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Spin label saturation transfer ESR studies of protein-lipid interactions in Photosystem II-enriched membranes

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Saturation transfer ESR has been used to study the dynamic behaviour of lipids in the appressed regions of thylakoid membranes from pea seedlings. Four different phospho- and galacto-lipid spin labels (phosphatidylcholine labelled at the 12 or 14 C-atom positions of the *sn*-2 chain, phosphatidylglycerol labelled at the 14-position of the *sn*-2 chain, and monogalactosyldiacylglycerol labelled at the 12-position of the *sn*-2 chain) were used to probe the lipid environment in photosystem II-enriched membranes prepared by detergent extraction. The ESR spectra show that the majority of the lipid in these preparations is strongly motionally restricted. Values for the effective rotational correlation times of the labelled chains were deduced from the lineheight ratios and integrals of the saturation transfer ESR spectra. The effective rotational correlation times were found to be in the 10^{-5} s range, indicating a very low lipid chain mobility which correlates with the low lipid content of these preparations. Comparison of the effective rotational correlation times deduced from the different diagnostic regions of the spectrum revealed little anisotropy in the chain mobility, indicating that the dominant motional mode was *trans-gauche* isomerization. The effective rotational correlation times deduced from the spectral integrals were similar to those deduced from the lineheight ratios, consistent with the absence of any appreciable fluid lipid component in these preparations. The results also indicate some selectivity of interaction between the lipid species, with phosphatidylcholine exhibiting appreciably slower motion than either phosphatidylglycerol or monogalactosyldiacylglycerol.

Introduction

The thylakoid membrane system of green plants is the site of all the photosynthetic light reactions (see, for example, Ref. 1). Thylakoids appear to consist of a series of stacked membranes which are either 'appressed' or 'non-appressed'. Although laterally contiguous, the appressed and non-appressed regions differ in their protein [1–3] and possibly in their lipid [4,5] compositions. The Photosystem II (PS II) protein-pigment complex is largely found in the appressed regions, whereas the Photosystem I and ATP synthetase complexes are found mainly in the non-appressed regions. The finding by some authors of lateral heterogeneity in the distribution of acyl lipids in thylakoid membranes may imply that there are specific associations between certain lipids and the heterogeneously distributed protein complexes such as PS I and PS II.

The acyl lipid population of thylakoid membranes is unusual, compared with that of membranes of non-plant origin, in the preponderance of neutral, polyunsaturated galactolipids, which make up 75% of the total lipids [1,6]. The remainder of the thylakoid lipid is composed largely of more saturated, anionic sulpholipids or phospholipids. Various roles have been proposed for these lipids in the stabilization of thylakoid structure [1,6,7]

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Abbreviations: *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; 14-PGSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; 12-MGDGSL, 1-oleoyl-2-[12-(4,4-dimethyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-galactose; EDTA, ethylenediamine tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid; PS II, Photosystem II; PS I, Photosystem I; LHC II, light-harvesting complex II; STESR, saturation transfer ESR; V_1, first harmonic ESR absorption signal detected in-phase with respect to the field modulation; V_2', second harmonic ESR absorption signal detected 90° out-of-phase with respect to the field modulation.

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or in the optimization of its photosynthetic function [1]. The possibility of specific lipid–protein interactions has been addressed both in membrane reconstitution studies, and in investigations of the effects of lipolytic cleavage on membrane function. The conflicting conclusions reached by these studies (reviewed in Refs. 1 and 6) point to the need for direct physical measurements on the protein-lipid associations.

The hydrophobic region of the thylakoid lipid bilayer acts as a channel for the passage of the electron carrier, plastoquinone, between Photosystem II and the cytochrome *b/f* complex [1,8]. The diffusion of plastoquinone/plastoquinol through the thylakoid membrane and/or the reoxidation of plastoquinol is believed to be the rate-limiting step in photosynthetic electron transport. The nature and influence of the hydrophobic core of the thylakoid membrane therefore deserves further attention. It is generally agreed that the fluidity of the lipid chains of thylakoid membranes is lower in the appressed than in the non-appressed regions [9–14]. Indeed, it has been found that the lipid chain motion in the hydrophobic regions of the appressed membranes approaches the rigid limit of conventional spin label ESR spectroscopy [11,15]

For the above reasons, we have undertaken a series of studies of the lipid chain mobility and lipid–protein interactions in thylakoid membranes and subthylakoid membrane fractions, using as probes spin-labelled galacto- and phospholipids which are structurally similar to their counterparts in thylakoid membranes [16,17]. These studies have shown that much of the lipid in PS II-enriched subthylakoid fractions prepared by detergent extraction is motionally restricted. Since these preparations retain oxygen-evolving capacity, the motional requirements for function merit further investigation. In the present work we have attempted to define the degree of chain motional restriction in greater detail, for the various lipid classes, by using saturation transfer ESR (STESR) spectroscopy. STESR extends the motional limit of conventional spin-label ESR spectroscopy to rotational correlation times in the 10^{-7} to 10^{-3} s range [18] and is therefore well suited to a study of the lipid chain motion in the appressed regions of the thylakoid membrane.

Materials and Methods

Spin-labelled phosphatidylcholines, *n*-PCSL, were prepared from the corresponding spin-labelled stearic acids and egg lysophosphatidylcholine as described in Ref. 19. Spin-labelled phosphatidylglycerol, 14-PGSL, was prepared from the corresponding spin-labelled phosphatidylcholine by headgroup exchange catalyzed by phospholipase D [19]. Spin-labelled monogalactosyldiacylglycerol, 12-MGDGSL, was a gift from Dr. I. Nishida, National Institute for Basic Biology, Okazaki, Japan, and was prepared as described in Ref. 20.

Pea seeds (*Pisium sativum* L, var. Kelvedon Wonder) were germinated directly in vermiculite, kept in the dark for the first 5 days at 25°C, and then transferred to a controlled growth room (20°C, 12 h, light/dark). Leaves were harvested after approximately 14 days of growth, following a period of 12 h dark (to reduce starch levels) and 1 h light. The leaves were homogenized in grinding medium (330 mM glucose, 50 mM Na_2HPO_4, 50 mM K_2HPO_4, 5 mM $MgCl_2$, 25 mM NaCl (p 6.5)) with two bursts of 2 s in a chilled blender. The homogenate was filtered through eight layers of muslin, and the filtrate centrifuged briefly (30 s, 2500 × *g*) to sediment the chloroplasts. The pellets were then resuspended and vortex mixed gently for 20–60 s in a small volume of 5 mM $MgCl_2$. The stacked thylakoids were then twice washed free of lysed chloroplast envelopes, by further centrifugation after resuspension in 330 mM sorbitol, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.6). The final pellets were resuspended in the same buffer and kept in the dark at 4°C until further use.

The chlorophyll *a/b* ratio of both leaves and thylakoid preparations was measured by the method of Arnon [21]. Values for the thylakoid preparations were similar to those for the intact leaves (approx. 2.4). Any preparations with appreciably lower values were discarded. The protein content of the thylakoid preparation was measured by the method of Markwell et al. [22] and the lipid content was determined by gas chromatographic and gravimetric analysis as described in Ref. 23. The lipid/chlorophyll/protein ratio was determined to be 33:8:58 (w/w). The intactness and purity of the thylakoid preparations were also checked by thin section electron microscopy, according to the methods of Telfer et al. [24]. The electron micrographs showed that the preparations were essentially free of contamination by other membranes and organelles.

Photosystem II-enriched membranes were prepared by detergent extraction according to Ref. 25. Thylakoid membranes were suspended in stacking medium (330 mM sorbitol, 5 mM $MgCl_2$, 15 mM NaCl, 50 mM Hepes (pH 7.0)). The suspension was then diluted to a concentration of 2 mg chlorophyll/ml and incubated in the dark for 25 min after addition of Triton X-100 at a concentration of 50 mg/ml. The PS II-enriched membranes were then harvested by centrifugation at 40000 × *g* for 30 min and the membrane pellets resuspended in 300 mM sucrose, 5 mM $MgCl_2$, 15 mM NaCl, 20 mM Hepes (pH 7.0). The lipid/chlorophyll/protein ratio of the PS II-enriched membranes was determined to be 16:17:67 (w/w).

The purity of the PS II membrane preparations was checked by polyacrylamide gel electrophoresis. The gel patterns were enriched in the PS II and LHC II centres,

but contained very little of the 18–25 and 70 kDa polypeptides characteristic of the PS I reaction centres. Thus there was little cross-contamination of the two photosystem complexes. The lipid compositions of the thylakoid and of the PS II membranes were determined as described previously [4].

For spin-labelling, PS II-enriched membranes comprising approx. 2 mg of polar lipids were suspended in 2–3 ml of 20 mM Mes, 50 mM KCl, 5 mM $MgCl_2$ (pH 6.5) and 20 μl of 1 mg/ml spin label solution in ethanol was added slowly. The sample was then vortex mixed briefly and incubated for 15–20 min in the dark at room temperature. The spin-labelled sample was centrifuged (45 min, 90000 × g, 4° C) and washed in 13 ml of the same buffer, using a Beckmann SW-40 swing-out rotor. The pellet from the final centrifugation was transferred with about 10 μl of buffer to a 100 μl capillary (1 mm i.d.) sealed at one end. The sample was then packed using a bench-top centrifuge, excess buffer removed, the sample trimmed to 5 mm in length, and the capillary flushed with argon prior to sealing. Argon-flushed buffers were used throughout the sample preparation and the capillary tubes were wrapped with aluminium foil to minimize light-induced ESR signals.

ESR spectra were recorded on a Varian Century Line 9 GHz spectrometer interfaced to a PDP 11/10 computer for data collection. The 5 mm samples in the 1 mm I.D. capillaries were carefully centered in the TE102 rectangular cavity, following the protocol of Ref. 26. The capillaries were accommodated within a standard 4 mm quartz ESR tube which contained light silicone oil for thermal stability. Temperature was regulated using a nitrogen gas flow system. Conventional in-phase absorption spectra (V_1 display) were recorded at a modulation amplitude of 1.6 G p-p, at the same microwave power as used for the STESR experiments. STESR spectra were recorded in the second harmonic, 90° out-of-phase, absorption mode (V_2' display) at a modulation frequency of 50 kHz and with a modulation amplitude of 5 G p-p. All STESR measurements were performed at a microwave power which gave an average H_1 field over the sample of 0.25 G, according to a standard protocol [26,27]. Details of the calibration of the spectrometer, along with rotational correlation time calibrations, are described elsewhere [26,28]. The first integrals of the V_2' STESR spectra, I_{ST}, were nor-

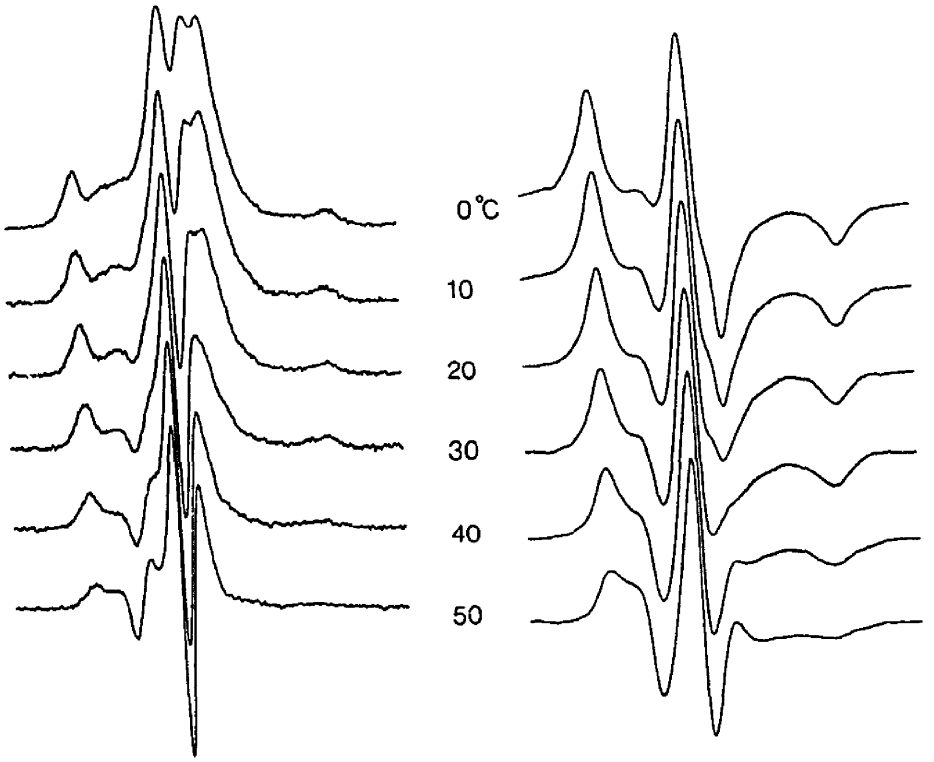

Fig. 1. Temperature dependence of the ESR specta of the 12-MGDGSL monogalactosyldiacylglycerol spin label in PS II-enriched membranes from pea thylakoids. Left-hand side: second harmonic, 90° out-of-phase, absorption STESR spectra (V_2' display). Right-hand side: first harmonic, in-phase, absorption ESR spectra (V_1 display). Total scan width = 100 gauss.

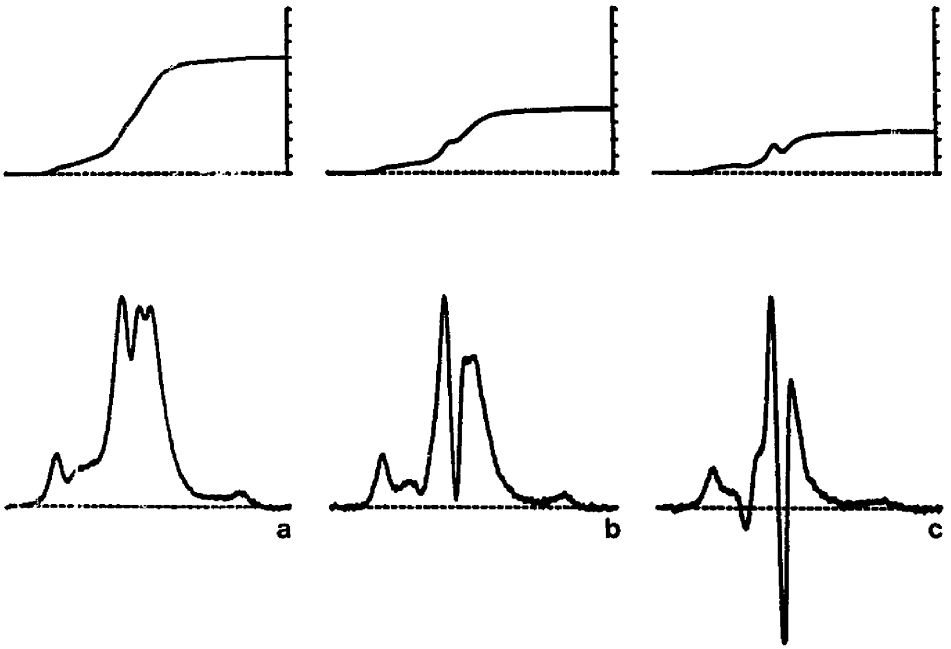

Fig. 2. Lower row: second harmonic, 90° out-of-phase, absorption ESR spectra (V_2' display) of the 12-MGDGSL monogalactosyldiacylglycerol spin label in PS II-enriched membranes from pea thylakoids. (a) Recorded at 0°C, (b) at 20°C, and (c) at 40°C. Upper row: first integrals of the STESR spectra in the lower row, normalized with respect to the double-integrated intensity of the conventional spectra. (Full scale = 10^{-2}, corresponding to an effective correlation time of 370 μs). Total scan width = 100 gauss.

malized with respect to the double integral of the conventional V_1 spectrum recorded under standard conditions as described in Ref. 29.

Results

Representative second harmonic 90° out-of-phase absorption STESR spectra, together with the corresponding conventional ESR spectra, for the 12-MGDGSL spin label in PS II-enriched membranes at different temperatures are shown in Fig. 1. Large changes are seen in the STESR spectra with increasing temperature, whilst the conventional spectra change relatively little, except at the highest temperatures measured. The changes observed in the conventional spectra at the higher temperatures are due to a small fluid population of lipids, which comprises approx. 30% of the total lipids in the PS II-enriched membrane preparations [17]. This fluid component affects the lineshapes of the STESR spectra, making the normal lineheight ratio method of determining the effective rotational correlation times * [18] inappropriate at the higher temperatures. However, the STESR integral method of determining the effective rotational correlation times, which is applicable to multi-component STESR spectra [29], can be applied at all temperatures.

Evaluation of the STESR integrals is illustrated in Fig. 2. Characteristic changes are seen in the total integral that correspond to decreasing rotational correlation times with increasing temperature. The fast motional fluid lipid spectral component contributes negli-

TABLE I

Effective rotational correlation times, τ_R^{eff} (μs) deduced from the lineheight ratios L''/L, C'/C and H''/H in the low-field, central and high-field regions of the STESR spectrum, respectively, and from the normalized STESR integral, I_{ST}, for the 12-MGDGSL spin label in PS II-enriched membranes

STESR parameter	τ_R^{eff} (μs)					
Temperature (°C):	0	10	20	30	40	50
I_{ST}	68	28	15	6	3.2	3.5
L''/L	23	15	7	–	–	–
C'/C	46	13	5	–	–	–
H''/H	55	5.5	14	–	–	–

* In the case of anisotropic motion, the correlation times derived from the isotropic haemoglobin calibrations can only be referred to strictly as 'effective' values [30–34,39]. The effects of motional anisotropy are manifested in different 'effective' correlation times being obtained from the different diagnostic regions of the STESR spectrum [30,32,33]. This point is addressed further under Discussion.

TABLE II

Effective rotational correlation times, τ_R^{eff} (μs), deduced from the normalized STESR integral, I_{ST}, for the 14-PGSL, 14-PCSL and 12-PCSL spin labels in PS II-enriched membranes

Spin label	τ_R^{eff} (μs)					
Temperature (°C):	0	10	20	30	40	50
14-PGSL	59	22	15	10	11	8
14-PCSL	97	55	36	11	7	6
12-PCSL	-	34	28	14	5	-

gibly to the total STESR integral and therefore does not interfere with the evaluation of the effective correlation time [29]. The effective rotational correlation times of the spin-labelled lipid chain have been evaluated by comparison with calibration spectra from isotropically rotating spin-labelled haemoglobin [28]. The values deduced from both the normalized STESR integral and the diagnostic lineheight ratios [16] are given for the 12 MGDGSL spin label in Table I. At low temperatures, all four STESR parameters yield effective rotational correlation times of a similar order of magnitude. Some differences are observed between the various parameters that can be attributed in part to the underlying incipient fluid component, and also to the different inherent sensitivity and precision of the parameters. As the temperature is increased these discrepancies get larger and the lineheight ratios are no longer reliable parameters, as explained previously.

The effective rotational correlation times deduced from the normalized STESR integral are given for the three phospholipid spin labels in PS II-enriched membrane preparations in Table II. As for the 12-MGDGSL label, all phospholipid labels yield values in the 10^{-5} s regime, indicating that the chain motion is remarkably slow. The values obtained for the two different positional isomers of the phosphatidylcholine spin label are of comparable magnitude, and somewhat larger than for either the 14-PGSL or 12-MGDGSL (Table I) spin labels.

Discussion

The results from all four spin labels indicate that the chain motion is very slow for the lipids directly associated with the Photosystem II-protein complex. The effective rotational correlation times are beyond the limits of motional sensitivity of conventional spin label ESR, indicating that analysis by saturation transfer ESR is essential. Whereas the bulk of the lipids (approx. 60%) in whole thylakoid membranes is in a fluid state [10,17], detergent extraction of the PS II centres yields preparations which are relatively low in lipid content, consisting primarily of the first lipid shell surrounding the protein complex. This preparation therefore provides a system well-suited for studying the mobility of the lipids directly associated with the protein.

Previous measurements of the exchange rates of lipids at the interface with integral proteins in membranes containing an excess of lipids have yielded off-rates in the region of 10^7 s^{-1} [30,31], which is considerably more rapid than the rates of chain rotation observed in the present study. However, in the present case, the population of fluid lipids with which exchange may take place is very limited and therefore exchange is unlikely to make an appreciable contribution to the effective rotational rates measured. It seems likely that exchange may be the predominant contribution to the mobility of first-shell lipids at the protein interface in lipid-rich membranes.

One striking feature of the STESR spectra recorded at the lower temperatures in Figs. 1 and 2 is the high intensity in the centre of the spectrum. This suggests that the chains are not undergoing a preferentially more rapid rotation about their long axes (cf. Refs. 32, 33). This conclusion is borne out by the effective correlation time deduced from the c'/C lineheight ratio given in Table I. The reasonable consistency of the correlation times deduced from the three different lineheight ratios suggests that the motion must approximate the isotropic rotational diffusion for which the calibrations were deduced. Considering the possible motional modes of the lipid chains, it therefore seems likely that these are dominated by *trans-gauche* isomerization, rather than a preferential axial rotation, at the lipid/protein interface.

The order of magnitude of the effective correlation times is much greater than that found for the chain rotational motions in the fluid phase of lipid membranes, which are typically in the 10^{-9} to 10^{-8} s range [34,35], and approaches much closer to that obtained in gel phase lipid bilayers [32]. The motions are, however, significantly faster than those of the closely packed chains in lipid gel phases which can have effective correlation times of the order of 10^{-4} s. It is of interest to compare the effective correlation times for lipid chain motion with those expected for protein rotational diffusion. Using the hydrodynamic approach of Saffman [36], the correlation time for protein rotation is related to the effective membrane viscosity, η, by:

$$\tau_{R\parallel} = 2\pi\eta a^2 h/(3kT) \quad (1)$$

where h is the height of the membrane-spanning region. For a PS II-LHC II protein complex of approximate mean diameter $2a = 133$ Å [1], the predicted rotational correlation time is $\tau_{R\parallel} \approx 55$ μs, assuming $\eta \approx 5$ P [37]. This estimate is comparable to the values given in Table I, however the STESR data do not suggest the characteristic uniaxial anisotropy expected for integral protein rotational diffusion. In addition, it is possible that the

TABLE III

Polar lipid composition (mol%) of thylakoid membranes and subthylakoid membrane fractions from pea seedlings

MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol; PC, phosphatidylcholine.

Lipid	Thylakoid	PS I fraction	PS II fraction
MGDG	40.3	38.4	46.0
DGDG	25.1	28.3	22.7
PG	11.7	11.6	10.1
SQDG	13.0	12.6	11.3
PC	5.6	4.0	4.3
Remaining	4.3	5.1	5.6

effective viscosity may be considerably higher in complexes with low lipid/protein ratio, and by the same token the lipid motions at the protein interface may be correspondingly slowed down. The detergent-extracted PS II-enriched membranes gave (2,5-dimethyl-*p*-benzoquinone)-dependent oxygen evolution of 180–190 μmol $\cdot s \cdot h^{-1} \cdot$(mg chlorophyll)$^{-1}$. This rate was not altered significantly following incorporation of the spin-labelled probes.

Although PS II-enriched membranes have a lower lipid/protein ratio, the distribution of lipid classes is not notably changed from that found in thylakoids (see Table III). Therefore, it is unlikely that there is inhibition as a result of preferential depletion of a single lipid class with a possible functional role (cf. Ref. 39). In addition, it is found in the present work that all lipid classes display a strong degree of motional restriction of the acyl chains. The question thus arises to what extent this inhibition of the chain motions influences the functional properties of the photosystem II reaction centre. This question is important, since models for thylakoid membrane function (see Ref. 39 for a review) require mobility of protein components, e.g. LHC II, and redox carriers, e.g. plastoquinone. This mobility would be strictly limited at the lipid/protein interface if the lipid acyl chain motion is restricted in this region. Under these circumstances, it is indeed possible that the electron transfer rate may be determined or influenced by the exchange rate at the lipid/protein interface.

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