakoid membrane has been the subject of several recent investigations [4,8,9] with the conclusion that a fluid environment is required for normal rates of electron flow between PS II and PS I to occur. The term fluidity is used here to describe the dynamic properties and structural order of the lipid matrix [10].

In this study, measurements of lipid fluidity have been made for granal and stromal lamellae separately. Indications of the fluidity have been obtained from fluorescence polarization studies using 1,6-diphenyl-1,3,5-hexatriene and from ESR measurements using the spin labels 5-doxyldecane and 12-doxylstearate. The fluidity measurements

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; PS, photosystem; Chl, chlorophyll.

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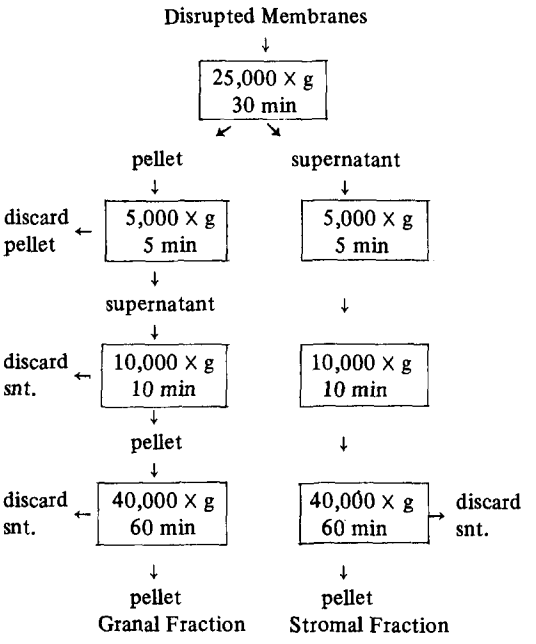
were complemented by analyses of fatty acid composition and of lipid/protein ratios for the two types of membrane fragments (granal and stromal).

Materials and Methods

Fresh pea and lettuce chloroplasts were prepared free of envelopes by the method described previously [9]. After washing, the membranes were suspended in phosphate buffer containing about 10 or 140 mM K^+ to cause unstacking or stacking. The unstacking medium (low-salt buffer) consisted of 5 mM K_2HPO_4/KH_2PO_4 (pH 7.5) plus 0.33 M sorbitol. The stacking medium (high-salt buffer) consisted of 5 mM K_2HPO_4/KH_2PO_4 (pH 7.5) plus 130 mM KCl and 0.2 M sorbitol. The membranes were incubated on ice in the respective media for at least 1 h before disruption using sonication or a Yeda pressure cell. For the sonic disruption of the thylakoid membranes, a 'soniprobe' sonicator (Dawe Instruments Ltd.) was used set at low power (setting 3, 1.5 A). Sonication of 15 ml of thylakoid suspension for 70 s on ice was found to give a reasonable yield of supernatant after the $25000 \times g$ centrifugal step. For mechanical fragmentation the thylakoid membranes were passed twice through a Yeda pressure cell packed in ice using a pressure of $95 \cdot 10^5$ $N \cdot m^{-2}$.

The separation of the fragmented membranes was achieved by differential centrifugation with a Beckman J21C centrifuge. All the fractions were subjected to the same centrifugation steps, as shown in the flow diagram (Scheme I). The first high-speed centrifugation step was used to assess the efficiency of the fractionation. If the supernatant was a very light green, the pellet was resuspended and the sonication or Yeda press treatments repeated.

The amount of chlorophyll and the Chl *a/b* ratio in the fractions were estimated using the method of Arnon [11]. The membranes were suspended at 100 μg Chl/ml and labelled with 7.5 μM 1,6-diphenyl-1,3,5-hexatriene which was added directly from a stock solution (3 mM diphenylhexatriene in tetrahydrofuran). About 40 min incubation at room temperature were used to ensure incorporation of the probe molecules into the membranes. The labelling was also carried out



Scheme I. snt., supernatant.

before the final centrifugation step in order to use the final spin to remove any non-incorporated diphenylhexatriene possibly dispersed in micellar form in the aqueous phase [12]. Fluorescence polarization values were estimated as described previously [8]. A small background signal was subtracted (about 5–10% of the diphenylhexatriene signal), being due to intrinsic fluorescence at 460 nm from the chloroplast membranes.

The measurements of the ESR spectra of the spin labels were taken using a Varian E 104A spectrometer fitted with a variable temperature accessory. The spin labels, dissolved in ethanol, were added to small glass test tubes, and dried down under nitrogen gas. Membranes equivalent to 2 mg/ml Chl suspended in 100 mM sorbitol and 1 mM Hepes-KOH, pH 7.5, were added to the tube and gently shaken. The amount of spin label added was generally 40 μg/mg Chl. DCMU in ethanol was added (5 μM) to avoid light-induced reduction of the spin labels, and the samples were protected from the light as much as possible. The concentration of ethanol was 0.25%. Incubation at room temperature for about 30 s was sufficient to allow incorporation. The spin-broadening agent, tris(oxalato)chromate(III) trihydrate, was added (35 mM) in order to remove the probe-in-water

signal [13]. Samples were placed in a Varian variable temperature aqueous cell. The magnetic field strength was 3280 G (microwave frequency 9.18 GHz). The microwave power was set at 5 mW. Modulation frequency and amplitude were set at 100 kHz and 1.0 G.

All measurements were made at room temperature. 5-Doxyldecane was obtained from Molecular Probes (Plano, TX). 12-Doxylstearate was obtained from Syva (Palo Alto, CA).

The estimate of the amount of protein in the membrane fractions was performed using the method of Lowry et al. [14]. The protein was precipitated, and the amount of chlorophyll determined, in a single step by treating the membranes with 80% ice-cold acetone containing 30 mM NaCl, and centrifugation at $6000 \times g$ for 5 min. Lipids were extracted from membrane preparations with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene and aqueous fractions were removed by the method of Williams and Merillees [15]. Quantities of lipids were determined from gas chromatography of fatty acid methyl esters prepared with boron trifluoride-methanol complex and analysed using methylpentadecanoate as internal standard on a column of 15% Reoplex 400 on Chromosorb W.H.P. 100/120 mesh.

Rates of electron transport were measured using a Rank Brothers (Clark-type) oxygen electrode and a standard assay medium containing 0.33 M sorbitol, 50 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA (disodium salt), pH 7.6. PS II activity was measured at saturating light intensities in the presence of benzoquinone (0.5 mM) and NH_4Cl (5 mM), and then DCMU (10 μM), D-isoascorbate (sodium salt) (1 mM), DCIP (150 μM) and methyl viologen (20 μM) were added to measure PS I activity.

Results

Details of some properties of membrane fragments obtained from the Yeda press treatment are given in Table I. The stromal fraction obtained by disrupting high-salt (stacked) membranes had high Chl *a/b* ratios and essentially only PS I activity. In contrast, the granal fraction had a lower PS I activity and Chl *a/b* ratio although they were not highly enriched in PS II. To obtain such membranes requires the use of phase partition methods which have recently been developed [2,16], but these methods require the use of high polymer concentrations which are likely to affect the normal physical properties of the membrane lipids [17]. Membrane fragments derived from thylakoids suspended in low-salt buffer were not enriched in either PS II or PS I activities.

The fluorescence polarization values of diphenylhexatriene incorporated into various membrane fractions prepared by either Yeda Press treatment or sonication are shown in Fig. 1. In general, membrane fragments with lower Chl *a!b* ratios had higher diphenylhexatriene polarization values, suggesting that fractions enriched in granal lamellae are less fluid than those enriched in stromal lamellae. When the thylakoids were suspended in low-salt buffer, prior to Yeda Press treatment, the

TABLE I

PS I AND PS II ACTIVITIES OF VARIOUS MEMBRANE FRAGMENTS

Rates of PS II-dependent oxygen evolution, and PS I-dependent oxygen uptake via methyl viologen for the pea thylakoid membrane fractions fragmented by the Yeda press. Values shown are the average of three experiments and represent μequiv. O_2/mg Chl per h.

Fraction	PS II activity (H_2O-benzoquinone)	PS I activity (DCIP/ascorbate-methyl viologen)	PS I/PS II	Chl *a/b*
Total thylakoid	250	670	2.7	3.2
High-salt/granal	170	350	2.1	2.9
High-salt/stromal	<10	680	>50	6.0
Low-salt/heavy fraction	140	710	5.0	3.1
Low-salt/light fraction	170	510	3.1	3.3

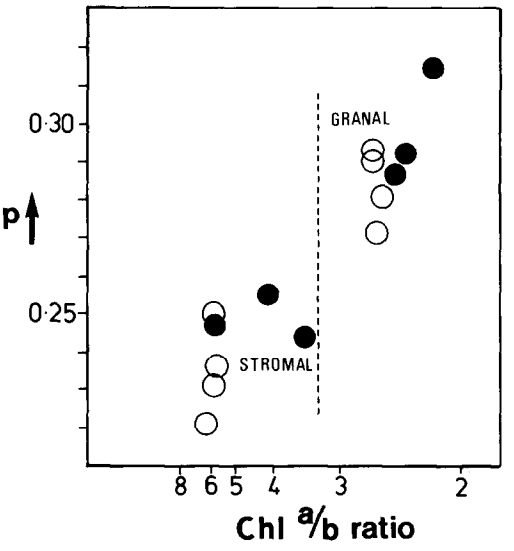


Fig. 1. Polarization values (p) at 20°C of diphenylhexatriene fluorescence in stromal and granal lamellae prepared by sonication (●) and Yeda press (○) treatments of pea chloroplasts. The Chl a/Chl b ratio is plotted on a reciprocal scale.

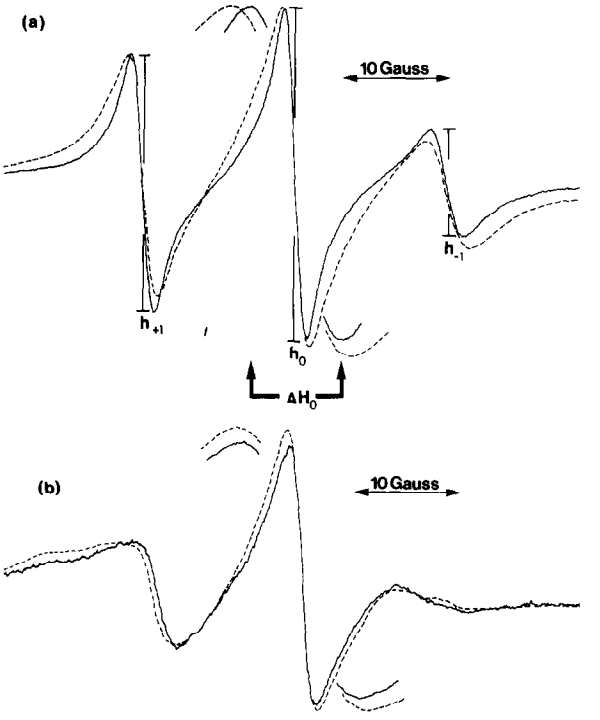


Fig. 2. (a) First-derivative ESR spectra at 20°C of granal and stromal membranes prepared from lettuce chloroplasts by sonication and labelled with 5-doxyldecane. Gain setting is the same for both spectra. Also shown is 4-fold expansion of field sweep to give a more accurate estimate of the centre line width (ΔH_0). The spectrum obtained with granal membranes is represented by the dashed line while the solid line is the spectrum obtained with stromal membranes. (b) As in a, but using 12-doxylstearate as the spin label.

resulting membrane fragments had diphenylhexatriene fluorescence polarization values which were intermediate between the values found for granal and stromal lamellae as shown in Table II.

To extend the above fluidity measurements, the apparent rotational correlation times of the spin labels 5-doxyldecane and 12-doxylstearate embedded in the various membrane fragments were calculated from the ESR spectra. The relative heights of the three peaks of the first-derivative ESR spectrum (low-field h_{+1}, centre h_0 and high-field h_{-1}) and the width of the centre line in gauss (ΔH_0) were used to calculate the line width parameters B and C using the following equations [18]:

$$B = \frac{1}{2}\,\Delta H_0\left[(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}\right]$$

$$C = \frac{1}{2}\,\Delta H_0\left[(h_0/h_{+1})^{1/2} + (h_0/h_{-1})^{1/2} - 2\right]$$

These parameters were then used to calculate apparent rotational correlation times for isotropic motion using the equations [18]:

$$\tau_B = -1.22\,B$$

$$\tau_C = 1.19\,C$$

TABLE II

DIPHENYLHEXATRIENE FLUORESCENCE POLARIZATION VALUES OF VARIOUS MEMBRANE FRAGMENTS

Fractions were obtained after the Yeda Press treatment of pea chloroplasts. Measurements were made at 20°C and the data are from four separate experiments.

Fraction	p (±S.E.)
Total thylakoid	0.262±0.002
High-salt/granal	0.290±0.005
High-salt/stromal	0.250±0.004
Low-salt/heavy fraction	0.285±0.004
Low-salt/light fraction	0.273±0.001

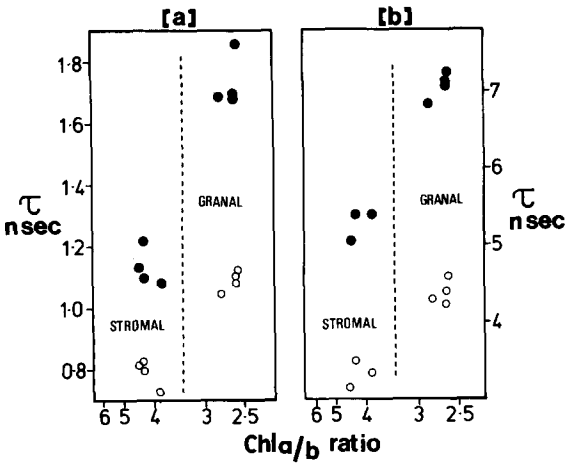


Fig. 3. (a) Rotational correlation times (τ), at room temperature, of 5-doxyldecane in stromal and granal lamellae prepared by sonication of lettuce chloroplasts. The parameters τ_B (open circles) and τ_C (closed circles) are plotted. (b) As in a, but using 12-doxylstearate. The Chl *a*/Chl *b* ratio is plotted on a reciprocal scale.

where τ_B, τ_C are in nanoseconds.

These equations are not strictly valid under the conditions used here [18] and the values given are only intended to provide a quantitative measure of the relative mobility of the spin label. Fig. 2a and b shows the first-derivative ESR spectra of lettuce stromal and granal membranes labelled with 5-doxyldecane and 12-doxylstearate, respectively. The calculated rotational correlation times (τ_B and τ_C) of the two probes in various lamellae types are presented in Fig. 3. The results show that both spin labels are more freely mobile in the fraction derived from the stromal membranes in agreement with the measurements with diphenylhexatriene.

TABLE III

PROTEIN/LIPID RATIOS OF VARIOUS MEMBRANE FRAGMENTS

Protein/acyl lipid weight ratios were measured with pea thylakoid membrane fractions obtained by Yeda press treatment. Ratios were calculated from protein/Chl and lipid/Chl ratios of six experiments except where stated.

Fraction	Protein/lipid (±S.E.)
Total thylakoid	1.98±0.33 (n=4)
High-salt/granal	1.84±0.16
High-salt/stromal	1.22±0.11
Low-salt/heavy fraction	1.65±0.13
Low-salt/light fraction	1.70±0.24

Protein/acyl lipid ratios in the thylakoid membrane fractions are shown in Table III. Values for stromal membranes are significantly different from those for granal fractions ($P<0.002$). At first approximation, the fatty acid compositions of the granal and stromal membranes are similar but there are some small consistent differences as shown in Table IV. Intact thylakoid membranes not subjected to mechanical disruption were found to have a protein/lipid ratio higher than those of the various fragments, suggesting that the Yeda press treatment removes some loosely bound extrinsic membrane proteins.

Discussion

The ordering effect of thylakoid membrane pro-

TABLE IV

FATTY ACID COMPOSITION OF THYLAKOID MEMBRANE AND FRAGMENTS

Mol% fatty acid composition (±S.E.) of thylakoid fractions. Average of three separate experiments (three duplicate analyses per experiment).

Fraction	Fatty acid type				
	16:0	18:0	18:1	18:2	18:3
Total thylakoid	10.2±0.2	1.6±0.1	1.6±0.2	8.8±0.9	77.8±0.7
High-salt/granal	9.4±0.1	1.8±0.2	1.6±0.1	8.0±0.8	79.3±0.6
High-salt/stromal	11.8±0.7	2.4±0.5	1.8±0.2	11.1±0.04	72.9±1.4
Low-salt/heavy	9.5±0.2	1.8±0.1	2.3±0.4	9.5±0.5	76.9±0.3
Low-salt/light	10.0±0.3	1.8±0.1	1.6±0.2	8.4±0.8	78.2±1.1

teins on their lipid environment has previously been investigated using spin-label studies [19,20]. Hiller and Raison [19] found that the fluidity of vesicles composed of extracted thylakoid lipids was higher than that of the natural membrane. Strzałka and Subczynski [20] used a different approach to come to a similar conclusion. They studied the effect of protein synthesis inhibitors on thylakoid membrane fluidity and found that chloroplasts isolated from 7-day-old wheat leaves treated with the inhibitors had a higher lipid fluidity than the untreated control chloroplasts. These workers also noted differences between 'heavy' and 'light' fractions in their chloroplast isolation. The light fraction, containing developing chloroplasts with few grana, had a higher membrane fluidity than the heavy fraction which contained chloroplasts having fully developed grana. They suggested that the fluidity differences observed between the heavy and light fractions were not due to grana formation, but due to differences in the protein/lipid ratios. The results presented in this present study, however, would suggest that the formation of grana is accompanied by an increase in the protein/lipid ratio of the thylakoids as a whole and thus emphasises a close relationship between the two phenomena.

Earlier studies on protein/lipid ratios in chloroplast fragments by Allen et al. [21] suggested that no differences existed between stromal and granal fractions. However, their results were for total lipid, including chlorophyll, which is now widely believed to be part of the light-harvesting protein complexes of the two photosystems [22]. When the weight due to chlorophyll is subtracted from the data given by these workers for both fractions, protein/acyl lipid weight ratios in granal and stromal membranes are approx. 1.9 and 1.7, respectively, the ratio for total thylakoid being 1.9. Thus, even from this earlier study, a lower protein/lipid ratio was indicated for stromal lamellae compared with grana.

Measurements of fluidity made using different probes and different techniques are difficult to compare quantitatively. Different probes may not distribute themselves in the same way in the membrane as well as behaving differently in response to changes in the structural order of the membrane lipids. Nevertheless, in this present study similar differences between the fluidity of membrane fractions were observed using three different probes for fluidity determinations, suggesting that qualitatively significant differences exist in the fluidity of stromal and granal regions.

Our results indicate that electron-transport reactions associated with PS I and located in the stromal lamellae occur in a relatively fluid lipid environment. It seems likely that changes induced by low-salt unstacking, and randomization of the thylakoid membranes [6], would cause a change in the lipid environment of PS I as well as PS II. This factor may be important for studies of the effect of salt-induced stacking changes on electron transport. The effect of salt-induced stacking changes on fluidity in thylakoid membranes has been investigated by Hiller and Raison [19]. They found no significant differences in the order parameters of stacked and unstacked membranes probed with 6-doxylstearate. The same results were obtained in this laboratory (Scoufflaire, C., Ford, R.C. and Barber, J., unpublished results) using diphenylhexatriene fluorescence polarization measurements. These findings are not inconsistent with the conclusions of data presented in this paper provided that an even distribution of the probe exists between granal and stromal regions.

In conclusion, it seems that the lipid matrix of the non-appressed membranes of higher plant chloroplasts is more fluid than that of the appressed membranes of the grana. The difference probably reflects the increased protein level in the partition region of the grana rather than differences in the fatty acid composition of the two membrane regions. Overall, the thylakoid membrane is a relatively fluid system at optimal temperature which presumably reflects the requirement for diffusional processes to occur in order to maintain the most efficient rates of electron transport.

Acknowledgements

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