Biochimica et Biophysica Acta, 725 (1983) 519–528
Elsevier

BBA 41421

SPIN-LABEL STUDIES OF THE LIPID REGIONS OF SPINACH THYLAKOIDS AND A DETERGENT-DERIVED OXYGEN-EVOLVING PHOTOSYSTEM II PREPARATION

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(Received May 31st, 1983)

Key words: Photosystem II; Oxygen evolution; Spin label; Membrane fluidity; Thylakoid membrane; (Spinach chloroplast)

We have compared the fluidity of thylakoid membranes with the membrane present in a Triton X-100-derived, oxygen-evolving Photosystem II (PS II) preparation using two different spin labels. Data obtained with 2,2,6,6-tetramethylpipiridine-*N*-oxyl (TEMPO) shows that the PS II preparation contains less fluid membrane than the thylakoid. The TEMPO partition parameter (f) is about 2.5-times greater for the thylakoids at 6 mg chlorophyll/ml than for the PS II preparation at the same chlorophyll concentration. Similarly, the rotational correlation time, τ, of TEMPO residing in the membrane of the PS II preparation is about 2-times longer than the τ for TEMPO in the thylakoid membrane. A spin label which partitions more completely into the bilayer, 2-heptyl-2-hexyl-5,5-dimethyloxazolidine-*N*-oxyl (7N14), indicates a much greater fluidity in the thylakoid membrane than the membrane of the PS II preparation. The PS II preparation appears to have a hydrocarbon phase which approaches the rigid limit of EPR detectable motion. These results are discussed in terms of possible lipid depletion in the PS II preparation and in terms of lateral heterogeneity of hydrocarbon fluidity in the thylakoid membrane caused by the lateral heterogeneity in protein components.

Introduction

A number of groups have provided evidence consistent with the hypothesis that proteins in the thylakoid membrane tend to decrease the fluidity of the hydrocarbon regions. Hiller and Raison [1] showed that spin labels in vesicles prepared from isolated thylakoid lipids displayed greater mobility than the same spin labels in the thylakoid membranes. Similarly, Strzalka and Subczynski [2] used spin labels to examine membrane fluidity in the thylakoids of control and antibiotic-treated wheat. They were able to manipulate experimentally the protein/lipid ratios in vivo and thereby show that proteins were associated with hindered spin-label mobility. Ford et al. [3] fractionated thylakoids with explosive decompression and sonication and probed the resulting grana and stroma fractions with fluorescent and spin probes. These investigators found differences in membrane fluidity which they attributed to lateral heterogeneity in protein/lipid ratios.

We have also fractionated the thylakoid, relying on the selective dissolution of stromal membrane by a mild detergent to produce an oxygen evolving PS II preparation [4,5]. Goodman et al. [5] have shown that these oxygen-evolving PS II preparations are membrane sheets which closely resemble

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Abbreviations: TEMPO, 2,2,6,6-tetramethylpipiridine-*N*-oxyl; 7N14, 2-heptyl-2-hexyl-5,5-dimethyloxazolidine-*N*-oxyl; chromium oxalate, potassium trioxalatochromiate; Tricine, *N*-tris(hydroxymethyl)methylglycine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll; PS II(I), photosystem II(I).

the appressed regions of thylakoid membrane found in grana stacks. This resemblance is based on the presence and distribution of the large (PS II) particles on the EFs (ectoplasmic fracture face, stacked region) and on the absence of visible unstacked regions in certain types of PS II preparation. We have chosen one of these PS II preparations (which shows no stromal contamination) as a model of granal membrane for use in our spin-label studies.

The hydrophobic regions of spinach thylakoids and an oxygen evolving PS II preparation were probed with two membrane spin labels. We show that the hydrophobic interior of the thylakoid has much different properties than the hydrophobic regions of the PS II preparation, suggesting that there may be significant differences in lipid mobilities in the hydrophobic interiors of granal and stromal membranes.

Materials and Methods

The spin label TEMPO was purchased from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. The spin label 7N14 was a generous gift from Dr. Philip D. Morse, II, who synthesized 7N14 by the methods of Williams et al. [6]. Chromium oxalate was purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.) and used without further purification. All other chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Typical EPR samples of 100 μl were prepared by vigorously mixing 89 μl of 6.75 mg chlorophyll/ml thylakoid or PS II preparation suspension with 1 μl of 100 mM spin label in methanol, and either 10 μl of 400 mM chromium oxalate or the buffer used to suspend the thylakoids (see below). A 60 μl aliquot of this suspension was taken up in a 1×100 mm capillary, which was then placed in a 4×300 mm quartz tube.

All EPR spectra were recorded on a Varian E-9 spectrometer using an E-231 cavity with temperature control. Rotational correlation times, τ, were calculated from the Kivelson equation [7–9] as we have described earlier [10], or by techniques of Mason and Freed [11] for 7N14 near the rigid limit. The relative amount of anisotropy was calculated by the method of Cannon et al. [12] (see Table II). Due to the anisotropy found in our thylakoid and PS II preparations, our calculations of τ should only be considered close approximations. Thus, we do not place emphasis on the actual values of τ calculated. Rather, we relate the τ back to systems of known viscosity such as water. To do this we calculate the apparent microviscosity, $\eta = \tau(\text{sample})/\tau(\text{water})$, or in the case of 7N14, which is not appreciably soluble in water, $\eta_0 = \tau(\text{sample})/\tau(\text{oleic acid})$. The partition parameter, f, was calculated as described by Hubble and McConnell [13], and was used to estimate the relative amount of fluid membrane in both the thylakoid and the PS II preparation.

Thylakoids were isolated from deveined leaves of market *Spinacea oleracea*. The washed leaves (200 g) were homogenized in 330 ml 0.4 M NaCl/5 mM $MgCl_2$/20 mM Tricine (pH 7.5) for about 20 s in a blender. The suspension was filtered through eight layers of cheesecloth and a green pellet was collected at $12\,000 \times g$ for 5 min. This pellet was resuspended in 80 ml 15 mM NaCl/5 mM $MgCl_2$/20 mM Tricine (pH 7.5). The resulting suspension was subjected to a mild centrifugation at $500 \times g$ for 20 s to pellet nuclei and cell-wall debris. The carefully decanted green suspension was then centrifuged at $18\,000 \times g$ for 5 min and the resulting pellet was resuspended in the 15 mM NaCl buffer above to 6.75 mg chlorophyll/ml.

The oxygen evolving PS II preparation was prepared by a modification of Berthold et al. [14]. Deveined spinach (250 g) was homogenized in about 300 ml 0.4 M NaCl/5 mM $MgCl_2$/20 mM HEPES (pH 7.5). The suspension was filtered through eight layers of cheesecloth and centrifuged at $17\,000 \times g$ for 5 min. The resulting pellet was resuspended in 80 ml 15 mM NaCl/5 mM $MgCl_2$/20 mM HEPES (pH 7.5) and subjected to centrifugation at $12\,000 \times g$ for 5 min. The resulting pellet was resuspended in 80 ml 15 mM NaCl buffer solution and then centrifuged at $500 \times g$ for 20 s to remove cell debris and nuclei. The carefully decanted supernatant suspension was then centrifuged at $12\,000 \times g$ for 5 min. The resulting pellet was resuspended in the 15 mM NaCl buffer to exactly 4 mg chlorophyll/ml and then an equal volume of the 15 mM NaCl/50 mg Triton X-100/ml was added. This suspension was stirred gently at 4°C for 25 min followed by centrifuga-

tion at 40 000 × *g* for 30 min. The resulting pellet was resuspended in the 15 mM NaCl buffer to 1 mg chlorophyll/ml and then an equal volume of the 15 mM NaCl buffer containing 5 mg Triton X-100/ml was added. The suspension was immediately centrifuged at 40 000 × *g* for 30 min. The resulting pellet was resuspended in the 15 mM NaCl/0.4 M sucrose to 6.75 mg chlorophyll/ml. This preparation could be stored at −70°C indefinitely without noticeable change in oxygen evolving activity.

Electron transport activity was measured as previously described by Ogilvie et al. [4] and is detailed in Table I. Chlorophyll concentrations were determined by the methods of Arnon [15].

Phospholipid vesicles were prepared from 1 g of egg phosphatidylcholine dissolved in chloroform/methanol, 2 : 1. This solution was rotoevaporated onto the walls of a 50 ml flask. 20 ml 5 mM $MgCl_2$/160 mM NaCl were added to flask along with twelve 4 mm glass beads. this mixture was rotated for 30 min followed by sonication to make predominantly single bilayer vesicles. The vesicles were centrifuged at 12 000 × *g* to a pellet which was resuspended in a minimal volume of 5 mM $MgCl_2$ with 160 mM NaCl solution. The vesicle suspensions were bubbled with nitrogen gas and frozen at −10°C under nitrogen.

Results

In this study, we use spin labels to probe the hydrocarbon regions of thylakoid membranes and a detergent-derived PS II preparation. In order to relate results obtained with the PS II preparation with those of thylakoids, grana membrane or stroma, one requires considerable characterization of the biochemical properties of the PS II preparation. Many PS II preparations have been described and characterized elsewhere [16], and so here we provide only the specific data characterizing the PS II preparation used in this report. Table I shows that sequential electron transport through both photosystems from water to methyl viologen is completely absent in the PS II preparation. Even the PS I-dependent partial reaction of reduced diaminodurene to methyl viologen is almost completely absent. Interestingly, the EPR-detectable Signal I shows that a significant amount (26%) of P-700 still remains in the PS II preparation in spite of the two Triton washes used in the isolation procedure. When quinones such as DCBQ are used as the electron acceptors, high rates of PS II-mediated [4,14] electron transport are still possible with the PS II preparation. The chlorophyll *a*/*b* ratios for our thylakoid and PS II preparation are 1.83 and 1.42, respectively, suggesting either a

TABLE I

BIOCHEMICAL CHARACTERIZATION OF THE OXYGEN-EVOLVING PS II PREPARATION

Thylakoids and PS II preparation were isolated as described in the section Materials and Methods. Electron transport was measured as oxygen production ($H_2O \rightarrow$ 2,6-dichloro-*p*-benzoquinone [DCBQ]) or methyl viologen (MV)-mediated oxygen uptake ($H_2O \rightarrow$ MV and diaminodurene (DAD)/ascorbate (ASC) → MV with a clark electrode in a thermostatically controlled 2 ml chamber. Actinic illumination was saturating. The reaction mixture contained, for the methyl viologen-mediated reactions: 100 mM sucrose, 25 mM KCl, 3 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N*-3-propanesulfonic acid (HEPES) (pH 8.0), 50 μM methyl viologen, 1 mM diaminodurene, 10 mM sodium ascorbate and 10 mM methylamine and thylakoids or PS II preparation equivalent to 200 μg chlorophyll/ml. The PSI reaction (DAD/ASC → MV) also contained 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to inhibit sequential electron transport from PS II. Electron transport mediated by 2,6-dichloro-*p*-benzoquinone (DCBQ) was measured as light-driven oxygen production in a reaction mixture containing: 5 mM $MgCl_2$, 50 mM NaCl, 20 mM Pipes (pH 6.6), and either thylakoids or PS II preparation equivalent to 100 μg chlorophyll/ml. P700 was determined as EPR-measurable Signal I [14]. Chlorophyll *a* and *b* concentrations were determined by the methods of Arnon [15].

	$H_2O \rightarrow$ MV (μeq·mg Chl^{-1}·h^{-1})	DAD/ASC → MV (μeq·mg Chl^{-1}·h^{-1})	$H_2O \rightarrow$ DCBQ (μeq·mg Chl^{-1}·h^{-1})	% EPR detectable Signal I (P700)	Chl *a* Chl *b*
Thylakoids	1100	1300	940	100%	1.83
PS II	0	18	620	26%	1.42

relative depletion of chlorophyll *a* or an enrichment of chlorophyll *b*. The biochemical characterization of the PS II preparation in Table I, together with our more detailed characterization [4,5], is consistent with a considerable enrichment for PS II relative to PS I and, depending on whether PS I is an integral part of granal association, could be consistent with the substantial isolation of the granal association away from stromal contamination.

Although ambiguities remain in the relationship between the thylakoid and the PS II preparation (especially with regard to the likelihood of partial lipid depletion during the detergent treatment), it is not our purpose to resolve them here and so we now direct our attention to the spin-label experiments. Fig. 1 shows the spectra obtained in our attempt to monitor the relative amounts of fluid

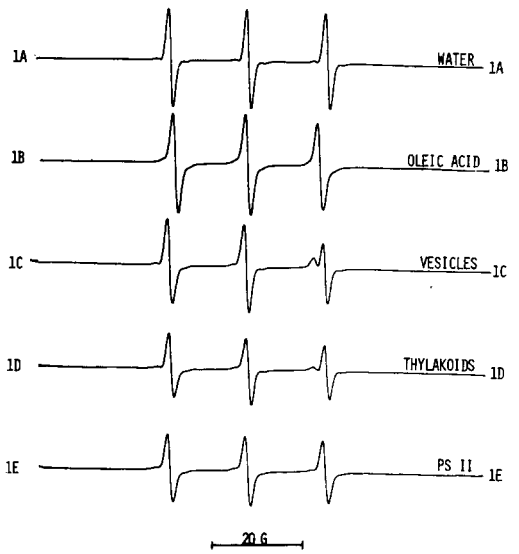

Fig. 1. 100 G spectra of TEMPO in various environments. All spectra were taken with the following instrument settings: field set, 3245 G; time constant, 0.5 s; scan time, 8 min; modulation amplitude, 0.5 G; modulation frequency, 100 kHz; microwave power, 5 mW; and microwave frequency, 91 GHz. The temperature in the EPR capillary was 19°C. The instrument gains for the spectra are: (A) 10^{+3}; (B) $3.2 \cdot 10^{+2}$; (C) $2.5 \cdot 10^{+3}$; (D) $8 \cdot 10^{+2}$; (E) $5 \cdot 10^{+2}$. Each sample except the water sample contained 2 mM TEMPO. The water sample contained 1 mM TEMPO. Samples were prepared as described in the section Materials and Methods and were contained in 100 μl capillaries inside 4×60 mm quartz NMR tubes. The thylakoids and the PS II preparation were both at a concentration equivalent to 6.75 mg chlorophyll/ml.

membrane both in our PS II preparation and in our Class II thylakoids. To do this, we employ the techniques of Hubble and McConnell [13] who have studied the partition of TEMPO, between the fluid hydrophobic regions of model membranes and the aqueous suspending medium. The solubility of TEMPO in the membrane is highly dependent on the relative fluidity of the hydrocarbon phase and thus, TEMPO partition has been used as an assay for the fraction of lipids present in the fluid state [17–19]. Fig. 1A shows the spectrum of TEMPO in water. It is characterized by a hyperfine coupling constant (A_n) of 17.2 G. Fig. 1B is the spectrum of TEMPO dissolved in oleic acid. The non-polar character of this solvent results in $A_n = 15.9$ G. When TEMPO is added to a suspension of vesicles made from egg phosphatidylcholine (Fig. 1C), the high-field line of the resultant spectrum has a small, clearly visible membrane component. By measuring the amplitude of the membrane (H) and the aqueous (P) components as described by Shimshick and McConnell [17,18], it is possible to calculate a TEMPO spectral parameter $f = H/(H + P)$. This spectral parameter is approximately equal to the fraction of the spin label dissolved in the membrane bilayer [17,18].

The addition of TEMPO to a thylakoid suspension at 6 mg chlorophyll/ml, results in a spectrum (Fig. 1D), which shows that a small amount of TEMPO partitions into the thylakoid membrane. From Fig. 1D we calculate an $f = 0.08$. Spectrum 1E shows that significantly less TEMPO ($f = 0.03$) will partition into the PS II preparation at the same chlorophyll concentration.

Spectra like 1D and 1E are not suitable for calculation of correlation times due to the large, superimposed aqueous signal which obscures the more interesting membrane signal. However, we have shown elsewhere [10] that it is possible to selectively remove the external aqueous signal. These techniques require the use of membrane impermeable broadening agents (such as chromium oxalate) to broaden the external aqueous signals to near invisibility. When broadening agents are added to various preparations, spin labelled with TEMPO, it is possible to observe the spectra of TEMPO residing in the hydrocarbon regions of the membrane, without significant contribution by

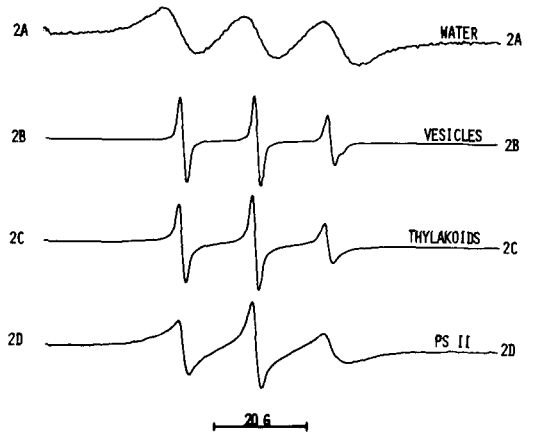

Fig. 2. EPR spectra of TEMPO in the presence of chromium oxalate, a broadening agent which was added to broaden the external aqueous signal to near invisibility. Instrument settings are as described in Fig. 1. The receiver gains were: (A) 10^{+5}; (B) 10^{+3}; (C) $8 \cdot 10^{+3}$; (D) $2 \cdot 10^{+4}$. Each sample contained 40 mM chromium oxalate and 1 mM TEMPO. Other sample conditions and contents were as described in Fig. 1.

the external aqueous signal.

Fig. 2 shows the spectra obtained when both TEMPO and chromium oxalate are present in the various preparations. Spectrum 2A is obtained when TEMPO and chromium oxalate are simply mixed in water. Note from the noise level that the instrument gain is much greater ($50\times$) than for the other spectra. Fig. 2B shows the spectrum obtained when chromium oxalate and TEMPO are added to a suspension of phospholipid vesicles. In contrast to spectrum 1C, we now see a major component with an $A_n = 15.8$ G and a minor component at approx. 17.2 G. The minor component arises from TEMPO which has partitioned from the membrane into the aqueous lumen of the phospholipid vesicles. When TEMPO and chromium oxalate are added to a thylakoid suspension (2C) the A_n obtained is 15.9 G and there is no evidence of an internal aqueous signal. Similarly, in spectrum 2D, the PS II preparation displays a spectrum with $A_n = 16.0$ G. However, the instrument gain required to produce this spectrum is higher than that required for either the thylakoid or vesicle preparations and thus, the aqueous broadened signal is more visible than in Fig. 2B and C.

Since we can observe the spectrum of the spin label residing in the membranes hydrophobic interior, it is possible to calculate motion parameters such as the rotational correlation time (τ) for TEMPO in the various preparations. By employing the formalisms of Kivelson [7–9] and Keith et al. [9], one finds that in oleic acid (1B) the $\tau = 9.2 \cdot 10^{-10}$ s. In phospholipid vesicles (Fig. 2B) the $\tau = 4.1 \cdot 10^{-10}$ s. The value of τ should be evaluated only in relative terms, since the formalisms apply only to isotropic motion, and there is clear evidence of anisotropic motion, especially in the PS II preparation. For convenience in comparing these numbers, we define the membrane fluidity index, $\eta_0 = \tau(\text{sample})/\tau(\text{oleic acid})$. Applying the definition to our vesicles, thylakoids, and PS II preparation, we find (for TEMPO) that $\eta_0 = 0.44$, 0.67, and 1.4, respectively.

The relative amount of anisotropy in the various preparations can be estimated with the formalisms of Cannon et al. [12]. The calculated anisotropy index, ΔT, is associated with spin-label anisotropy and it increases from 0 in isotropic solutions, to larger values when spin-label motion

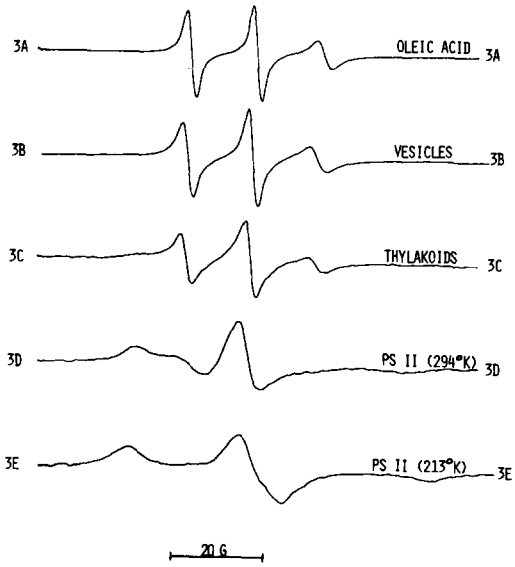

Fig. 3. EPR spectra of 7N14 in various environments. Instrument settings were as described in Fig. 1 except that the time constant was increased to 1 s and the modulation amplitude was increased to 2 G. All samples were prepared by mixing 99 μl sample with 1 μl 25 mM 7N14 (in methanol). Receivers gains were: (A) $1.25 \cdot 10^{+3}$; (B) $6.3 \cdot 10^{+3}$; (C) $2 \cdot 10^{+4}$; (D) $3.2 \cdot 10^{+4}$; (E) $3.2 \cdot 10^{+4}$. All spectra were obtained at 294 K except (E) which was obtained at 213 K.

TABLE II

EPR PARAMETERS MEASURED OR CALCULATED FROM THE SPECTRA SEEN IN FIGS. 1–3

The relative microviscosity η cannot be calculated for 7N14, since 7N14 does not partition appreciably into water. The f parameter is a measure of spin-label partition into the membrane and was calculated by the method of Hubble and McConnell [13,17]. A_n is the hyperfine coupling constant measured from the spectra. τ is the rotational correlation time calculated by the equations of Kivelson [7,8]. τ^* is the rotational correlation time estimated from the immobilized spectrum 3D by the methods of Mason and Freed [11]. ΔT is associated with the anistropy of probe motion and is calculated by the methods of Cannon et al. [12]. η is the microviscosity of the spin label environment relative to water. η_0 is an index of probe motion relative to the probe motion in oleic acid.

	Spin label	40mM chromium oxalate	Spectrum number	f parameter	A_n (G)	τ (s)	τ^* (s)	ΔT	$\eta = \frac{\tau(\text{sample})}{\tau(\text{water})}$	$\eta_0 = \frac{\tau(\text{sample})}{\tau(\text{oleic acid})}$
Water	TEMPO	–	1A	–	17.2	$0.24 \cdot 10^{-10}$	–	0.0081	1	–
	TEMPO	+	2A	–	17.5	$0.24 \cdot 10^{-10}$	–	$0.008 \cdot 10^{-10}$	–	–
Oleic acid	TEMPO	–	1B	–	15.9	$9.2 \cdot 10^{-10}$	–	0.032	38	1
	7N14	–	3A	–	14.4	$13 \cdot 10^{-10}$	–	$2.8 \cdot 10^{-10}$	–	1
Vesicles	TEMPO	–	1C	0.24	17 and 16	–	–	–	–	–
	TEMPO	+	2B	–	17 and 16	$4.1 \cdot 10^{-10}$	–	$0.97 \cdot 10^{-10}$	17	0.44
	7N14	–	3B	–	14.2	$17 \cdot 10^{-10}$	–	$6.6 \cdot 10^{-10}$	–	1.3
Thylakoids	TEMPO	–	1D	0.081	17 and 16	–	–	–	–	–
	TEMPO	+	2C	–	15.9	$6.2 \cdot 10^{-10}$	–	$2.7 \cdot 10^{-10}$	26	0.67
	7N14	–	3C	–	14.0	$23 \cdot 10^{-10}$	–	$9.8 \cdot 10^{-10}$	–	1.8
PS II	TEMPO	–	1E	0.031	17.1	–	–	–	–	–
	TEMPO	+	2D	–	16.0	$13 \cdot 10^{-10}$	–	$9.2 \cdot 10^{-10}$	54	1.4
	7N14	–	3D	–	–	–	1×10^{-7}	–	– *	77 *

is anisotropic. As can be seen in Table II, ΔT varies over three orders of magnitude from $0.008 \cdot 10^{-10}$ s for water to $9.2 \cdot 10^{-10}$ s for the PS II preparation. The anisotropy index calculated for TEMPO in the various preparations varies such that the PS II preparation anisotropy > thylakoid > vesicle > oleic acid > water.

As discussed above, observation of a partitioning spin label in the membrane requires the use of a membrane impermeable broadening agent to reduce the aqueous signal to near invisibility. However, other spin labels are available which partition almost completely into the hydrocarbon phase, obviating the broadening agent. One of these spin labels is 7N14. Fig. 3 shows the spectra obtained when 7N14 is applied to the various preparations which we are considering. The spectra 3A, 3B, and 3C all lend themselves to estimation of τ, η, and ΔT by the formalisms used above for TEMPO. The membrane fluidity index, η_0, for thylakoids and vesicles are 1.3 and 1.77, respectively (Table II). These values are higher than those seen for TEMPO, but the order of increasing viscosities is the same. Similarly, the estimation of anisotropy with ΔT shows that the anisotropy of thylakoids > vesicles > oleic acid.

When 7N14 is applied to the PS II preparation (3D) a spectrum is obtained which approaches the rigid limit of EPR detectable motion. In order to estimate the motional parameters responsible for such a spectrum, one must apply techniques different from those applied to the more freely moving spin labels described above. Mason and Freed [11] have developed a simple method for estimating rotational correlation times in the microsecond region by using the widths of the outer EPR hyperfine extrema, relative to the widths of the outer hyperfine extrema of samples at the rigid limit. When the sample giving rise to spectrum 3D was cooled from 294 to 213 K, no further change in the spectrum was noted and thus, spectrum 3E was taken as the empirical rigid limit spectrum. Using these two spectra it is possible to estimate $\tau = 1 \cdot 10^{-7}$ s. This allows us to estimate that the microviscosity is about 4000-times greater than water.

Discussion

We chose to probe thylakoids and the detergent derived PS II preparation with the spin label TEMPO, because this spin label has been shown to partition readily into regions of greater membrane fluidity [19]. The extent of partition depends on a number of parameters, including the amount of fluid membrane available and its relative fluidity. We chose to normalize our thylakoid and PS II preparations to 6 mg chlorophyll/ml rather than to a constant lipid concentration. Since all chlorophyll is thought to be bound to protein [20,21], we actually normalize the protein concentrations in the two preparations. Thus, the difference in the f parameter between the thylakoids and the PS II preparation could be interpreted in terms of greater membrane fluidity in the thylakoid relative to the PS II preparation or in terms of less membrane lipid being available for partition in the PS II preparation. Spectral analysis speaks to this issue, since τ of TEMPO in the PS II preparation is about twice as long ($13 \cdot 10^{-10}$ s vs. $6.2 \cdot 10^{-10}$ s) as that of TEMPO in the thylakoid. Thus, at least part of the difference in f should be due to reduced membrane fluidity in the PS II preparation.

As discussed above, many studies have related the presence of integral membrane proteins with the reduced membrane fluidity in thylakoids [1–3,22–26]. Thus, it is conceivable that the more hindered motion in the PS II preparation relative to the thylakoid could be explained by a greater protein/lipid ratio in the PS II preparation. A number of studies have shown that the protein/lipid ratios for grana preparations are greater than those of stroma preparations [3,27], and so it would not be surprising if our PS II preparation also had a higher protein/lipid ratio. A larger protein/lipid should produce a preparation with a greater density, and this was observed by Ogilvie et al. [4], when the PS II preparation was subjected to sucrose buoyant density gradient analysis. Thus, we believe that the PS II preparation does have a larger protein/lipid ratio than the thylakoid, and this larger ratio would be expected to be manifest in reduced membrane fluidity and a decreased f parameter under conditions of normalized protein concentration. These are exactly the results observed in this communication. Spectra

characteristic of highly immobilized spin labels have also been reported in a number of other membrane systems such as *Halobacterium cutirubrum* membrane [22]; Sindbis virus membrane [23]; purple membrane from *Halobacterium halobium* [24]; and chromatophores from *Rhodopseudomonas sphaeroides* [25]. All these systems display large protein/lipid ratios, confirming the general hypothesis that proteins profoundly affect the mobility of their adjacent lipids.

Another concern is related to intramembrane partitioning. Once the spin label enters the hydrocarbon phase by partition from the aqueous phase, it must also satisfy its required partition between regions of more fluid membrane (stroma?) and regions of less fluid membrane (grana?). Hubble and McConnell [13] have shown that we must expect this type of intramembrane partitioning with spin labels. Thus, questions arise as to the extent of this intramembrane partition. For instance, in Yeda press-produced grana preparations like those used by Ford et al. [3], what fraction of more fluid stroma membrane remains? When one probes these preparations with fluorescent or spin probes, what fraction of the reporting molecules partition preferentially into the more fluid stroma contamination? How does one interpret the resulting spectra, which are certainly a summation of all possible environments of the probe? These types of consideration could explain the more modest differences in EPR spectra between stroma and grana preparations reported by others [2,3].

In the PS II preparation which we employ, there is no stromal contamination and the preparation appears highly homogeneous [5], but new ambiguities arise. The preparation is produced by gentle detergent treatment of thylakoids in the presence of sufficient Mg^{2+} to maintain the appressed regions of membranes in tight association [5]. The use of detergents may selectively remove fluid membrane lipids from between the closely associated PS II complexes, leaving primarily the more tightly associated boundary lipids, or the detergent may simply replace the natural galactolipids and give rise to highly reduced mobility which our spin labels are reporting. In thin-layer chromatographic studies of the polar lipids of the PS II preparation (unpublished data), we have noted a substantial difference in lipid quantity (relative to chlorophyll), but not in lipid type, between thylakoids and the PS II preparation. This is curious, since the electron microscopy has revealed no large change in the spacing of the EFs particles in the PS II preparation. The spacing remains the same regardless of whether one washed with Triton once (see Ref. 5) or twice as described in this paper. Thus, if substantial lipid depletion has occurred, there must be concomitant detergent replacement of the lost lipids. However, it is interesting to note that Stewart and Bendall [28] have reported that significant amounts of mono- and digalactosyldiacylglycerols remained associated with their PS II preparation isolated with detergents from a cyanobacterium.

The second probe (7N14) was chosen for its almost complete partition into the hydrocarbon phase of the membrane. The difference in partition is due to the long, highly hydrophobic hexyl and heptyl groups on the 7N14 molecule. Studies with related spin labels (doxyl derivatives of stearic acid) have shown that the nitroxyl group is deeply buried in the hydrocarbon matrix [29], and so we are confident that the 7N14 is probing the hyphobic interior of the membrane. This conclusion is strongly supported by the observation that the hyperfine coupling constant of 7N14 in oleic acid is actually slightly greater than that observed in either phospholipid vesicles or thylakoid membranes. The carboxyl group of the stearic acid spin labels orients these probes in the membrane. Thus, the nitroxyl is placed at a fixed distance from the aqueous interface [29]. 7N14 has no strongly ionizable group to orient it in the membrane and so it probably varies in location and samples the entire hydrophobic phase.

The results obtained with 7N14 are entirely consistent with those obtained with TEMPO, except that the magnitude of the fluidity differences is exaggerated with 7N14. Thus, when one calculates the relative membrane fluidity (η_0) of thylakoid membranes from the spectra of TEMPO and 7N14, one consistently observes higher apparent viscosity with 7N14 (see Table II). This is particularly apparent in the PS II preparation, where 7N14 reports an apparent microviscosity which is 55-times greater than that reported by TEMPO. This difference is presumably due to hexyl and heptyl groups of the 7N14 which must

interact strongly with either the immobilized boundary lipids surrounding the protein complexes buried in the membrane, or perhaps with the hydrophobic side groups of the proteins themselves. These interactions must greatly hinder the motion of 7N14 relative to TEMPO. The hexyl and heptyl groups of 7N14 also have profound effect on the measurement of the anisotropy of the system. The index of anisotropy, ΔT, is in vesicles 120-times greater than in water as observed in experiments using TEMPO, but 7N14 results give the anisotropic character as 825-times greater than water. Again, we believe that the extra hydrocarbon groups of 7N14 strongly influence the information which is derived from the spectra of the two spin labels.

These differences are important, however, since they suggest that molecules with long hydrocarbon groups may experience considerable motional restraint when they reside in close juxtaposition with integral membrane proteins. This type of interaction could strongly influence the relative mobility of molecules such as plastoquinone residing in close associations with protein complexes. Since plastoquinone mobility is a critical component of recent models of photosynthetic electron transport, which laterally separate PS II from PS I [30], one would like to be assured that the lateral diffusion coefficients of plastoquinone in grana as well as stroma, are adequate to explain observable rates of electron transport. Using TEMPO and 7N14 we cannot speak directly to these diffusion coefficients of plastoquinone, but experiments are underway to measure these coefficients directly.

Our results do suggest, however, that the spin label 7N14 is highly immobilized in the PS II preparation. The clear similarity between spectrum 3D at 294 K and the spectrum obtained when the same sample is cooled to the rigid limit at 213 K (spectrum 3E), strongly suggests a highly hindered motion. Estimation of the correlation time [11], confirms the immobilization. The correlation time is about 10^{-7} s, or about 40-times more hindered than the same spin label in the thylakoid membrane or about 75-times more hindered than the same spin label in oleic acid. These correlation times are not necessarily inconsistent with the lateral mobility of plastoquinone suggested by Anderson [30], since the rate limiting step of sequential electron transport is probably the oxidation of plastohydroquinone, and this reaction has a half-time of 20 ms [31]. Our results do, however, suggest that diffusion coefficients for grana and stroma regions may be different, and the relative mobility of plastoquinone may be altered by adjacent proteins.

Acknowledgements

This work was supported by grant No. 82-CRCR-1-1125 from the Competitive Research Grants Office of the United State Department of Agriculture to S.P.B. The authors are grateful to Professor Gareth Eaton of the Department of Chemistry at the University of Denver for the use of his EPR facility. The authors also thank Dr. Philip D. Morse, II, for his generous gift of the 7N14 used in this study.

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